Catalytic Mechanism of Glucoamylase Probed by Mutagenesis in Conjunction with Hydrolysis of α-D-Glucopyranosyl Fluoride and Maltooligosaccharides†

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ABSTRACT: The catalytic mechanism of glucoamylase (GA) is investigated by comparing kinetic results obtained using α-D-glucosyl fluoride (GF) and maltooligosaccharides as substrates for wild-type and four active site mutant GAs, Tyr116→Ala, Trp120→Phe, Asp176→Asn, and Glu400→Gln. These replacements decreased the activity (k_{cat}/K_M) toward maltose by 6−320-fold. Toward GF, however, Tyr116→Ala and Trp120→Phe GAs, showed wild-type and twice wild-type level activity, while Asp176→Asn and Glu400→Gln GAs had 22- and 665-fold lower activity, respectively. Glu400, the catalytic base, is suggested to strengthen ground-state binding in subsite 1, and Asp176 does so at subsites 1 and 2. Tyr116 and Trp120 belong to an aromatic cluster that is slightly removed from the catalytic site and not critical for GF hydrolysis, but which is probably involved in maltooligosaccharide transition-state stabilization. Since the mutation of groups near the catalytic site decreased activity for both GF and maltose, but substitution of Tyr116 and Trp120 decreased activity only for maltose, interaction with the substrate aglycon part may be implicated in the rate-limiting step. Rate-limiting aglycon product release was suggested previously for GA-catalyzed hydrolysis [Kitahata, S., Brewer, C. F., Genghof, D. S., Sawai, T., & Hehre, E. H. (1981) *J. Biol. Chem. 256,* 6017−6026]. For Glu400→Gln and wild-type GA complexed with GF, the pH−activity (k_{cat}) profile shows a pK_a of 2.8. When these two enzymes were complexed with maltose, however, only wild-type GA had a titrating base group, assigned to Glu400 [Frandsen, T. P., Dupont, C., Lehmbeck, J., Stoffer, B., Sierks, M. R., Honzatko, R. B., & Svensson, B. (1994) *Biochemistry 33*, 13808−13816]. Thus, GF binding to Glu400→Gln GA presumably elicits the deprotonation of a carboxyl group that facilitates catalysis.

Site-directed mutagenesis has been very useful in providing insight into structure−function relationships in glycosylases [for reviews, see Sinnott (1990), Svensson and Søgaard (1993), McCarter and Withers (1994), and Svensson (1994)]. Fluorinated glycosides have served to help researchers gain information regarding the catalytic mechanisms (Kitahata et al., 1981; Street et al., 1986; Sinnott, 1990; Konstantinidis & Sinnott, 1991; Withers et al., 1992; Matsui et al., 1993; Tanaka et al., 1994). To explore the role in the mechanism of selected side chains at the active site, the present work addresses the hydrolysis of α-D-glucosyl fluoride (GF)[1] and the maltooligosaccharides maltose through maltoheptaose by using wild type and four mutants of glucoamylase (GA, 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3).

