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Active Site-directed Inhibition by Optically Pure Epoxyalkyl Cellobiosides Reveals Differences in Active Site Geometry of Two 1,3-1,4-β-D-Glucan 4-Glucanohydrolases

THE IMPORTANCE OF EPOXIDE STEREOCHEMISTRY FOR ENZYME INACTIVATION*

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Peter Bordier Høj‡, Evelyn B. Rodriguez§¶, Joanne R. Iser‡, Robert V. Stick§, and Bruce A. Stone‡

From the ‡Department of Biochemistry and Commonwealth Special Research Centre for Protein and Enzyme Technology, La Trobe University, Bundoora, Victoria 3083, Australia and the §Department of Organic Chemistry, The University of Western Australia, Nedlands 6009, Western Australia

1,3-1,4-β-D-Glucan 4-glucanohydrolases (EC 3.2.-1.73) from *Bacillus subtilis* and barley (*Hordeum vulgare*) with identical substrate specificities but unrelated primary structures have been probed with (*R,S*)-epoxyalkyl (-propyl, -butyl, -pentyl) β-cellobiosides and with optically pure (3*S*)- and (3*R*)-3,4-cellobiosides as active site-directed inhibitors. The optimal aglycon length for inactivation differs for the two enzymes, and they are differentially inhibited by the pure epoxybutyl β-cellobioside diastereoisomers. The (3*S*)-epoxybutyl β-cellobioside inactivates the *B. subtilis* enzyme much more efficiently than does the (3*R*)-isomer, whereas the reverse is true for the barley enzyme. Both enzymes are inactivated by a mixture of the stereoisomers at a rate intermediate of that observed with the individual isomers. The two β-glucan endohydrolases may therefore employ sterically different mechanisms to achieve glycoside bond hydrolysis in their common substrate. The efficiency and specificity of epoxide-based "suicide" inhibitors may be enhanced significantly by the use of inhibitors bearing only one stereoisomeric form of the epoxide group.

Irreversible, active site-directed inhibitors have been used in a number of studies to define the mechanism and specificity of enzyme action (1). One class of widely used inhibitors incorporates a substrate analogue covalently linked to an epoxide group. This epoxide is susceptible to nucleophilic attack from catalytic amino acid residues located at the active sites of kinases (2, 3), proteases (4, 5), and glycoside hydrolases (6–21). Recently, we employed a series of epoxyalkyl oligo-β-D-glucosides for the irreversible, active site-directed inhibition of a number of related β-glucan endohydrolases with distinct, but overlapping, substrate specificities (6). It was found that the linkage and length of the glycon part of the inhibitor greatly influenced the rate of inactivation. It was concluded that differences in the kinetics of inactivation of the enzymes by the various inhibitors reflected differences in active site structures. Moreover, the results showed very clearly that the structure of the epoxide-bearing aglycon significantly influences the potency of a given inhibitor.

In the analysis of the action of epoxy-activated substrate analogue inhibitors, the effect of the chirality of the epoxide group on the inhibitor has attracted only little attention (16, 22). In the generally employed route of synthesis of epoxyalkyl oligo-β-D-glucosides, an alkene (*e.g.* 3-butenyl β-cellobioside) is oxidized to give a mixture of diastereoisomers, (3*R*)- and (3*S*)-G4G-O-C_4 [1] (see Fig. 1). Recently, however, we reported the synthesis of optically pure epoxyalkyl β-D-glucosides and cellobiosides (23). We have now extended our earlier observations (6) by comparing (3*R*)- and (3*S*)-G4G-O-C_4 as active site-directed inhibitors of *Bacillus subtilis* and barley 1,3-1,4-β-glucanases. These enzymes exhibit identical substrate specificities and action patterns (24, 25) but have completely unrelated primary structures (26, 27). The experiments describe, to our knowledge for the first time, a strict stereospecificity requirement for inhibition by the epoxyalkyl group of the oligoglucoside.

MATERIALS AND METHODS

Chemicals—MES was a product of Sigma. Barley 1,3-1,4-β-glucan was a product of Biocon (Australia) and Amberlite AG-X8 was supplied by Bio-Rad.

