Enzymatic Properties of the Cysteinesulfinic Acid Derivative of the Catalytic-Base Mutant Glu400→Cys of Glucoamylase from *Aspergillus awamori*†

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ABSTRACT: The pK_a of the catalytic base was lowered and its distance to the general acid catalyst, Glu179, was increased in the glucoamylase from *Aspergillus awamori* by replacing the catalytic base Glu400 with cysteine followed by oxidation to cysteinesulfinic acid [Fierobe, H.-P., Mirgorodskaya, E., McGuire, K. A., Roepstorff, P., Svensson, B. and Clarke, A. J. (1998) *Biochemistry 37*, 3743−3752. ^{1}H NMR spectroscopy demonstrated that the oxidized mutant Glu400→Cys-SO_2H glucoamylase, like the wild-type, catalyzed hydrolysis with inversion of the anomeric configuration of the product. Relative to the catalytic base mutant Glu400→Cys, the Cys400-SO_2H glucoamylase had 700 times higher k_{cat} toward maltose, while K_m was unchanged. Compared to wild-type glucoamylase, the Cys400-SO_2H derivative had k_{cat} values of 150−190% and 85−320% on malto- and isomaltooligosaccharides, respectively, while K_m values were similar to those of wild-type with the two disaccharides and 3.5−5.5- and 1.8−2.5-fold higher for the longer malto- and isomaltooligosaccharides substrates, respectively. The pH−activity dependence at saturating concentration of maltose indicated that the pK_a of the catalytic base Cys400-SO_2H was about 0.5 pH unit lower than that of wild-type Glu400. The K_i of Cys400-SO_2H glucoamylase for the pseudotetrasaccharide and potent inhibitor acarbose increased more than 10^4-fold, but K_i values of the mono- and disaccharide analogues 1-deoxynojirimycin and *β*-*O*-methylacarviosinide were unchanged, suggesting perturbation at binding subsites beyond the catalytic center. A distinct property of Cys400-SO_2H glucoamylase was the catalysis of the condensation of *β*-D-glucopyranosyl fluoride and subsequent hydrolysis of the product to *β*-glucose, under conditions where this was not detected for the wild-type enzyme.

Glucoamylase (GA)[1] from *Aspergillus awamori* (identical to *A. niger* GA, *1*−*3*) has been subject to extensive protein engineering (*4*). GA catalyzes the hydrolytic release of glucose from nonreducing ends of starch and related oligo- and polysaccharides with inversion of anomeric configuration, and is used in the industrial production of high-glucose syrups. The preferred GA substrates are α-1,4-linked; the α-1,6 bond is also hydrolyzed, but typically with 500-fold lower efficiency (*5*). The crystal structure has been solved of the catalytic domain of GA from *A. awamori* var. *X100* in the free state (*6*) and in complex with the inhibitor 1-deoxynojirimycin and two pseudotetrasaccharides, acarbose and D-*gluco*-dihydroacarbose (*7*−*10*). The 15 different GA sequences reported establish family 15 of glycosyl hydrolases (*11*). Recently, improved activity toward α-1,6-linked substrates and higher thermostability of *A. awamori* GA were obtained by protein engineering (*4*, *12*−*14*). To enhance the activity toward isomaltooligosaccharides, conserved α→α loops 3 and 5 in *A. awamori* GA $(\alpha/\alpha)_6$-barrel (*6*) were replaced with short homologous sequences from *Hormoconis resinae* GA (*13*), the most active GA on α-1,6-linked substrates (*15*).

A highly active GA derivative, Cys400-SO_2H GA, has been generated by specific mild oxidation of Cys400 in the catalytic base Glu400→Cys mutant of the *A. awamori* GA to the corresponding cysteinesulfinic acid (*16*). This enabled investigation of the kinetic and mechanistic consequences of both increasing the distance between the general acid Glu179 and the base catalyst at position 400 and lowering the pK_a of the catalytic base. According to a computer-generated model, the average distance between Glu179 and Glu400 in wild-type GA is 9.2 Å, which increased to around 10.4 Å in Cys400-SO_2H GA. Such a wide catalytic gap is unusual for a carbohydrase (*17*, *18*). Interestingly, the Cys400-SO_2H GA derivative has approximately 1.6-fold higher activity (k_{cat}) than wild-type toward maltose and maltoheptaose (*16*), which motivated the present thorough characterization of its catalytic properties toward malto- and

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[1] Abbreviations: GA, glucoamylase; Cys400 GA, Cys320→Ala/Glu400→Cys GA; Cys400-SO_2H GA, cysteinesulfinic acid derivative of Cys320→Ala/Glu400→Cys GA; DP, degree of polymerization; G2 through G7, maltose through maltoheptaose; iG2 through iG7, isomaltose through isomaltoheptaose (α-1,6-linked throughout).

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isomaltooligosaccharides, various disaccharides, natural polysaccharides, and nonnatural substrates such as α-D-glucopyranosyl fluoride. This analysis also included tests of potent pseudooligosaccharide inhibitors, and of the ability of the Cys400-SO_2H to catalyze the reverse condensation reaction.