GA catalyzes the release of β-D-glucose from the nonreducing ends of starch and related saccharides (Weill et al., 1954; Hiromi et al., 1983) and is important in the industrial production of glucose syrups. Kinetic analysis indicates that the active site of GA contains seven consecutive subsites, each accommodating a substrate glucosyl residue, with the catalytic site located between subsites 1 and 2 (Hiromi et al., 1983; Meagher et al., 1989; Sierks et al., 1989). Both α-1,4- and α-1,6-glucosidic bonds are attacked by GA at the same catalytic site, although the α-1,4-linkage is much preferred (Hiromi et al., 1966b). GA hydrolyzes GF extremely quickly, but has poor affinity for this unnatural substrate (Konstantinidis & Sinnott, 1991). The transition state is proposed to resemble gluconolactone at subsite 1 (Laszlo et al., 1978; Hiromi et al., 1983), and the α-secondary isotope effect for GA-catalyzed hydrolysis (Firsov, 1978; Matsui et al., 1989; Tanaka et al., 1994) suggests that the glucopyranosyl intermediate has substantial oxocarbonium ion character and is well separated from the incoming nucleophile and the leaving group, which is consistent with an S_N1 rather than an S_N2 mechanism (Koshland, 1953). Different GAs utilize a common mechanism (Savel'ev & Firsov, 1982; Hiromi et al, 1983; Meagher et al., 1989; Sierks et al., 1989; Ohnishi et al., 1990), as reflected by the presence of 6 short stretches of high similarity in the 16 known GA sequences (Itoh et al., 1987; Coutinho & Reilly, 1994; Henrissat et al., 1994). These conserved regions make up interhelix segments of the characteristic $(\alpha/\alpha)_6$-fold and form the substrate binding and catalytic areas seen in structural models of GA−inhibitor complexes (Aleshin et al., 1992, 1994; Harris et al., 1993; Stoffer et al., 1995).

Glu179 and Glu400 in *Aspergillus niger* GA have been identified as general acid and base catalysts (Sierks et al., 1990; Svensson et al., 1990; Harris et al., 1993; Aleshin et al., 1994; Frandsen et al., 1993, 1994) that donate a hydrogen to the oxygen of the scissile bond or promote the nucleophilic

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[1] Abbreviations: GA, glucoamylase; GF, α-D-glucosyl fluoride.

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attack of water on C-1 of the substrate intermediate, respectively. The backbone NH groups of Glu179 and Glu180 are hydrogen bonded with Asp176 (Harris et al., 1993), the mutation of which to Asn influences the catalytic properties (Sierks et al., 1990; Olsen, 1994). The functional roles of Glu400 and Asp176 are investigated further in the present work.

Pre-steady-state kinetic studies using fluorescence spectroscopy showed that the GA mechanism includes very fast initial maltooligosaccharide binding and a subsequent slower step, presumably involving a unimolecular rearrangement of the initial enzyme−substrate complex, followed by hydrolysis and product release (Hiromi et al., 1983; Olsen et al., 1992). Several tryptophans are important in *A. niger* GA activity, in particular the conserved Trp120, which is located near subsites 3 and 4 (Clarke & Svensson, 1984b; Aleshin et al., 1994; Stoffer et al., 1995) and is associated with the conformational change of the initial binding complex (Svensson & Sierks, 1992; Olsen et al., 1993) required for transition-state stabilization (Sierks et al., 1989). A short sequence around Trp120 is recognized in other amylolytic enzymes, suggesting that this region is important in starch degradation (Svensson, 1988). To further characterize the function of this motif, the effects of two mutations at conserved residues, Tyr116→Ala and Trp120→Phe GAs, are examined using GF and maltooligosaccharides as substrates.

MATERIALS AND METHODS

Mutant Preparation. The Tyr116→Ala GA gene was constructed by using the mutagenic primer, 5′-CAGATGCGGCCGCCCCCCAAGAACCAGT**AGC**GGCAGTCTCCCGTC-3′ (underlined nucleotides represent the mutated codon; replacements are in boldface type). The mutation was verified by DNA sequencing of the subcloned region. Cassette mutagenesis, plasmid purification, subcloning, sequencing, yeast transformation, fermentation, and protein purification were performed as described previously (Sierks et al., 1989). In the *Saccharomyces cerevisiae* expression system only G1, the full-length 616-residue form (Svensson et al., 1983) of *A. niger* GA, is obtained. The Trp120→Phe (Sierks et al., 1989), Asp176→Asn (Sierks et al., 1990), and Glu400→Gln GAs (Frandsen et al., 1994) were produced in either yeast or *A. niger*, as described previously.