Inhibitors—The mixtures of diastereoisomers of epoxyalkyl β-D-glycosides were prepared as previously described (28). These inhibitors were the same as used previously (6). Analysis by ^{13}C NMR spectroscopy established the ratio of (*R*)- and (*S*)-stereoisomers of the epoxyalkyl glycosides. The synthesis of the optically pure epoxyalkyl β-cellobiosides has been reported (23). All inhibitors were deacetylated as previously described (6) except that the sodium methoxide concentration was held at 50 mM. The deacetylation reaction was followed by TLC as described by Clarke (17).

Enzymes—The *B. subtilis* 1,3-1,4-β-glucanase was purified as in Ref. 29. Barley 1,3-1,4-β-glucanase isozyme II was purified to homogeneity essentially as described (30) and judged to be free of contamination from barley 1,3-1,4-β-glucanase isozyme I by probing Western blots with isozyme-specific monoclonal antibodies (31).

Assays for Enzyme Activity—All activities were measured as in Ref. 6 by determining the amount of reducing sugar equivalents released on incubation of the β-glucanases with 0.5% (w/v) barley 1,3-1,4-β-glucan at 40 °C in 50 mM sodium maleate buffer at pH 6.5 and in 50 mM sodium acetate buffer at pH 5.0 for the *B. subtilis* and barley 1,3-1,4-β-glucanases, respectively. All other analytical techniques were as described previously (6).

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¶ Present address: Inst. of Chemistry, University of the Philippines, Los Banos, Laguna, Republic of the Philippines.

[1] The abbreviations used are: G4G-O-C_4, 3,4-epoxybutyl β-cellobioside; G4G-O-C_3, 2,3-epoxypropyl β-cellobioside; G4G-O-C_5, 4,5-epoxypentyl β-cellobioside; MES, 2-(*N*-morpholino)ethanesulfonic acid; *B. subtilis* 1,3-1,4-β-glucanase, *Bacillus subtilis* 1,3-1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73); barley 1,3-1,4-β-glucanase, barley (*Hordeum vulgare* cv. Clipper) 1,3-1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73).

RESULTS AND DISCUSSION

From studies of irreversible, active site-directed inactivation of the *B. subtilis* 1,3-1,4-β-glucanase by a series of structurally related epoxyalkyl oligo-β-D-glucosides (6) it was concluded that the aglycon residues of the inhibitors span the glycosyl binding subsite occupied by the 3-substituted glucosyl residue involved in the glucosidic linkage cleaved in the natural 1,3-1,4-β-glucan substrate (see Scheme 1; adapted from Ref. 6). It is not known whether this also applies to the barley enzyme or whether this enzyme, in contrast to the *B. subtilis* 1,3-1,4-β-glucanase, is inactivated more efficiently by epoxyalkyl laminaribiosides than by epoxyalkyl cellobiosides. For the purpose of comparison of the two enzymes, however, the barley enzyme was challenged with G4G-O-C_5, G4G-O-C_4, and G4G-O-C_3 only. The inactivation of the barley enzyme by the mixture of diastereoisomers of G4G-O-C_3, G4G-O-C_4, and G4G-O-C_5 was compared with that for the *B. subtilis* 1,3-1,4-β-glucanase (Fig. 2). Despite the indistinguishable substrate specificity and product formation of these two enzymes (30, 31), there exists a clear difference in the dependence on the aglycon chain length for maximum inactivation. Such differences in the dependence on the aglycon chain length must reflect subtle differences in the geometry of the active sites of these two endohydrolases. To probe the relationship between the structure and potency of epoxide-activated inhibitors further, we took advantage of the recent synthesis of stereochemically pure (3*S*)- and (3*R*)-3,4-epoxybutyl β-D-cellobiosides (23) and compared their action on the *B. subtilis* and barley 1,3-1,4-β-glucanases (Fig. 3). The two enzymes were differentially inhibited by the diastereoisomers. Thus, (3*S*)-G4G-O-C_4 inactivated the *B. subtilis* 1,3-1,4-β-glucanase much more efficiently than did (3*R*)-G4G-O-C_4, whereas the reverse is true for the inactivation of the barley enzyme. The mixture of the stereoisomers had an intermediate rate of inactivation as compared with the individual isomers. In other studies Shulman *et al.* (18) found the (*R*)- and (*S*)-diastereoisomers of the epoxyalkyl C-glucosides differed only slightly in the rate at which they inhibited sweet almond β-glucosidase, whereas the D- but not the L-enantiomer of conduritol B epoxide inactivated *Aspergillus wentii* β-glucosidases (22) and rabbit small intestine sucrase-isomaltase (32). On the other hand, a yeast β-fructosidase was inactivated only by the L-enantiomer (33, 34).