MATERIALS AND METHODS

Enzymes and Reagents. Maltooligosaccharides (G2 to G7[1]), isomaltose, isomaltotriose, the glucose oxidase kit, α-D-glucopyranosyl fluoride, β-D-glucopyranosyl fluoride, 4-nitrophenyl α-D-glucoside, 4-nitrophenyl β-D-glucoside, panose, α,α-trehalose, nigerose, amylopectin (from potato), glycogen (from oyster), and soluble starch were from Sigma (St. Louis, MO); maltose monohydrate was from Merck (Darmstadt, Germany). Isomaltotetraose through isomaltoheptaose and kojibiose were generous gifts of the late Dr. B. S. Enevoldsen (Scandinavian Brewmaster School, Copenhagen). D-Glucal was a generous gift of Dr. Inge Lundt (DTU, Lyngby). Acarbose, 1-deoxynojirimycin, and β-*O*-methylacarviosinide were generous gifts of Drs. D. Schmidt and E. Truscheit, Bayer AG (Wuppertal, Germany). Purification of wild-type and Glu400→Cys/Cys320→Ala GAs (*19*) and the preparation of Cys400-SO_2H GA were done as previously described (*16*).

Enzyme Kinetics. Initial rates of hydrolysis were determined at 45 °C in 50 mM sodium acetate, pH 4.5, using up to 15 substrate concentrations, from $0.1K_m$ to $8K_m$, of maltooligosaccharides, isomaltose, and isomaltotriose. Since limited amounts of isomaltotetraose through isomaltoheptaose, nigerose, kojibiose, and α-D-glucopyranosyl fluoride were available, only eight substrate concentrations from $0.1K_m$ to $5K_m$ were used. The kinetic measurements were performed using various enzyme concentrations adjusted to the substrate in question in the range of 1nM−2.5×10^3 nM for wild-type and 1−610 nM for Cys400-SO_2H GA. The glucose released was quantified by the glucose oxidase method monitored in microtiter plates with a CERES UV900 (Bio-Tek Instruments, Inc., Winooski, VT) scanning autoreader (*20*−*22*). The enzymatic hydrolysis of the various substrates was followed from 5 to 30 min. Values of k_{cat} and K_m were determined by a direct fit to the Michaelis−Menten equation using ENZFITTER (Biosoft, Cambridge, U.K.). The change in transition-state stabilization energy, $\Delta\Delta G^{\ddagger}$, was calculated using the equation $\Delta\Delta G^{\ddagger} = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{WT}]$ (*23*). The pH−activity dependence was determined using 50 mM citrate phosphate buffer at 10 different pH values ranging from 2.3 to 7.2. Maltose was used in experiments at 0.2 mM (approximately $0.1K_m$) and 30 mM (approximately $10K_m$), and the pK_a values of the catalytic groups of the free and the substrate-complexed enzyme, respectively, were calculated from the theoretical equation $V_{max} = (V_{max})_m/\{1 + ([H^+]/K_{a1}) + (K_{a2}/[H^+])\}$ where $(V_{max})_m$ is the maximum rate at the optimum pH, V_{max} is the rate at a given pH, and K_{a1} and K_{a2} are the dissociation constants of the two ionizable groups. Calculation of the pK_a values was also performed using $P = P_{max}/\{1 + ([H^+]/K_{a1})\}$ for pH <4 and $V_{max} = (V_{max})_m/\{1 + (K_{a2}/[H^+])\}$ for pH >5.5, where P is the kinetic parameter (k_{cat} or k_{cat}/K_m) and P_{max} the kinetic parameter at optimum pH (*24*).

For α-D-glucopyranosyl fluoride, the amount of glucose liberated in the absence of enzyme by spontaneous hydrolysis at 45 °C was used to correct amounts measured in experiments with enzyme catalysis. The activity toward 4-nitrophenyl α-D-glucoside and 4-nitrophenyl β-D-glucoside was measured using total assay volumes of 100 μL and initiating the hydrolysis by addition of enzyme to a final concentration of 0.61−10 μM. Aliquots (20 μL) were removed and mixed with 0.1 M sodium borate, pH 9.4 (200 μL), in microtiter wells to quench the reaction. The absorbance was measured at 405 nm and quantitated using 4-nitrophenol as a standard.

K_i values for the competitive inhibitors acarbose, 1-deoxynojirimycin, and β-*O*-methylacarviosinide were determined with maltohexaose as substrate in the absence and in the presence of inhibitor at three different concentrations using the equation $v = [E]_0[S]k_{cat}/([S] + K_m(1 + [I]/K_i))$, where v is the initial rate of hydrolysis, $[E]_0$ is the total enzyme concentration, and [S] and [I] are the substrate and inhibitor concentration, respectively.

Concentrations of stock enzyme solutions were determined by the aid of amino acid analysis and spectrophotometrically using $\epsilon_{280} = 1.37 \times 10^5\ M^{-1}\ cm^{-1}$ (*25*).

^1H NMR Analysis. To determine the anomeric configuration of the products of wild-type and Cys400-SO_2H GAs, 1.2 nmol of enzyme was twice dissolved in 50 μL of 50 mM sodium acetate, pH 4.5 in 99% D_2O, and lyophilized. A spectrum of maltohexaose (10 mM) in 600 μL of 50 mM sodium acetate, pH 4.5 in 99% D_2O, was recorded on a 600 MHz Bruker spectrometer at 45 °C (time zero). Stock solutions of wild-type or Cys400-SO_2H GA (50 μL) were added to a final concentration of 2 μM, and spectra were recorded with 5 min intervals, until the mutarotation equilibrium was reached after about 3 h. Trace amounts of H_2O (about 1%) allowed efficient turnover.