Enzyme Assays. α-D-Glucosyl fluoride (GF) was a generous gift of Professor C. Pedersen (Lyngby); maltose was from Merck; and maltooligosaccharides and isomaltose were from Sigma. Values of k_{cat} and K_M for substrate hydrolysis were obtained by plotting initial rates (v) vs v/[S] to the Eadie−Hofstee equation using the software Enzfitter (Elsevier-BIOSOFT, Amsterdam). Assays were conducted essentially as described previously (Sierks et al., 1989). The hydrolysis of GF in 0.05 M sodium acetate (pH 4.5) at 45 °C was measured by using two concentrations (1.5 and 3.0 mM) sufficiently low to ensure that the rate was proportional to k_{cat}/K_M. The activity was determined at different pH values for Glu400→Gln and wild-type GAs, essentially as described previously (Sierks et al., 1990), at 288 mM and 1 mM GF to obtain values representing k_{cat} and k_{cat}/K_M, respectively. The average value of two trials (typically within 10% of the two values) at each concentration was used. The concentration ranges of wild-type, Tyr116→Ala, and Trp120→Phe GAs used were 0.008−0.150 μM and those of Asp176→Asn and Glu400→Gln GAs were 0.012−0.950 μM. For substrate-complexed enzyme, a graphical procedure (Cornish-Bowden & Wharton, 1988) was used to determine the pK_a's of catalytic groups, since the limited data at high GF concentrations excluded accurate fitting by nonlinear regression. pK_a values for the uncomplexed enzyme were fitted by nonlinear regression of a plot of $\log(k_{cat}/K_M)$ vs pH using the following equation (Dixon & Webb, 1979):

$$\log(k_{cat}/K_M) = \log(k_{cat}/K_M)_o - \log(1 + [H^+]/(K_{E1} + K_{E2}/[H^+]))$$

Rates of spontaneous hydrolysis of GF at various pH values were measured and confirmed to be negligible (Konstantinidis & Sinnott, 1991).

Kinetic parameters, k_{cat} and K_M, for maltooligosaccharides were determined using at least seven substrate concentrations ranging from $^1/_8K_M$ to $8K_M$. In the case of Tyr116→Ala GA, due to the extremely low K_M, maltotriose concentrations were (0.2−20) K_M. The concentration of Tyr116→Ala GA used varied from 10^{-3} to 12 μM.

GA concentrations were measured spectrophotometrically at 280 nM using $\epsilon_m = 1.37 \times 10^5$ M^{-1} cm^{-1} (Clarke & Svensson, 1984a) and $\epsilon_m = 1.30 \times 10^5$ M^{-1} cm^{-1} for Trp120→Phe GA. The G1 form was used throughout. G1 wild-type GA has been purified from a commercial preparation of AMG 200L (Novo Nordisk, Bagsvaerd, Denmark) from *A. niger* (Svensson et al., 1982; Stoffer et al., 1993).

RESULTS AND DISCUSSION

Preparation of Tyr116→Ala GA. Tyr116 of *A. niger* GA is conserved, being either Tyr or Phe in the 16 known GA sequences (Itoh et al., 1987; Coutinho & Reilly, 1994). Tyr116 furthermore has been recognized in Taka-amylase A, cyclodextrin glucanotransferases, and different amylolytic enzymes by sequence alignment (Svensson, 1988; MacGregor & Svensson, 1989; Jespersen et al., 1993). In Taka-amylase A, the corresponding residue was indicated to be involved in sugar binding in a distant subsite (Matsuura et al., 1984). Ala was chosen as a nonaromatic replacement to study the role of Tyr116 in GA activity. The Tyr116→Ala GA was produced in yeast and purified from the culture supernatant by affinity chromatography on acarbose−Sepharose (Clarke & Svensson, 1984b) essentially as described previously (Sierks et al., 1989).

