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The E358S mutant of *Agrobacterium* sp. β-glucosidase is a greatly improved glycosynthase

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Abstract Glycosynthases are nucleophile mutants of retaining glycosidases that catalyze the glycosylation of sugar acceptors using glycosyl fluoride donors, thereby synthesizing oligosaccharides. The 'original' glycosynthase, derived from *Agrobacterium* sp. β-glucosidase (Abg) by mutating the nucleophile glutamate to alanine (E358A), synthesizes oligosaccharides in yields exceeding 90% [Mackenzie, L.F., Wang, Q., Warren, R.A.J. and Withers, S.G. (1998) J. Am. Chem. Soc. 120, 5583–5584]. This mutant has now been re-cloned with a His_6-tag into a pET-29b(+) vector, allowing gram scale production and single step chromatographic purification. A dramatic, 24-fold, improvement in synthetic rates has also been achieved by substituting the nucleophile with serine, resulting in improved product yields, reduced reaction times and an enhanced synthetic repertoire. Thus poor acceptors for Abg E358A, such as PNP-GlcNAc, are successfully glycosylated by E358S, allowing the synthesis of PNP-β-LacNAc. The increased glycosylation activity of Abg E358S likely originates from a stabilizing interaction between the Ser hydroxyl group and the departing anomeric fluorine of the α-glycosyl fluoride.

Key words: Enzymatic oligosaccharide synthesis; Glycosyl transfer

1. Introduction

Oligosaccharides have considerable potential as therapeutics but are only now slowly assuming this important role [1–3]. One of the reasons for their slow development has been the considerable difficulty in synthesizing oligosaccharides on the scale necessary for their clinical evaluation. Enzymatic synthesis has emerged as the method of choice for such large scale syntheses, there being two major approaches. Glycosyl transferase-catalyzed synthesis using nucleotide phosphosugar donors is a logical choice, but one that until recently has been limited by the availability of both enzyme and substrates [4–7]. The alternative method involving the use of glycosidases has a much longer history [8–11] and comprises two separate approaches. The equilibrium approach relies upon the use of high concentrations of sugars to generate products, but only offers very modest (typically $< 15\%$) yields, and suffers from almost complete lack of regiochemical control. The kinetic approach requires a retaining glycosidase and uses a relatively reactive glycosyl donor, typically a nitrophenyl glycoside or a glycosyl fluoride, to maintain a steady state concentration of the glycosyl-enzyme intermediate that is intercepted by an added sugar moiety bound productively in the aglycone site [12]. This approach provides better yields (typically 10–40%) and greater regiochemical control, but still is not generally economical for large scale syntheses.

A new variation on this approach that eliminates the hydrolytic reaction was introduced recently [13]. A specifically mutated retaining β-glycosidase is used in which the glutamic or aspartic acid group of the catalytic nucleophile, itself readily identified using the 2-fluorosugar strategy previously developed [14,15], is replaced by the methyl group of an alanine residue. Such mutants are incapable of hydrolyzing oligosaccharides, but can transfer a sugar moiety from a glycosyl fluoride donor of the opposite anomeric stereochemistry to that of the normal substrate. Thus the E358A mutant of *Agrobacterium* sp. β-glucosidase (Abg) will transfer the glucose moiety from α-glucosyl fluoride to a suitable acceptor sugar in high (typically $> 80\%$) yields without any hydrolysis of product. The approach was demonstrated with several different glycosyl fluoride donors and a range of different acceptor sugars; good yields being obtained in each case. A second example has since been published involving an endoglycanase [16]. Although effective as catalysts and producing excellent yields, the reactions are still somewhat slow, therefore requiring substantial quantities of mutant enzyme and/or extended incubation times. This led us to search for mutants with improved properties. Here, we report the generation and evaluation of a mutant of Abg of significantly improved properties, catalyzing glycoside formation with rate constants approximately 24-fold greater than those of the Ala mutant.