To test for the occurrence of a condensation reaction (*26*), enzyme was lyophilized and dissolved to final concentrations of 7.3 and 3.65 μM for wild-type and Cys400-SO_2H GA, respectively, in 650 μL of 50 mM sodium acetate, pH 4.5 in D_2O (99%). At time zero, the enzyme solution was mixed with 3 mg of β-D-glucopyranosyl fluoride (final concentration 25 mM) and incubated at 45 °C for 50 min. Spectra were recorded on a 600 MHz Bruker spectrometer at 10 min intervals. As a control, spontaneous hydrolysis of β-D-glucopyranosyl fluoride was followed in parallel. In the case of Cys400-SO_2H GA, an additional experiment was performed in the presence of 1 mM acarbose.

Finally, enzymatically catalyzed hydroxylation of D-glucal was tested by using 6.1 μM wild-type or Cys400-SO_2H GA in 50 mM sodium acetate, pH 4.5 in D_2O (99%) (final volume 650 μL). At time zero, 5.3 mg of D-glucal (final concentration 55 mM) was added to the enzyme, and the mixture was incubated at 45 °C. The reaction was monitored for 2 h by recording spectra every 10 min.

RESULTS

Kinetic Parameters for Hydrolysis of Maltooligosaccharides. Values for k_{cat} and K_m of the Cys400-SO_2H derivative and the parent Cys400 GA mutant were determined for maltooligosaccharides of DP 2 to DP 7 (Table 1). Cys400 GA had a very low k_{cat} for all substrates, being reduced approximately 450-fold compared to wild-type, while the K_m of Cys400, Cys400-SO_2H, and wild-type GAs for maltose was essentially the same. K_m values for the Cys400-SO_2H

Table 1: Kinetic Parameters[a] for Hydrolysis of Maltooligosaccharides by Wild-Type, Cys400, and Cys400-SO_2H GAs

	WT			Cys400			Cys400-SO_2H		
substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
G2	6.8 ± 0.1[b]	3.0 ± 0.1	2.3 ± 0.11	0.015 ± 0.0002	2.70 ± 0.13	$0.0055 \pm 3 \times 10^{-4}$	10.4 ± 0.01	2.5 ± 0.02	4.2 ± 0.03
G3	28 ± 0.3	0.29 ± 0.016	95 ± 5.3	0.062 ± 0.008	1.1 ± 0.04	0.058 ± 0.008	42 ± 1.5	0.96 ± 0.09	44 ± 4.4
G4	45 ± 1	0.18 ± 0.013	250 ± 18	0.11 ± 0.003	0.61 ± 0.03	0.19 ± 0.01	76 ± 0.6	0.68 ± 0.02	110 ± 3.4
G5	44 ± 2	0.10 ± 0.021	440 ± 95	0.12 ± 0.004	0.49 ± 0.07	0.24 ± 0.034	83 ± 0.9	0.58 ± 0.02	140 ± 5.2
G6	49 ± 0.7	0.11 ± 0.006	450 ± 25	0.12 ± 0.001	0.51 ± 0.02	0.23 ± 0.009	89 ± 0.8	0.55 ± 0.015	160 ± 4.7
G7	54 ± 0.8	0.12 ± 0.008	440 ± 30	0.12 ± 0.002	0.45 ± 0.02	0.26 ± 0.012	86 ± 0.8	0.45 ± 0.01	190 ± 4.6

[a] Determined at 45 °C in 50 mM sodium acetate, pH 4.5. [b] Standard deviation.

derivative for the longer maltooligosaccharides (DP 3 to DP 7) were 3.5−5.5-fold higher than of wild-type GA. The catalytic efficiency (k_{cat}/K_m) of the catalytic base mutant Cys400 GA was thus reduced by 400−1900-fold.

Oxidation of Cys400 to Cys400-SO_2H restored activity. With the maltooligosaccharide substrates (DP 2 to DP 7), k_{cat} thus increased dramatically by 680−770-fold. The k_{cat} values of Cys400-SO_2H GA correspond to 150−190% of the wild-type GA values. The increase in transition-state stabilization energy $\Delta\Delta G^{\ddagger}$ was found to be 16.0 and 19.7 kJ mol^{-1} (see Materials and Methods) for maltose and maltoheptaose, respectively, which according to the litterature is in the same range as the increase associated with the loss of an important charged hydrogen bond in the transition-state complex (*27*). The Glu400→Cys mutation is likely to result in loss of an important enzyme−substrate interaction involving the γ-COOH of Glu400 (*22*). Upon oxidation to Cys400-SO_2H GA, however, the $\Delta\Delta G^{\ddagger}$ was reduced by 17.6 kJ mol^{-1} to −1.6 kJ mol^{-1} for maltose and by 17.7 kJ mol^{-1} to 2.2 kJ mol^{-1} for maltoheptaose hydrolysis, as calculated from the k_{cat}/K_m values given in Table 1, indicating that a lost enzyme−substrate interaction was either compensated or reestablished by the oxidation of Cys400.