Maltooligosaccharide Hydrolysis. The mutation of Tyr116 decreases k_{cat} for maltose hydrolysis by about 15-fold and K_M by about 2.5-fold compared to wild-type GA, while k_{cat} for isomaltose decreases by only 3.7-fold with K_M remaining essentially unchanged (Table 1). In contrast, the previously described substitution of the conserved Trp120 from the same structural element (Svensson, 1988) by Tyr reduced the k_{cat} value for both maltose and isomaltose by about 100-fold (Sierks et al., 1989).

Besides the distinctly different effects toward the α-1,4- and α-1,6-linked disaccharide substrates, Tyr116→Ala GA displayed an unusual pattern in k_{cat} and K_M for the hydrolysis of maltooligosaccharides (Table 2). K_M decreased by around 20-fold for maltotriose compared to maltose, while k_{cat} decreased slightly. Surprisingly, K_M for maltotriose is still 10-fold lower than that for the longer substrates, which normally bind even better (Hiromi et al., 1983; Sierks et al.,

Table 1: Kinetic Parameters for Hydrolysis of Maltose and Isomaltose by Wild-Type and Mutant GAs

	maltose			isomaltose		
enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	KM (mM)	k_{cat}/K_M($s^{-1}mM^{-1}$)
Tyr116→Ala	0.62 ± 0.02	0.57 ± 0.05	1.09	0.111 ± 0.005	23.2 ± 3.0	0.00476
Trp120→Phe	0.178 ± 0.012	0.909 ± 0.147	0.195	nd	nd	nd
Asp176→Asn	0.728 ± 0.029	6.22 ± 0.62	0.117	0.052 ± 0.005	135 ± 25	0.000385
Glu400→Gln	0.299 ± 0.011	14.8 ± 0.9	0.020	nd	nd	nd
wild type[a]	9.2 ± 0.4	1.4 ± 0.2	6.4	0.41 ± 0.01	19.8 ± 1.2	0.0207

[a] Data from Sierks and Svensson (1993).

Table 2: Kinetic Parameters for Maltooligosaccharide Hydrolysis by Wild-Type and Mutant GAs at pH 4.5 and 45 °C

	enzyme							
	Tyr116→Ala		Trp120→Tyr[a]		Glu400→Gln[b]		wild type[b]	
substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}(s^{-1})	K_M(mM)
maltose	0.62 ± 0.02	0.57 ± 0.05	0.118 ± 0.006	0.634 ± 0.095	0.299 ± 0.011	14.8 ± 0.9	10.7 ± 0.6	1.21 ± 0.14
maltotriose	0.53 ± 0.02	0.028 ± 0.004	0.256 ± 0.009	0.121 ± 0.013	0.561 ± 0.001	1.27 ± 0.015	33.5 ± 0.7	0.28 ± 0.04
maltotetrose	16.6 ± 1.1	0.51 ± 0.09	0.568 ± 0.017	0.132 ± 0.009	0.923 ± 0.021	0.512 ± 0.032	41.3 ± 1.4	0.12 ± 0.01
maltopentose	19.1 ± 0.5	0.24 ± 0.02	0.534 ± 0.30	0.0748 ± 0.0111	1.01 ± 0.06	0.443 ± 0.028	48.04 ± 3.1	0.11 ± 0.01
maltohexose	22.2 ± 0.7	0.26 ± 003	0.466 ± 0.020	0.0742 ± 0.0071	1.15 ± 0.03	0.445 ± 0.021	56.2 ± 5.0	0.11 ± 0.01
maltoheptose	23.6 ± 1.2	0.29 ± 0.01	0.440 ± 0.016	0.059 ± 0.0077	1.05 ± 0.31	0.380 ± 0.043	59.7 ± 1.6	0.12 ± 0.01

[a] From Sierks et al. (1989); at 50 °C. [b] From Frandsen et al. (1994).