2. Materials and methods

2.1. Reagents, enzymes and bacterial strains

Plasmid-containing strains were grown in Luria–Bertani broth containing 50 mg/ml kanamycin (LB_{kan}) or in TYP (16 g/l tryptone, 16 g/l yeast extract, 5 g/l NaCl, 2.5 g/l K_2HPO_4) containing 50 mg/ml kanamycin (TYP_{kan}). *Pwo* DNA polymerase and deoxynucleoside triphosphates were from Boehringer Mannheim. Restriction endonu-

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Abbreviations: Abg, *Agrobacterium* sp. β-glucosidase; DNPG, 2,4-dinitrophenyl-β-D-glucopyranoside; α-GalF, α-D-galactopyranosyl fluoride; α-GlcF, α-D-glucopyranosyl fluoride; PNPG, *para*-nitrophenyl β-D-glucopyranoside; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; ESI-MS, electrospray ionization mass spectrometry

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cleases and T4 DNA ligase were from New England BioLabs. Electrocompetent *Escherichia coli* Topp10 cells and the pZeroBlunt cloning kit were from Invitrogen. The pET-29b(+) expression vector, electrocompetent *E. coli* BL21(DE3) cells and His-bind metal chelation resin were from Novagen. Preparation of oligonucleotide primers and DNA sequencing was performed at the Nucleic Acids and Peptide Service (NAPS) Unit, University of British Columbia.

2.2. Construction of His_6-tagged Abg

The gene encoding the Abg (*abg*) was amplified by the polymerase chain reaction (PCR) and subsequently subcloned into the His_6 fusion protein expression vector, pET-29b(+) (Novagen). The PCR mixture contained 10 μM oligonucleotide primers (shown below), 1 mM concentrations of the four deoxynucleoside triphosphates in 100 ml of DNA polymerase buffer, and 25 ng of plasmid pTug10Nabg, carrying the entire *abg* gene [17]. After heating the mixture to 95°C, the reaction was started by adding 5 U of *Pwo* DNA polymerase (Boehringer Mannheim). Thirty PCR cycles (45 s at 94°C, 45 s at 56°C and 70 s at 72°C) were performed in a thermal cycler (Perkin Elmer, GeneAmp PCR System 2400). Agarose gel electrophoresis of the PCR product revealed a single DNA fragment of approximately 1380 bp. The primers were as follows: 5′-AA CAT ATG GGA CCG TTA TGG CTA GAC-3′ (forward primer) and 5′-GAG AAC CTC GAG CCC CTT GGC AAC CCC ATG GTT CC-3′ (reverse primer). Underlined are the restriction sites *Nde*I and *Xho*I introduced by the two primers. The PCR product was purified and subsequently subcloned into the cloning vector pZero2.0 as well as the expression vector pET-29b(+) using procedures described elsewhere [18].

2.3. Construction of Abg E358S by site-directed mutagenesis

A 'megaprimer' PCR approach [19] was used to introduce a serine residue at the position of the catalytic nucleophile (E358). The oligonucleotide primers were as follows. Mutagenic reverse primer, 5′-TT GTA GCA GGC GCC GTT GCT GGT GAT GTA GCA CTC CGG-3′ (the mutated codon is underlined), forward primer, 5′-GGT CTT CAA GGG CGA ATA TCC-3′, reverse primer, 5′-TCA CCC CTT GGC AAC CCC ATG-3′. In a first PCR, a 300 bp fragment was amplified using the mutagenic and the forward primer. The PCR product was purified and subsequently used in a second PCR as megaprimer. The conditions for the first PCR were as described above, but pTug10NabgE358C [20] was the template. The mixture for the second PCR contained 2.5 μg megaprimer, 10 mM concentrations of reverse primer as well as 25 ng of plasmid pTug10NabgE358C. The following PCR protocol was used: 5 cycles (60 s at 95°C, 180 s at 72°C), then 25 cycles (45 s at 95°C, 45 s at 58°C and 80 s at 72°C). The major band of about 600 bp was purified by agarose gel electrophoresis. The purified PCR fragment was ligated into the vector pZero2.0 and subsequently subcloned into pET29-abgHis6. The cloned product, called pET29abgE358S, was sequenced to verify the induced mutation. The vector pET29abgE358A was prepared in a similar manner (data not shown).