pH−Activity Dependence. In the case of the Cys400-SO_2H GA, the pH profile of the enzyme−substrate complex differed significantly from that of wild-type GA. Whereas the pK_a values of 2.4 and 6.1 for the wild-type GA−substrate complex were similar to those of the free wild-type GA (*22*, *28*), the pK_a value of the catalytic base in Cys400-SO_2H GA was estimated using two different procedures (*24*) to be 0.5 pH unit lower than that of both the wild-type GA and the uncomplexed Cys400-SO_2H GA. The pK_a of the general acid catalyst was slightly increased in the GA derivative in the complexed state and essentially the same for free wild-type (Figure 1A,B). The pH−activity profile of the uncomplexed GA derivative was closer to that of wild-type GA, although the pK_a of the catalytic base seems slightly higher (2.6 versus 2.35 for wild-type GA); the basic limbs of profiles of GA derivatives indicated a pK_a of 6.1 for the general acid catalyst as previously reported for the wild-type GA (*22*). Cys400 GA was only investigated at high substrate concentration because of the poor activity, and as with the pH−activity dependence of the catalytic base mutant Glu400→Gln GA (*22*), no titrating group corresponding to the catalytic base, Glu400 in wild-type GA, was detected for Cys400 GA (Figure 1A).

Kinetic Parameters for Isomaltooligosaccharides. The Cys400-SO_2H GA had essentially the same value for K_m for the isomaltooligosaccharides of DP 2 through DP 7, but while the K_m for isomaltose was similar to that of wild-type GA, K_m increased 2−3-fold for the longer isomaltooligosaccharides. In contrast, the effect of the oxidation on k_{cat} with these substrates was most remarkable. Cys400-SO_2H GA thus showed 85% of the wild-type k_{cat} value toward isomaltose, but elevated k_{cat} for the higher oligosaccharides up to 320% of the wild-type value for isomaltopentaose hydrolysis (Table 2).

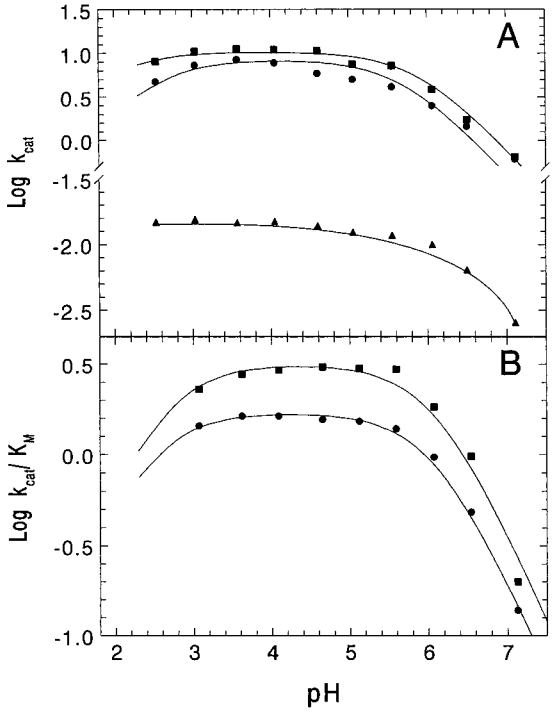

FIGURE 1: Dependence of kinetic parameters on pH for the hydrolysis of maltose by (●) wild-type, (▲) unmodified Cys400, and (■) Cys400-SO_2H. The pH−activity profiles for (A) maltose-complexed and (B) free GA derivatives were determined using 30 and 0.2 mM substrate, respectively, in 50 mM citrate phosphate buffers. The solid lines through each data set represent the theoritical curves generated using the corresponding calculated values of pK_{a1}, and pK_{a2}.

A conspicuous difference between the Cys400-SO_2H and wild-type GA was noted by the ratio $k_{cat,derivative}/k_{cat,wild-type}$, which increased significantly from 0.9 to 3.2 with the degree of polymerization of isomaltooligosaccharides, whereas for all maltooligosaccharides this ratio remained approximately 1.7. Thus, with increasing DP from 2 to 7 of isomaltooligosaccharides, k_{cat} increased 5-fold for the wild-type, but 20-fold for Cys400-SO_2H GA. The k_{cat} and K_m for the α-1,6-linked substrates were not determined for Cys400 GA because of its very poor activity.

Activity toward Disaccharides and Panose. The kinetic parameters for the Cys400-SO_2H and wild-type GAs were determined on different disaccharides (Table 3) to expand the analysis of the difference in the bond-type specificity of

Table 2: Kinetic Parameter[a] for Hydrolysis of Isomaltooligosaccharides by Wild-Type and Cys400-SO_2H GAs

	WT			Cys400-SO_2H		
substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
iG2	0.27 ± 0.009[b]	38 ± 3.0	$0.0071 \pm 6 \times 10^{-4}$	0.23 ± 0.009	32 ± 3.9	$0.0072 \pm 9 \times 10^{-4}$
iG3	0.64 ± 0.06	18 ± 1.4	0.036 ± 0.003	1.4 ± 0.064	32 ± 4.2	0.043 ± 0.006
iG4	0.99 ± 0.009	15 ± 5.3	0.069 ± 0.002	2.6 ± 0.13	36.0 ± 5.0	0.073 ± 0.011
iG5	1.2 ± 0.05	16 ± 2.0	0.077 ± 0.010	3.9 ± 0.3	38 ± 4.9	0.102 ± 0.015
iG6	1.5 ± 0.08	18 ± 2.0	0.081 ± 0.010	4.3 ± 0.21	42 ± 4.3	0.102 ± 0.012
iG7	1.3 ± 0.06	17 ± 1.7	0.077 ± 0.008	3.9 ± 0.3	37 ± 5.1	0.107 ± 0.017

[a] Determined at 45 °C in 50 mM sodium acetate, pH 4.5. [b] Standard deviation.