The implications of these findings are severalfold. They demonstrate that very subtle differences in the structure of these mechanism-based epoxide-bearing inhibitors greatly influence their potency. Thus, designers of epoxy-activated enzyme inhibitors must pay strict attention to the stereo-

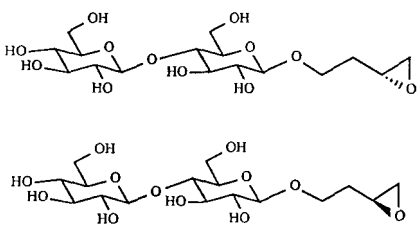
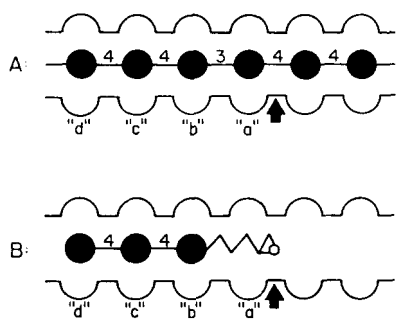


FIG. 1. **The structure of (3*R*)-G4G-O-C_4 (*above*) and (3*S*)-G4G-O-C_4 (*below*) used in this study.**



SCHEME 1. *A*, schematic representation of the glucosyl requirement of *B. subtilis* (30) and barley (31) 1,3-1,4-β-glucanases for hydrolysis. The point of cleavage is indicated by the *arrow*; *a–d*, glycogen-binding subsites. *B*, the deduced mode of binding of G4G-O-C_4 inhibitor to the *B. subtilis* 1,3-1,4-β-glucanase (6). Glucose residues are represented by *closed circles* and (1,3)- and (1,4)-β-glucosidic linkages by *3* and *4*, respectively.