2.4. Overexpression of the gene and purification of His_6-tagged Abg358S

Recombinant Abg E358S was purified from IPTG-induced cultures of *E. coli* BL21(DE3) cells, carrying the plasmid pET29abgE358S. The cell suspension was passed two times through a French press at 5°C, centrifuged at $10\,000 \times g$ for 30 min and the soluble cell extract purified by Ni^{2+} chelation chromatography (His-bind resin, Novagen). A 20 l culture of Abg E358S processed in this way yielded 3.5 g of pure mutant enzyme.

2.5. Rescue and transglycosylation kinetics

Rescue of the glycosidic bond cleaving activity of each nucleophile mutant using anionic nucleophiles was performed as reported previously [21]. Concentrations of mutant enzyme solutions were quantified directly by absorbance at 280 nm ($\varepsilon_{280}^{0.1\%} = 2.20$). Curve-fitting was performed with GraFit 3.0 [22].

All transglycosylation kinetic studies were performed at 25°C. Assay solutions consisted of α-D-galactopyranosyl fluoride (α-GalF) and *para*-nitrophenyl β-D-glucopyranoside (PNPG) in 150 mM NaPi, 150 mM NaCl, 1 mg/ml bovine serum albumin, pH 7. An Orion fluoride electrode (model 96-09BN), interfaced with a Fischer Scientific Accumet 925 pH/ion meter, was used to monitor fluoride release following addition of a small aliquot of Abg E358A or E358S. Final concentrations of enzyme were 0.108 mg/ml (2.12 μM) E358S and 1.94 mg/ml (37.9 μM) E358A. All enzymatic rates were corrected for the spontaneous hydrolysis rate of α-GalF. In the first studies, a fixed concentration of α-GalF (57 mM) was used to determine an initial rate profile for PNPG. A maximal rate was observed at 22 mM PNPG followed by substrate inhibition at higher concentrations of PNPG (see Fig. 2a). These data were fit according to Eq. 1 using GraFit.

$$V_o = \frac{V_{max}[S]}{K_m + [S] + \frac{[S]^2}{K_i}} \quad (1)$$

K_i represents the inhibition constant for the substrate (in this case PNPG) binding in a non-productive mode. The complementary experiment was carried out in which PNPG was kept at a constant concentration of 22 mM and the concentration of α-GalF was varied (see Fig. 2b). Apparent values of k_{cat}, K_m and k_{cat}/K_m for GalF and PNPG were also derived using GraFit.

In studies of the dependence of reaction rate upon pH, the following buffers were used: pH 5–6, 100 mM sodium citrate; pH 7–8, 100 mM NaPi; pH 8–9, 100 mM 3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropanesulfonic acid. Apparent values of k_{cat}/K_m for α-GalF were determined from the linear dependence of rates ($V_o = k_{cat}E_o/K_m$) measured at three low concentrations of α-GalF (5, 10 and 15 mM) at a fixed concentration of PNPG (22 mM).

2.6. Oligosaccharide synthesis with Abg E358S

In a typical reaction, the acceptor glycoside was dissolved in approximately 2.5 ml 150 mM ammonium hydrogen carbonate (pH 7.9) to which an aliquot of donor glycosyl fluoride (α-D-glucopyranosyl fluoride (α-GlcF) or α-GalF) and Abg E358S, all in buffered solutions of ammonium hydrogen carbonate, were added. Acceptor concentrations ranged from 20 to 40 mM, donor concentrations from 30 to 80 mM, and Abg E358S concentrations from 0.25 mg/ml (4.85 μM) to 1 mg/ml (20 μM). Reaction times ranged from 2 h to 7 days. Reactions were monitored by thin layer chromatography (TLC) (silica gel 60 F_{254}, aluminum-backed, Merck) using 7:2:1 ethyl acetate/methanol/water. Plates were visualized by exposure to 10% sulfuric acid in methanol followed by charring.