Table 3: Kinetic Parameters[a] for Hydrolysis of Disaccharides and Panose by Wild-Type and Cys400-SO_2H GAs

		wild-type			Cys400-SO_2H		
substrate	linkage	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
α,α-trehalose	α-1,1		ND[b]			ND[b]	
kojibiose	α-1,2	0.13 ± 0.009[c]	150 ± 11	$8.4 \times 10^{-4} \pm 8 \times 10^{-5}$	0.14 ± 0.005	120 ± 9	$1.2 \times 10^{-3} \pm 1 \times 10^{-4}$
nigerose	α-1,3	0.14 ± 0.007	32 ± 3.5	$4.4 \times 10^{-3} \pm 5 \times 10^{-4}$	0.28 ± 0.002	92 ± 9	$3.1 \times 10^{-3} \pm 4 \times 10^{-4}$
maltose[d]	α-1,4	6.8 ± 0.11	3.0 ± 0.1	2.3 ± 0.11	10.4 ± 0.01	2.56 ± 0.02	4.2 ± 0.03
isomaltose[e]	α-1,6	0.27 ± 0.009	38 ± 3	$7.1 \times 10^{-3} \pm 6 \times 10^{-4}$	0.23 ± 0.009	32 ± 4	$7.2 \times 10^{-3} \pm 9 \times 10^{-4}$
panose	α-1,6/α-1,4	6.9 ± 0.16	17 ± 1.2	0.41 ± 0.030	14 ± 0.16	42 ± 1.3	0.33 ± 0.011

[a] Determined at 45 °C in 50 mM sodium acetate, pH 4.5. [b] Not detected. [c] Standard deviation. [d] Data from Table 1. [e] Data from Table 2.

Table 4: Activity toward Natural Polysaccharides

	activity[a]	
substrate	wild-type	Cys400-SO_2H
soluble starch	42	91
amylopectin	39	89
glycogen	36	95
pullulan	1.1	1.4

[a] Determined at 45 °C in 50 mM sodium acetate, pH 4.5. Expressed in micromoles of glucose released from 1% substrate per second and per micromole of enzyme.

the GA derivative, which became apparent from the analysis of α-1,4- and α-1,6-linked oligosaccharide substrates. Except for α,α-trehalose, these disaccharides were all hydrolyzed by the wild-type enzyme, as earlier reported (*29*), and by the GA derivative. A similar relationship between k_{cat} values was obtained for the GA derivative, as found with the malto- and isomaltooligosaccharides. Thus, toward the α-1,2-linked kojibiose, K_m values for the GA derivative and wild-type were similar, and the ratio $k_{cat,derivative}/k_{cat,wild-type}$ was intermediary to the ratio for maltose and isomaltose. A different effect was seen with the α-1,3-linked nigerose for which k_{cat} was doubled and K_m was 3-fold higher for Cys400-SO_2H than for wild-type GA (Table 3). Nigerose was the only disaccharide for which the K_m values of wild-type and the GA derivative differed significantly, but Cys400-SO_2H GA with the trisaccharide panose (α-1,6-glucosyl maltose), as with maltotriose and isomaltotriose, had a 2−3-fold increase of K_m, and a 2-fold increase in k_{cat} (Table 3).

Activity on Polysaccharides. Consistently, more than 2-fold increases in activity were found for the Cys400-SO_2H GA derivative compared to wild-type acting on natural α-1,4-linked polysaccharide substrates with various contents of α-1,6 linkages (Table 4). At saturating substrate concentration, the rate of hydrolysis by the derivative and wild-type GA, however, was not influenced by modest structural differences between the polysaccharide substrates. Although the activity was low on pullulan (Table 4), Cys400-SO_2H GA still displayed enhanced activity compared to wild-type GA.

Kinetic Parameters on Fluoro and Aryl Glucosides. The impact of the aglycon leaving group ability on catalysis was investigated by using α-D-glucopyranosyl fluoride and 4-nitrophenyl α-D-glucoside (Table 5). The k_{cat} for wild-type GA toward α-D-glucopyranosyl fluoride was very high, as previously reported (*30*, *31*), and even 3 times higher for the GA derivative compared to wild-type (Table 5). This level of k_{cat} of Cys400-SO_2H GA was thus 12-fold higher than that of the longest maltooligosaccharide (see Table 1, maltoheptaose); in comparison, the k_{cat} was only 5.5 times higher than on maltoheptose in the case of wild-type GA. A similar trend was apparent toward 4-nitrophenyl α-D-glucoside, despite the much lower k_{cat} value. The Cys400-SO_2H GA derivative apparently responded more favorably to substrates with good leaving groups than wild-type GA, as found earlier with Trp120→Phe and Tyr116→Ala GA acting on α-D-glucopyranosyl fluoride (*31*).