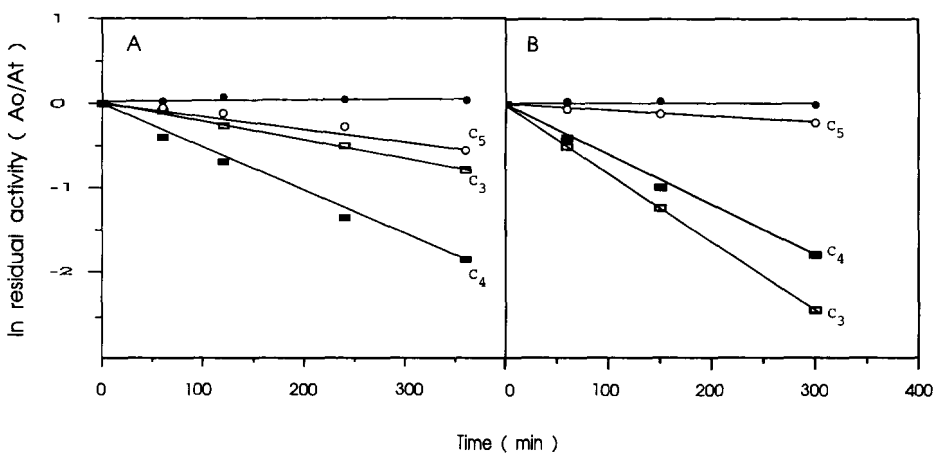


FIG. 2. **Inactivation of *B. subtilis* and barley (isozyme II) 1,3-1,4-β-glucanases by (*R,S*)-epoxyalkyl cellobiosides with varying aglycon chain lengths.** *A*, *B. subtilis* 1,3-1,4-β-glucanase (155 μg/ml) and (*R,S*)-epoxyalkyl β-cellobioside (22 mM) were incubated at 35 °C and pH 6.6 in 14 mM sodium acetate and 153 mM MES buffer. Residual enzyme activity was measured at appropriate time intervals and apparent rate constants for inactivation determined by semilogarithmic plots. G4G-O-C_3 (□, K_{app} = 2.22 × 10^{-3} min^{-1}), G4G-O-G_4 (■, k_{app} = 5.12 × 10^{-3} min^{-1}), G4G-O-C_5 (○, k_{app} = 1.52 × 10^{-3} min^{-1}), and control with no inhibitor (●, k_{app} = 9.05 × 10^{-5} min^{-1}) are shown. *B*, barley 1,3-1,4-β-glucanase (isozyme II) 95 μg/ml and (*R,S*)-epoxyalkyl β-cellobioside (34 mM) were incubated at 25 °C and pH 5.0 in 50 mM sodium acetate buffer. Residual enzyme activity was measured at appropriate time intervals and apparent rate constants for inactivation determined by semilogarithmic plots. G4G-O-C_3 (□, k_{app} = 8.08 × 10^{-3} min^{-1}), G4G-O-C_4 (■, k_{app} = 5.91 × 10^{-3} min^{-1}), G4G-O-C_5 (○, k_{app} = 6.91 × 10^{-4} min^{-1}), and control with no inhibitor (●, k_{app} = 2.2 × 10^{-5} min^{-1}) are shown.

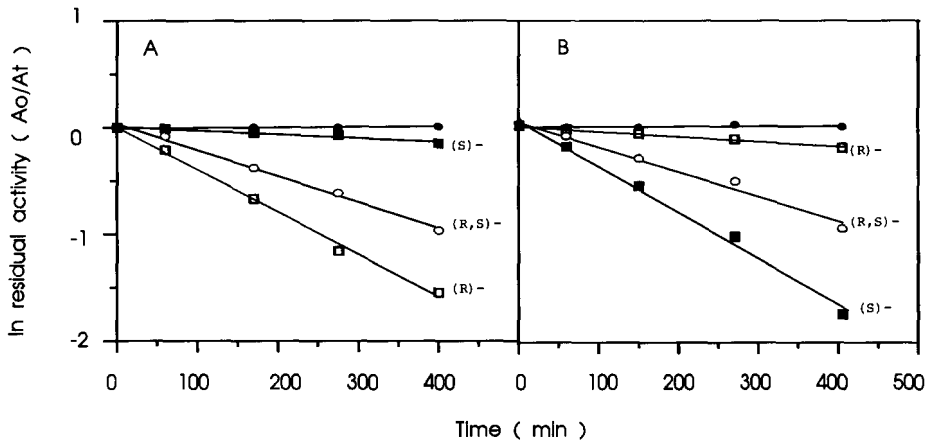


FIG. 3. **Inactivation of *B. subtilis* and barley isozyme II 1,3-1,4-β-glucanases by optically pure (3*R*)-G4G-O-C_4 and (3*S*)-G4G-O-C_4.** *A*, *B. subtilis* 1,3-1,4-β-glucanase (300 μg/ml) and epoxyalkyl β-cellobiosides (14 mM) were incubated at 30 °C and pH 6.5 in 100 mM MES buffer. Residual enzyme activity was measured at appropriate time intervals and apparent rate constants for inactivation determined by semilogarithmic plots. (3*S*)-G4G-O-C_4 (■, $k_{app} = 3.61 \times 10^{-4}$ min^{-1}), (3*R*)-G4G-O-C_4 (□, $k_{app} = 3.99 \times 10^{-3}$ min^{-1}), (3*R*,3*S*)G4G-O-C_4 (○, $k_{app} = 2.46 \times 10^{-3}$ min^{-1}), and control with no inhibitor (●, $k_{app} = 4.41 \times 10^{-5}$ min^{-1}) are shown. This experiment has also been performed in the presence of bovine serum albumin, and the same results have been obtained. *B*, barley isozyme II 1,3-1,4-β-glucanase (80 μg/ml), bovine serum albumin (0.8 mg/ml), and epoxyalkyl β-cellobiosides (14 mM) were incubated at 20 °C and pH 5.0 in 20 mM sodium acetate buffer. Residual enzyme activity was measured at appropriate time intervals and apparent rate constants for inactivation determined by semilogarithmic plots. (3*S*)-G4G-O-C_4 (■, $k_{app} = 4.28 \times 10^{-3}$ min^{-1}), (3*R*)-G4G-O-C_4 (□, $k_{app} = 4.66 \times 10^{-4}$ min^{-1}), (3*R*,3*S*)-G4G-O-C_4 (○, $k_{app} = 2.31 \times 10^{-3}$ min^{-1}), and control with no inhibitor (●, $k_{app} = 2.62 \times 10^{-5}$ min^{-1}) are shown. This experiment has also been performed in the absence of bovine serum albumin, and identical results have been obtained except for a minor loss of activity in the control.