2.7. Oligosaccharide purification and characterization by electrospray ionization mass spectrometry (ESI-MS)

Upon completion, reaction solutions were lyophilized, re-dissolved in 1 ml water, and the mutant enzyme removed by ultrafiltration (Centricon-30, Amicon). Reaction products were purified by preparative high performance liquid chromatography (HPLC) on a TosoHaas Amide-80 column (21.5 mm × 30 cm, # 14460). A BioSepra ProSys workstation (Beckman Coulter) was used to generate a linear gradient of 80:20 to 60:40 acetonitrile/water at a flow rate of 5 ml/min. Products were detected using a UV/Vis detector. Product fractions were concentrated in vacuo then lyophilized. Reaction yields were determined by integration of the peaks within the HPLC chromatograms using an analysis program developed by BioSepra. Molecular weights of oligosaccharide products were confirmed by ESI-MS (Perkin Elmer API 300, Sciex, Thornhill, Ont., Canada).

3. Results and discussion

While the E358A mutant of Abg successfully catalyzed the desired oligosaccharide synthesis reaction without observable product hydrolysis, a more effective mutant with higher catalytic activity was highly desirable, particularly to improve reactivity with the 'slower' acceptors. Substitution of a more polar side chain than the methyl group of alanine seemed a reasonable aim since this should provide a more favorable binding site for the anomeric fluoride as it departs during the glycosyl transfer reaction. Such a side chain must be smaller than that of the original glutamic acid to allow binding of the α-anomeric fluoride. An aspartic acid is not a useful substitution since we had shown previously that the E358D mutant of Abg catalyzes slow hydrolysis via the normal path-

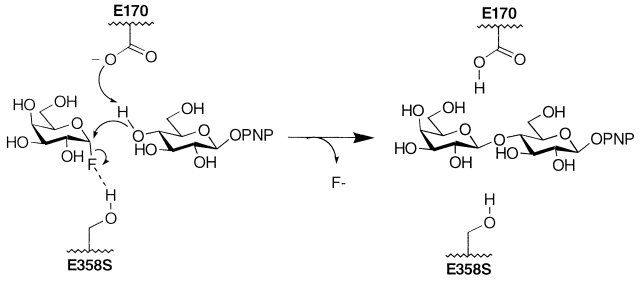


Fig. 1. Mechanism for Abg E358S acting as a glycosynthase.

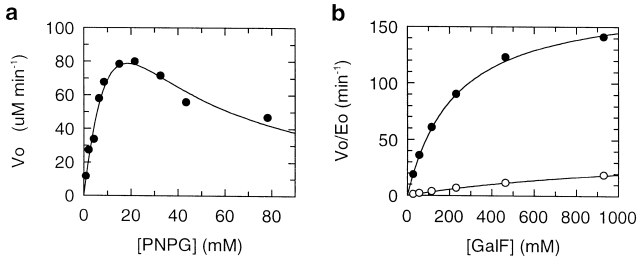


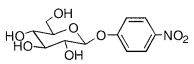
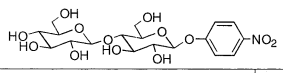
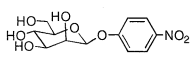
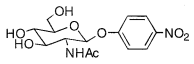
Fig. 2. Transglycosylation kinetics with Abg E358S. (a) Variation of PNPG with α-GalF fixed at 57 mM. The curve shown is a fit of the data according to Eq. 1. (b) A comparison of the transglycosylation kinetics of Abg E358S (●) and Abg E358A (○). PNPG is the acceptor in both cases, fixed at 22 mM.

way [23]. Further, substitution of cysteine could cause problems since we had also shown previously that this mutant reacts with the substrate 2,4-dinitrophenyl-β-D-glucopyranoside (DNPG) to generate a stable α-glucosyl cysteine adduct that hydrolyzes only very slowly ($t_{1/2} \sim 15$ h) [20]. Substitution with serine seemed to be a good alternative since the hydroxymethyl side chain should be sufficiently small to accommodate the axial fluorine and might even hydrogen bond to the departing fluoride (Fig. 1). Moreover, its alcohol moiety should be considerably less nucleophilic than the thiol of cysteine, thus unwanted adduct formation should be less likely.