^1H NMR Spectrometry. The stereochemical course of the reaction catalyzed by the Cys400-SO_2H GA derivative was determined by aid of ^1H NMR spectroscopy using maltohexaose as substrate. Upon enzyme addition, the doublet centered at 4.65 ppm, which corresponds to the anomeric proton of β-glucose, increased, clearly indicating that the hydrolysis of the glucosidic bond occurred with inversion of the anomeric configuration, as in the case of wild-type GA (spectra not shown).

Action on β-D-Glucopyranosyl Fluoride. Glucoamylase is reported to catalyze the condensation of β-D-glucopyranosyl fluoride to β-D-maltosyl fluoride which is subsequently hydrolyzed to yield β-D-glucose (*26*). Even though this condensation activity is low and requires high enzyme concentration for the product to be detected (25 μM was used in ref *26*), we investigated the ability of the Cys400-SO_2H GA to catalyze the condensation by monitoring glucose released by hydrolysis of the condensation product β-glucopyranosyl fluoride using the glucose oxidase method. At an enzyme concentration of 6 μM, Cys400-SO_2H GA seemed

Table 5: Kinetic Parameters[a] for the Hydrolysis of α-D-Glucopyranosyl Fluoride and 4-Nitrophenyl α-D-Glucoside by Wild-Type and Cys400-SO_2H GAs

	α-D-glucopyranosyl fluoride			4-nitrophenyl α-D-glucoside		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
wild-type	300 ± 17[b]	70 ± 8	4.2 ± 0.5	0.25 ± 0.010	3.4 ± 0.40	0.073 ± 0.010
Cys400-SO_2H	970 ± 13	110 ± 13	8.9 ± 1.0	0.73 ± 0.030	4.0 ± 0.41	0.18 ± 0.020

[a] Determined at 45 °C in 50 mM sodium acetate, pH 4.5. [b] Standard deviation.

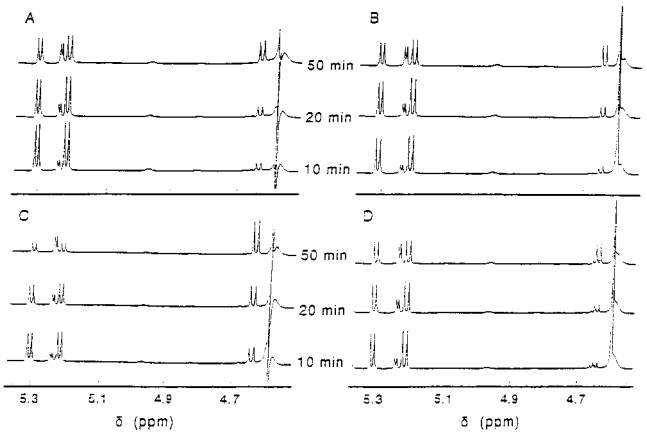

FIGURE 2: Determination of the sterochemical course of hydrolysis of β-D-glucopyranosyl fluoride, in the absence of enzyme (A) or in the presence of wild-type (B), Cys400-SO_2H (C), and Cys400-SO_2H GAs + 1 mM acarbose (D).

to catalyze glucose formation; however, because of a fast spontaneous hydrolysis of the substrate under the conditions used, the method was not appropriate for quantification of this activity. ^1H NMR spectrometric analysis was used therefore to follow and distinguish between glucose produced by spontaneous hydrolysis and glucose generated by the action of the enzyme (Figure 2). The quadruplet centered at 5.24 ppm was attributed to the anomeric proton of β-D-glucopyranosyl fluoride (*26*), and the doublets centered at 5.23 and 4.65 ppm to H-1 of α- and β-glucose, respectively (Figure 2A). The sample was allowed to equilibrate at 45 °C for 10 min before recording the first spectrum. At 20 min, the doublet corresponding to α-glucose had increased, whereas the doublet assigned to the β-glucose anomeric proton remained constant and began to increase only after 50 min because of the mutarotation equilibrium. Therefore, the spontaneous hydrolysis of β-D-glucopyranosyl fluoride led to formation of α-glucose.

Wild-type GA (7.3 μM) gave rise to a very similar set of spectra (Figure 2B), α-glucose being produced first and at a rate comparable to spontaneous hydrolysis. Thus, at the concentration of wild-type GA used, evidence for condensation activity was not obtained. In contrast, Cys400-SO_2H GA (3.65 μM) led to rapid production of the β-glucose (Figure 2C); the consumption of β-D-glucopyranosyl fluoride was indeed fast as indicated by the rapid decrease of the quadruplet at 5.24 ppm assigned to the anomeric proton of β-D-glucopyranosyl fluoride. To exclude the possibility that the activity was due to contaminating enzyme, e.g., a β-glucosidase, the experiment was performed in the presence of 1 mM acarbose of which the K_i for Cys400-SO_2H GA is 3.9×10^{-8} M (see Table 6). In that experiment, no initial production of β-glucose was observed (Figure 2D), and the spectra of the time progress closely resembled those obtained by spontaneous hydrolysis (Figure 2A). It was concluded, therefore, that the fast production of β-glucose observed with Cys400-SO_2H GA stemmed from the enzymatic reaction. From the spectra recorded, a catalytic velocity of 7 s^{-1} ± 1 was calculated. To exclude the highly unlikely possibility that glucose was produced directly by catalysis of the hydrolysis of β-D-glucopyranosyl fluoride involving a retaining mechanism, 4-nitrophenyl β-D-glucoside (15 mM) was incubated with Cys400-SO_2H GA (10 μM) at 45 °C and was followed for 2 h without any detectable hydrolysis.