chemistry of the epoxide function, not only in synthetic work, but also in later structure-function studies of inhibitors and enzymes. For example, the inactivation rates as a function of the aglycon chain length referred to in Fig. 2 are only meaningful if the ratio of the (3*R*)- and (3*S*)-isomers is identical in each inhibitor preparation employed. In this respect, it should be noted that all our mixtures of stereoisomers of inhibitors tested herein and in our earlier publication (6) have been analyzed by ^{13}C NMR spectroscopy and found to have practically identical proportions of (3*S*)- and (3*R*)-stereoisomers (data not shown).

Our results also imply that the efficiency and specificity of epoxide-based inhibitors may be greatly enhanced by presenting only one chiral form of the compound. For example if the two endohydrolases used in this study coexisted in the same experimental system, probing with (3*S*)- and (3*R*)-G4G-O-C_4 in separate experiments may selectively inactivate only one of the glucanases and thereby allow an elucidation of the role of the individual enzymes in the system.

The inactivation of the endohydrolases by irreversible, active site-directed inhibitors can be described by the equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*I \xrightarrow{k_2} E\text{–}I$$

where E^*I is a reversible complex between enzyme and inhibitor, and E–I is the enzyme with covalently attached inhibitor (35). The differences in effect of the (3*R*)- and (3*S*)-G4G-O-C_4 inhibitors against the two endohydrolases could be due to differences in the affinity for the active sites (K_i) and/or differences in the rate of irreversible inactivation of the noncovalent enzyme-inhibitor complex (k_2). Due to the high K_i of epoxyalkyl oligo-β-D-glucosides for endohydrolases (6, 9, 16, 17) (*e.g.* K_i of 63 mM for (3*R*,3*S*)-G4G-O-C_4 against the *B. subtilis* 1,3-1,4-β-glucanase; Ref. 6) and the very limited supply of the optically pure inhibitors, it was not possible to determine the dissociation constants for the enzyme-inhibitor complexes. However, since the two enzymes bind to and hydrolyze exactly the same substrate, it is likely that the K_i values for the (3*R*)- and (3*S*)-inhibitors do not differ markedly and the observed differences in inhibitor potency may be directly related to the stereochemical course of catalysis in the two enzymes, and therefore reflects changes in k_2. This in turn means that rotation around the C_2–C_3 bond of the aglycon must be restricted, possibly due to hydrogen bonding between the enzyme and the epoxide group. The strict stereospecificity of inhibitor action observed in this study shows that, although the unrelated polypeptide chains of the *B. subtilis* and barley 1,3-1,4-β-glucanases can fold to conformations that achieve the same substrate specificity and yield the same products, they are likely to employ sterically different mechanisms to achieve glycoside bond hydrolysis. Such apparent differences in active site geometry may be related to the mechanism of catalysis. Recently Post and Karplus (36) suggested an alternative to the generally accepted exocylic mechanism for hen egg white lysozyme-mediated hydrolysis of oligoglycosides, based on molecular dynamic simulations. The present results would suggest that stereochemically different pathways may be employed by glucanohydrolases. Further experiments would have to be performed to test this intriguing possibility.

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