Therefore, the E358S mutant of Abg was generated by side-directed mutagenesis by a 'megaprimer' PCR method using three oligonucleotide primers, one of which carried the mutation. The product of the first PCR reaction served, after purification, as a primer in the second PCR reaction (megaprimer). High megaprimer concentration is the critical factor in this approach [19]. A 295 bp gene fragment, carrying the mutation, was exchanged with the corresponding fragment in the wild-type (wt) gene. This procedure minimized the possibility of other mutations arising during the gene amplification by PCR.

Although systems were constructed for production in *E. coli* of Abg wt, and the E358A and E358C mutants, and high yields were obtained, purification remained a relatively lengthy process [24]. Therefore, wt Abg and the mutants E358S, E358A and E358C were produced as His_6-tagged-fusions, after subcloning the genes into an appropriate vector. Purification was achieved in a single step on the His-bind resin; 150–250 mg of each enzyme was obtained from a 1 l culture. The enzyme preparations were judged to be pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. A large scale preparation of Abg E358S from a 20 l culture yielded 3.5 g of purified protein (data not shown).

Table 1
Abg E358S transglycosylation reactions using α-GalF as donor

#	Acceptor	Yield Disaccharide (Observed *m/z*)	
		di-	tri-
1		98% (486, $[M+Na]^+$)	-
2			98% (648, $[M+Na]^+$)
3		80% (486, $[M+Na]^+$)	-
4		63% (527, $[M+Na]^+$)	-

The His_6-tagged Abg E358S and E358A were assayed with the highly reactive substrate DNPG and were found to be completely devoid of glycosidic bond cleaving activity, as expected [21]. However, significant activity was recovered in the presence of 2 M azide ($k_{cat}/K_m = 33.9$ min^{-1} mM^{-1} for E358A, 9.62 min^{-1} mM^{-1} for E358S) or 5 M formate ($k_{cat}/K_m = 1616$ min^{-1} mM^{-1} for E358A, 900 min^{-1} mM^{-1} for E358S). This rescue occurs by a mechanism in which the anion attacks directly at the anomeric center from the α-face, in place of the catalytic nucleophile, thereby cleaving the substrate with net inversion of configuration to give an α-glycosyl azide (or formate) product. Such behavior is a standard feature of retaining glycosidase nucleophile mutants as reported by this group [21,25] and others [26,27]. Of note here is the significantly lower activity of the serine mutant with azide or formate relative to the alanine mutant, which may well reflect the greater steric bulk of the serine side chain. No activity was detected with either mutant in the presence of molar concentrations of sodium acetate, indicating that only small nucleophiles can access a catalytically active position in the active site when DNPG is bound.

Abg E358S proved to be an effective glycosynthase when presented with α-glucosyl or α-galactosyl fluoride donors and a variety of acceptors (Tables 1 and 2). The kinetics of glycosylation of PNPG with α-GalF using E358S or the 'original' glycosynthase, E358A, were compared using a fluoride electrode to monitor fluoride release. Because transfer to an acceptor with an axial 4-hydroxyl group is not observed with either E358S or E358A, the use of α-GalF as donor for kinetic studies ensured that the observed release of fluoride corresponded to a single galactosyl transfer, the product itself being a poor acceptor. Indeed, TLC analysis of assay mixtures indicated the formation of a single disaccharide product. Higher concentrations of PNPG at a fixed concentration of α-GalF inhibited transglycosylation by E358S (Fig. 2a), indicating that the acceptor can adopt a non-productive binding mode at high concentrations. An apparent K_m of 22 ± 5 mM and an apparent K_i of 16 ± 4 mM, respectively, were obtained from a fit of the data in Fig. 2a with Eq. 1. Considering that PNPG is an excellent substrate for the Abg wt ($k_{cat} = 169$ s^{-1}, $K_m = 78$ μM) [24], this type of inhibition is not unexpected because PNPG can be envisioned to bind readily to the −1 subsite in the mutant enzyme and prevent binding of the donor galactosyl fluoride in a productive mode. Variation of the