Table 6: K_i[a] for Various Inhibitors of Wild-Type and Cys400-SO_2H GAs

	K_i (M)	
inhibitor	wild-type	Cys400-SO_2H
1-deoxynojirimycin	4.6×10^{-5}	1.8×10^{-5}
β-*O*-methylacarviosinide	9.8×10^{-8}	1.5×10^{-6}
acarbose	10^{-12} [b]	3.9×10^{-8}

[a] See Materials and Methods. [b] Data from *35*.

Hydration of D-*Glucal*. The mechanism of retaining α- and β-glycosyl hydrolases has been investigated using D-glucal (*32*). With inverting enzymes, hydration of the C-1−C-2 unsaturated bond in D-glucal was either not observed or happened at extremely high enzyme concentration (*33*, *34*), contrary to retaining enzymes. Since the Cys400-SO_2H GA shows distinct differences in enzymatic properties compared to wild-type GA, its possible capacity to catalyze the hydration of D-glucal was investigated by using ^1H NMR (see Materials and Methods). As reported for wild-type GA, however, no hydration of D-glucal was detected in the presence of Cys400-SO_2H GA (data not shown) under the conditions used.

Affinity for Inhibitors. The effect of three inhibitors, 1-deoxynojirimycin, β-*O*-methylacarviosinide, and acarbose (Figure 3), was investigated using maltohexaose as substrate. The three compounds were found to be competitive inhibitors of both Cys400-SO_2H and wild-type GA. However, while K_i (Table 6) of 1-deoxynojirimycin was slightly lower for the derivative compared to wild-type GA, K_i increased 10-fold for methyl-β-*O*-acarviosinide, and (4×10^4)fold, from 10^{-12} M (*35*−*37*) to 4×10^{-8} M, for acarbose. This decrease in affinity of acarbose conveniently enabled determination of K_i by kinetic analysis of the inhibition of maltohexaose hydrolysis, whereas the high affinity of wild-type GA was measured earlier by an inhibitor−displacement titration calorimetric analysis (*36*).

DISCUSSION

Despite the presumed increased distance between the two catalytic acid groups Glu179 and the Cys400-SO_2H, the Cys400-SO_2H GA derivative (*16*) exhibited excellent activity. This suggested that the position of the catalytic base in GA is highly flexible. The present detailed characterization

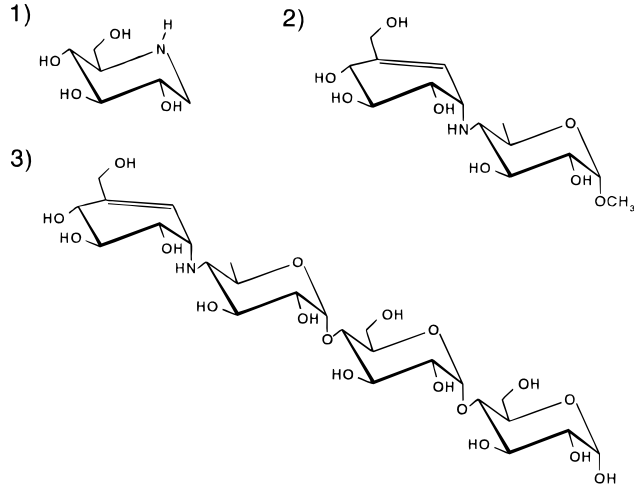

FIGURE 3: Structure of 1) 1-deoxynojirimycin, 2) *β*-*O*-methylacarviosinide, and 3) acarbose.

of the enzymatic properties of Cys400-SO_2H GA shows, however, that dramatic changes occurred not only relative to the Cys400 GA mutant but also when compared to the parent wild-type *A. awamori* GA. Most intriguing was the substantial improvement of the catalytic activity of the Cys400 GA cysteinesulfinic acid derivative, involving both hydrolysis and condensation reactions. This probably is a consequence of Cys-SO_2H being a stronger acid than the *γ*-COOH group of Glu400; the pK_a of the SO_2H of cysteinesulfinic acid is reported to be 1.50 (*38*).