Table 3: Second-Order Rate Constant for Hydrolysis of α-D-Glucosyl Fluoride by Wild-Type and Mutant GAs

	k_{cat}/K_M (s^{-1} mM^{-1})	
enzyme	GF	maltose
wild type	13.3 ± 2.0	6.3 ± 0.3
Tyr116→Ala	13.8 ± 0.9	0.93 ± 0.10
Trp120→Phe	24.4 ± 1.0	0.25 ± 0.05
Asp176→Asn	0.60 ± 0.04	0.12 ± 0.02
Glu400→Gln	0.020 ± 0.002	0.015 ± 0.001

1989; Frandsen et al., 1994). The k_{cat} and K_m values for maltotetrose and longer substrates, in sharp contrast to maltose and maltotriose values, are similar to wild-type values (Table 2). Due to this irregular pattern in the kinetics for oligosaccharide hydrolysis, a classic GA subsite map of individual binding affinities (Hiromi et al., 1970, 1983) cannot be calculated for Tyr116→Ala GA.

Tyr116→Ala GA seems to adopt a special mechanism for maltose and maltotriose hydrolysis. While short substrates form tight complexes that turn over slowly, longer ones have decreasing affinity and are more readily hydrolyzed by Tyr116→Ala GA. Tyr116 has no direct contact with substrate (Aleshin et al., 1994), but is part of an aromatic cluster, including Tyr50, Trp52, and Trp120, critical for transition-state stabilization of short substrates, but less so for longer substrates. Two disparate binding modes with GA involving the aromatic cluster are demonstrated for glucose rings three and four at the reducing end in two pseudotetrasaccharide inhibitors acarbose and D-*gluco*-dihydroacarbose (Stoffer et al., 1995). One of these may reflect a nonproductive complex favored in the Tyr116→Ala mutation for hydrolysis of short substrates, but not for longer ones.

Hydrolysis of GF. For wild-type GA, the activity (k_{cat}/K_M) toward the structurally simple substrate GF is comparable to that for maltose (Table 3), which, according to an earlier work, was found to involve around an 80-fold increase in k_{cat} and a 30-fold increase in K_M (Konstantinidis & Sinnott, 1991). Here only k_{cat}/K_M values, not the individual parameters, were determined, since GF has very poor affinity for GA (K_M = 34 mM; Konstantinidis & Sinnott, 1991) and was available in limited amounts. In contrast to results obtained with GA, remarkably smaller differences in k_{cat} were found for the hydrolysis of GF than in the natural substrates with other inverting enzymes: glucodextranase (1.5-fold decrease; Kitahata et al., 1981; Matsui et al., 1989), trehalase (2-fold increase; Hehre et al., 1982; Matsui et al., 1993), or glycogen debranching enzyme (6-fold increase; Liu et al., 1991).

Compared to wild-type GA, Asp176→Asn GA displays 22-fold and Glu400→Gln GA displays 665-fold lower activities (k_{cat} /K_M) in GF hydrolysis. These two mutants had similarly decreased activities toward maltose of around 2% and 0.2%, respectively (Table 3), and presumably used the same catalytic mechanism on the two substrates. In contrast, Tyr116→Ala and Trp120→Phe GAs show very substantial differences in retained activity toward GF and maltose. Tyr116→Ala GA thus has wild-type activity for GF, but only 15% activity for maltose (Table 3), while Trp120→Phe GA has 2-fold wild-type activity toward GF and just 4% activity toward maltose. Substitution of Tyr116 or Trp120 slightly enhances the affinity for maltooligosaccharides except for maltotriose, which has substantially increased affinity toward the Tyr116→Ala GA. These increased affinities, however, are dominated by greater decreases in the catalytic activity (Tables 1 and 2). These residues therefore play an important role in the transition-state stabilization of maltooligosaccharide substrates.

The two mutants Tyr116→Ala and Trp120→Phe GAs provide new insight into the GA mechanism. The activity of Tyr116→Ala and Trp120→Phe GAs toward GF (Table 3) corresponds to around 15- and 100-fold increases relative to maltose, which can be attributed primarily to elevated k_{cat} rather than to the reduced K_M evident in the enhanced affinity for maltose for these mutants and the very high K_M value of 34 mM for GF of wild-type GA (Konstantinidis & Sinnott, 1991). The decrease in activity for maltose hydrolysis seen for Tyr116→Ala and Trp120→Phe GAs thus can be attributed to the substrate glucose ring accommodated at subsite 2.