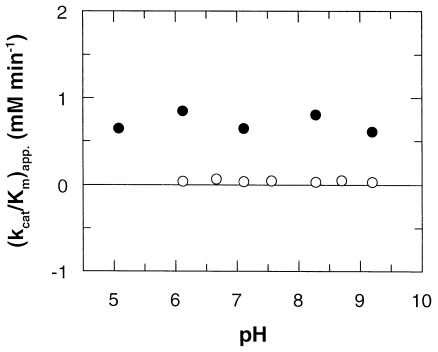


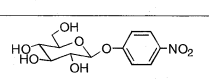
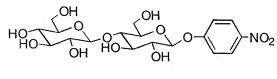
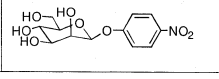
Fig. 3. The pH dependence of the apparent k_{cat}/K_m for Abg E358S (●) and Abg E358A (○) catalyzed transglycosylation of α-GalF and PNPG.

α-GalF concentration at a fixed concentration of PNPG (22 mM) afforded standard saturation kinetics for E358S (Fig. 2b) with apparent kinetic parameters of $K_m = 220 \pm 12$ mM, $k_{cat} = 177$ min^{-1} and $k_{cat}/K_m = 0.800$ min^{-1} mM^{-1}. By contrast, saturation with α-GalF ([PNPG] = 22 mM) was not observed with E358A (Fig. 2b). The data gave a value of 0.0338 min^{-1} mM^{-1} for k_{cat}/K_m, a value that is 24-fold less than the corresponding rate constant for E358S. The significantly higher activity of the Ser mutant, together with the obtaining of a measurable K_m for α-GalF with E358S but not with E358A, suggest that a specific interaction is formed between the Ser side chain and GalF, most likely through a hydrogen bond to the anomeric fluorine (Fig. 1). Although OH–fluorine hydrogen bonds are known to be weak at best in fluorocarbons, an OH–fluoride hydrogen bond will be considerably stronger [28]. Thus the hydrogen bond from serine would facilitate stabilization of the glycosylation transition state by neutralizing developing negative charge on the departing fluoride.

The pH dependence of the transglycosylation reaction was also examined for E358S and E358A (Fig. 3). No significant effect on $(k_{cat}/K_m)_{app.}$ was observed for either mutant over a range of pH 9–5. Although one might have expected a titration corresponding to the acid/base catalyst E170, this profile is analogous to the very broad pH rate profile observed for the wt Abg [24].

The synthetic utility of Abg E358S was investigated using α-GlcF or α-GalF as donors to various acceptors (Tables 1 and 2). The yields of oligosaccharides (determined by HPLC, with masses confirmed by ESI-MS) were better than reported previously for E358A [13]. An increased rate of glycosylation is expected to improve yields as a result of less of the donor glycosyl fluoride being lost to spontaneous hydrolysis. It is also significant that glycosides such as PNP-GlcNAc that are weak acceptors for E358A were glycosylated by E358S to form, in this example, *para*-nitrophenyl β-*N*-acetyllactosamine (PNP-LacNAc), a valuable precursor to various cell surface antigens, in a reasonable yield of 63% (Table 1, entry 4). Such syntheses could easily be done on a large scale since the E358S mutant can be produced in gram quantities.

Table 2
Abg E358S transglycosylation reactions using α-GlcF as donor

#	Acceptor	Yield of oligosaccharides (observed *m/z*)			Total Yield
		di-	tri-	tetra-	
1		0%	83% (648, $[M+Na]^+$)	13% (810, $[M+Na]^+$)	96%
2			88% (648, $[M+Na]^+$)	7.0% (810, $[M+Na]^+$)	95%
3		17% (486, $[M+Na]^+$)	25% (648, $[M+Na]^+$)	17% (810, $[M+Na]^+$)	59%

It has been demonstrated that the glycosylation rates of the glycosynthase Abg E358A are improved considerably by substitution of the nucleophile with serine (E358S) rather than alanine. Although this mutation reduces the free space between the anomeric carbon of a substrate and the serine hydroxyl group relative to the alanine methyl (as indicated by rescue studies with anionic nucleophiles), a favorable interaction with the anomeric fluorine of a glycosyl fluoride may occur in the ground state, and more strongly in the glycosylation transition state, resulting in enhanced catalysis. Presumably a similar mutation in other retaining glycosidases should enhance their glycosynthase activity, or even elicit such activity where the corresponding alanine mutation had failed, thereby extending the glycosynthase methodology. Likewise, as demonstrated by Abg E358S, the synthetic repertoire of existing glycosynthases may be broadened as a result of greater glycosylation activity.