Enhancement of the stabilization of oxocarbenium ion transition states is compatible with engineering to strengthen the catalytic base anion acting in water activation for nucleophilic attack at C-1. This is borne out in the present study where increases in k_{cat} were observed with the increase in the acidity of the cysteinesulfinic acid side chain at position 400 of GA, regardless of both the type of glucosidic linkage and the size of the substrate. Thus, up to 3-fold increase in k_{cat} was observed with Cys400-SO_2H compared to wild-type GA. This increased activity (k_{cat}) is reflected in the catalytic efficiency (k_{cat}/K_m) of Cys400-SO_2H GA acting on maltose, but not with the longer maltooligosaccharides for which the 2−3-fold loss in catalytic efficiency clearly is entirely a consequence of reduced affinity. The similarly increased K_m values of the enzyme derivative for isomaltooligosaccharides, panose, and the disaccharides kojibiose and nigerose were in the same range as for wild-type GA, but they were essentially compensated by increased k_{cat} values to maintain the catalytic efficiency. The increases in K_m also found for Cys400 GA suggested that the mutation at position 400 caused the loss of an interaction at the third subsite, which was not restored in the Cys400-SO_2H derivative. That significant perturbation of subsite −1 did not occur is indicated by the very minor decrease in K_i obtained for inhibition of Cys400-SO_2H with 1-deoxynojirimycin (Table 6). In fact, certain mutations of glucoamylase do lead to significant loss of affinity for this inhibitor (*37*).

In the absence of a three-dimensional structure of Cys400-SO_2H GA, it is not possible to fully interpret the important increase in k_{cat}. Nevertheless, it is known from the crystal structures of wild-type GA (*6*) and its complexes with 1-deoxynojirimycin (*7*), acarbose (*8*), and D-*gluco*-dihydroacarbose (*10*) that the catalytic base Glu400 and its bound water, Wat500, are involved in an extensive hydrogen-bonding network that extends through the innermost binding site pocket to subsite +2. The postulated alteration of the side-chain length at residue 400 will likely perturb this network, which involves Tyr48, Asp55, Gln401, Arg305, Asp309, Ser310, and Tyr311, and conceivably alters the binding properties of the component subsites. However, in view of the K_m obtained for all substrates of DP ≥3, it clearly appears that the affinity at subsite +2 has been slightly altered. Whereas the affinity of Cys400-SO_2H GA for the monomeric inhibitor 1-deoxynojirimycin increased marginally compared to that of wild-type GA, a decrease in affinity was observed for the potent pseudotetrasaccharide acarbose. Alteration of subsite +2 may again account for some of this loss in binding affinity for acarbose, but a change at subsite −1 may also contribute to the weakened association of the inhibitory pseudooligosaccharide. Despite large differences in affinity, acarbose (K_d, 10^{-12} M) and D-*gluco*-dihydroacarbose (K_d, 3×10^{-7} M) (*35*, *36*) bind almost identically to subsites −1 and +1 of GA (e.g., root-mean-square deviation of all carbon and oxygen atoms of residue a or the first ring of acarbose, i.e., at subsite −1, is 0.1 Å; *9*). The difference in affinity was ascribed by these authors, in part, to a perturbation of Wat500 by 0.2 Å from its site of optimal hydrogen bonding, as reflected in an increase of the thermal parameter of Wat500 by 7.9 Å^2. Hence, it is not too surprising that replacement of Glu400 with Cys400-SO_2H imparted significant changes to the binding of the pseudotetrasaccharide. Moreover, if this slight shift of the water at the catalytic site induces such drastic changes in the affinity of GA for two very similar inhibitors, it follows that a structural change to residue 400, which has to retain contact to Wat500 for catalytic activity, would alter the affinity of the different GA derivatives for the various substrates. This situation would be further exaggerated for nigerose (D-glucopyranosyl-α-1,3-D-glucopyranose) in view of its structure, and hence would account for the increase in K_m of the GA derivative for this substrate.

In addition to the increase in k_{cat} on maltooligosaccharides, of particular note, the GA derivative appeared to be more sensitive to the aglycon leaving group ability than wild-type GA (Table 6). Even though the overall mechanism of action (i.e., inverting) has not changed, this suggests that intermediate steps might differ significantly in structure and/or reactivity as compared to wild-type GA catalysis. Another difference in the enzymatic properties is the ability of Cys400-SO_2H GA to catalyze a condensation reaction using *β*-D-glucopyranosyl fluoride as substrate. Based on the reports of studies with other GAs (*26*), it is assumed that *A. awamori* wild-type GA could catalyze condensation reactions. In the current study, Cys400-SO_2H GA clearly catalyzes condensation; however, at the same concentrations of substrate and enzyme, wild-type GA did not. This suggested that catalysis of the reverse reaction of hydrolysis was enhanced by increasing the acidity of the catalytic base acid group at position 400.

These interesting and potentially useful properties described above have not been observed previously among the many GA mutants that we and others have generated. For example, replacement of seven residues in *A. awamori* GA to mimic the active site α→α loops 3 and 5 of *Hormoconis resinae* GA, the most active GA on α-1,6-linked substrates,

improved the k_{cat} for isomaltose by 1.8-fold (*13*), whereas Cys400-SO_2H GA is characterized by approximately 3-fold increase of k_{cat} for isomaltooligosaccharides and in addition has significantly enhanced activity toward maltooligosaccharides. The approach taken in the present study to introduce a sulfinic acid group at the position of the catalytic base has thus proven to be a useful tool for the engineering of a new GA derivative with enhanced properties (*16*).

The high activity obtained in the Cys400-SO_2H derivative seems to reflect that the geometry of the catalytic site of GA is quite flexible, even though perturbation of hydrogen bonds to the general acid catalyst leads to a great decrease in activity (*39*, *40*). This is in contrast to the strong demand for accurate positioning of a catalytic nucleophile in the retaining enzymes (*41*, and references cited therein).

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