The side chains of Tyr116 and Trp120 are indirectly and directly, respectively, in contact with subsites 2−4 via the aromatic cluster containing Tyr50, Trp52, Tyr116, and Trp120 stacking onto Lys108 (Aleshin et al., 1994; Svensson et al., 1994; Stoffer et al., 1995). Trp120 NE1 hydrogen bonds with OE2 of Glu179 (Aleshin et al., 1992; Harris et al., 1993) and, thus, from a remote position interacts with the catalytic acid, as suggested previously on the basis of kinetic analyses (Sierks et al., 1989). However, the wild-type activity of Trp120→Phe GA toward GF strongly excludes this interaction from being essential in the chemical step. Replacement of Trp120 results in decreased activity toward maltooligosaccharides due to an adverse effect on the formation of a productive GA−substrate complex (Olsen et al., 1993). The lower K_M for maltose with Trp120 and Tyr116 mutant GAs indicates enhanced complex formation, which could be reflected by a decreased rate of release of the reducing-end product, resulting in an increased concentration of GA−product complex. This effect is not realized during GF hydrolysis since F^- cannot mimic the sugar leaving group. As a consequence, the rate constants for GF hydrolysis are much higher than those for maltose hydrolysis by wild-type, Tyr116→Ala, and Trp120→Phe GAs. Since the measured rate for GF hydrolysis by GA is fast (k_{cat} = 730 s^{-1}) (Konstantinidis & Sinnott, 1991), the release of the reducing-end or aglycon product during maltooligosaccharide hydrolysis may be rate limiting, as suggested previously (Kitahata et al., 1981), with k_{cat} values ranging from 10 to 70 s^{-1} (Hiromi et al., 1983; Sierks et al., 1989, 1990). In contrast, Asp176→Asn and Glu400→Gln GAs, which are both involved in subsite 1 interactions, decrease the rate of hydrolysis of GF compared to wild-type GA.

The high K_M for maltose hydrolysis with these mutant GAs stems from the combination of a decreased concentration of the complex of enzyme-bound product and a decreased affinity toward substrate. Since Glu400→Gln GA has similar k_{cat}/K_M values for maltose and GF hydrolysis, Glu400 affects both the catalytic mechanism and binding/product release at subsite 1, consistent with its functional role and location in the crystal structure (Aleshin et al., 1994). Asp176→Asn, however, hydrolyzes GF faster than maltose, suggesting a partial interaction with other subsites in agreement with the previously observed perturbation of subsite 4 affinity by this mutation (Sierks et al., 1990).

pH Profiles of GF Hydrolysis. The k_{cat} and k_{cat}/K_M values obtained for GF hydrolysis by Glu400→Gln GA provide pH profiles similar to those for hydrolysis by wild-type GA (Figures 1 and 2), despite the greatly reduced activity of this mutant compared to wild-type GA. The effect of pH on the second-order catalytic rate constant, k_{cat}/K_M (Figure 1), reflects changes in the uncomplexed enzyme and an effect on the first-order rate constant, k_{cat} (Figure 2), changes in the enzyme−GF complex. For the uncomplexed enzyme, the pH dependence of k_{cat}/K_M indicates only one titratable group: the catalytic acid Glu179, having a pK_a of 5.8 in wild-type GA and 5.0 in Glu400→Gln GA. Surprisingly, two titratable groups are indicated in both mutant and wild-type GA complexes with GF (Figure 2). For the enzyme−GF complex, pK_a values of 5.4 and 3.0 were estimated graphically for wild-type GA and values of 6.1 and 2.8 for Glu400→Gln GA. Because concentrated GF was required and the available amount was limited, these values are determined from fewer experiments and thus are not as precise. The associated error in the pK_a was ±25% of a pH unit. With maltose, both complexed and uncomplexed Glu400→Gln showed titration only of the catalytic acid GA (Frandsen et al., 1994).