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References

[1] Simon, P.M. (1996) Drug Discov. Today 1, 522–528.
[2] Sears, P. and Wong, C.H. (1996) Proc. Natl. Acad. Sci. USA 93, 12086–12093.
[3] Zopf, D. and Roth, S. (1996) Lancet 347, 1017–1021.
[4] Ichikawa, Y., Wang, R. and Wong, C.H. (1994) Methods Enzymol. 247, 107–127.
[5] Ichikawa, Y., Look, G.C. and Wong, C.H. (1992) Anal. Biochem. 202, 215–238.
[6] Witte, K., Sears, P., Martin, R. and Wong, C.H. (1997) J. Am. Chem. Soc. 119, 2114–2118.
[7] Thiem, J. (1995) FEMS Microbiol. Rev. 16, 193–211.
[8] Crout, D.H.G. and Vic, G. (1998) Curr. Opin. Chem. Biol. 2, 98–111.
[9] Wong, C.H., Halcomb, R.L., Ichikawa, Y. and Kajimoto, T. (1995) Angew. Chem. Int. Ed. Engl. 34, 521–546.
[10] Watt, G.M., Lowden, P.A. and Flitsch, S.L. (1997) Curr. Opin. Struct. Biol. 7, 652–660.
[11] Bourquelot, E. and Bridel, M.J. (1912) J. Pharm. Chim. (Paris) 7, 569–573.
[12] Prade, H., Mackenzie, L.F. and Withers, S.G. (1997) Carbohydr. Res. 305, 371–381.
[13] Mackenzie, L.F., Wang, Q., Warren, R.A.J. and Withers, S.G. (1998) J. Am. Chem. Soc. 120, 5583–5584.
[14] Tull, D., Miao, S., Withers, S.G. and Aebersold, R. (1995) Anal. Biochem. 224, 509–514.
[15] Withers, S.G. and Aebersold, R. (1995) Protein Sci. 4, 361–372.
[16] Malet, C. and Planas, A. (1998) FEBS Lett. 440, 208–212.

[17] Graham, R.W., Greenwood, J.M., Warren, R.A.J., Kilburn, D.G. and Trimbur, D.E. (1995) Gene 158, 51–54.
[18] Vocadlo, D.J., Mayer, C., He, S. and Withers, S.G. (1999) Biochemistry (in press).
[19] Smith, A.M. and Klugman, K.P. (1997) Biotechniques 22, 438–442.
[20] Lawson, S.L., Warren, R.A.J. and Withers, S.G. (1998) Biochem. J. 330, 203–209.
[21] Wang, Q., Graham, R.W., Trimbur, D., Warren, R.A.J. and Withers, S.G. (1994) J. Am. Chem. Soc. 116, 11594–11595.
[22] Leatherbarrow, R.J. (1996) GraFit 3.09b Erithacus Software Ltd.
[23] Withers, S.G., Rupitz, K., Trimbur, D. and Warren, R.A. (1992) Biochemistry 31, 9979–9985.
[24] Kempton, J.B. and Withers, S.G. (1992) Biochemistry 31, 9961–9969.
[25] MacLeod, A.M., Tull, D., Rupitz, K., Warren, R.A.J. and Withers, S.G. (1996) Biochemistry 35, 13165–13172.
[26] Moracci, M., Trincone, A., Perugino, G., Ciaramella, M. and Rossi, M. (1998) Biochemistry 37, 17262–17270.
[27] Viladot, J.-L., de Ramon, E., Durany, O. and Planas, A. (1998) Biochemistry 37, 11332–11342.
[28] Emsley, J. (1980) Chem. Soc. Rev. 9, 91–124.