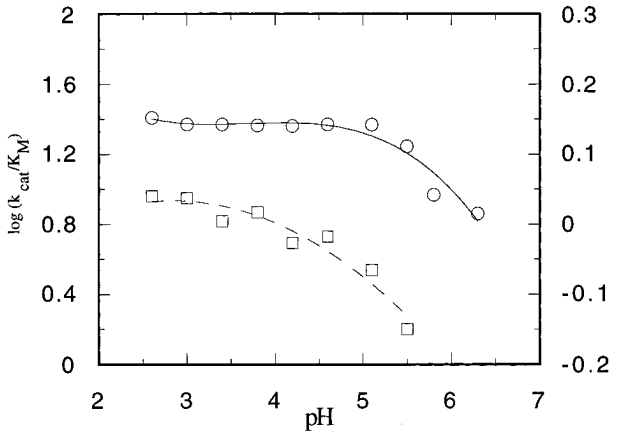

FIGURE 1: Effect of pH on k_{cat}/K_M (s^{-1} mM^{-1}) at 45 °C for GF hydrolysis by wild-type (○) and Glu400→Gln (□, right ordinate) glucoamylases.

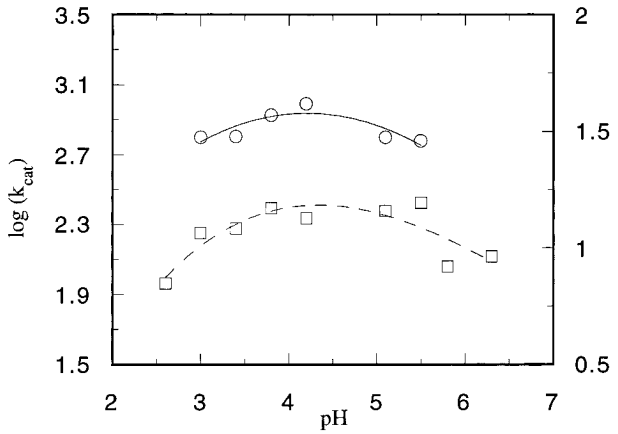

FIGURE 2: Effect of pH on k_{cat} (s^{-1}) at 45 °C for GF hydrolysis by wild-type (○) and Glu400→Gln (□, right ordinate) glucoamylases.

Because GF possibly may occupy only subsite 1, the presence of a group of low pK_a important in activity for wild-type and Glu400→Gln GAs, suggests that in the complex with GF water may obtain access to the catalytic site. If Glu400 functions to orient and activate a water molecule to exert nucleophilic attack at C-1 with GF as a substrate, even though replacement of this group causes significant loss of activity (Table 1), base catalysis seems to persist (Figure 2). The possibility that just one catalytic acid may suffice to secure enzymatic glycoside bond hydrolysis in certain cases is indicated by the recently reported crystal structure of hevamin (Terwisscha van Scheltinga et al., 1994), where the catalytic site has only a single carboxylic acid, Glu127, and a putative hydrolytic water molecule fixed in a hydrogen bond network established via two conserved residues, Asp125 and Gln181. This example supports the idea that just one catalytic acid suffices to secure enzymatic glycoside bond hydrolysis.

For maltose hydrolysis, the pK_a of the general acid catalyst is 5.9 in both free and complexed GA, while the catalytic base has pK_a values of 2.3 and 2.7 in the free and complexed states, respectively (Hiromi et al., 1966a; Sierks et al., 1990). These pK_a values indicate the dependence on pH of the rate-limiting step. Both k_{cat} and k_{cat}/K_M display maximum activity

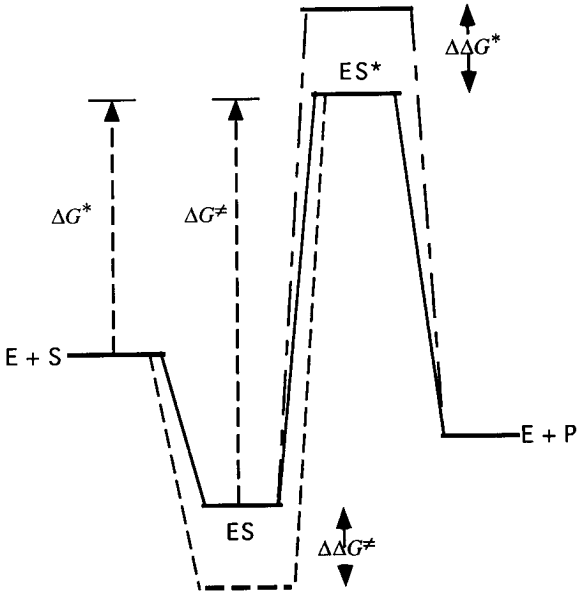

FIGURE 3: Schematic diagram depicting the change in free energy for the rate-limiting step of glucoamylase hydrolysis. E represents free enzyme, S unbound substrate, P unbound product, ES the bound complex preceding the rate-limiting step, and ES* the high-energy state for the rate-limiting step.

over a broad pH range, reflecting a general lack of pH effect on the postulated rate-limiting product release rather than providing any insight into the catalytic mechanism. A significant decrease in activity is observed only at more extreme pH values, when the charge of a higher fraction of the catalytic groups is altered. The pH dependence for GF hydrolysis may more closely reflect the actual pH dependence of the hydrolytic step and provide direct information on the pK_a values of the catalytic groups.

Ground-State vs Transition-State Energies. The difference in pH profiles of the first- and second-order rate constants for hydrolysis of GF provides additional insight into the GA mechanism. A decrease at low pH in k_{cat}, but not in k_{cat}/K_M, thus demonstrates counteraction by a similar decrease in K_M. This balance between k_{cat} and K_M suggests that the low k_{cat} is attributable to enhanced ground-state binding affinity and not to a loss of transition-state stabilization.

As shown schematically in Figure 3, the activation energy for k_{cat} can be represented by $\Delta G^{\ddagger}$ and that for k_{cat}/K_M by ΔG^* (Fersht, 1985). As long as the transition-state energy level remains unchanged, the value of k_{cat}/K_M remains constant at ΔG^*. However, if ground-state binding is stabilized (illustrated by the dotted line in Figure 3), the activation energy for k_{cat} ($\Delta G^{\ddagger}$) will increase by $\Delta\Delta G^{\ddagger}$. This appears to be the case for the hydrolysis of GF at low pH by wild-type and Glu400→Gln GAs. As the pH drops, the enzyme−substrate ground-state complex becomes more stable, while the transition-state complex remains unaltered. The opposite is seen for the hydrolysis of maltose, with K_m increasing by 2-fold at pH 2.4 compared to pH 4.5 for Glu400→Gln GA (Frandsen et al., 1994). A possible explanation for improved ground-state binding of GF at low pH is an increase in the positive charge that would adversely affect oxycarbonium ion transition-state binding. The α-secondary tritium kinetic isotope effect is high over the lower pH range (Tanaka et al., 1994), indicating that the commitment to GF hydrolysis is small compared to dissociation of the complex.

In the three-dimensional structure of GA, Arg54 binds substrate in subsite 1 and Arg305 does so in subsites 1 and 2 (Harris et al., 1993; Aleshin et al., 1994; Stoffer et al., 1995), so that the protonation of carboxylate groups is a likely source of such enhanced positive charge. Since only ground-state binding—not transition-state stabilization—is improved, the change in binding energies is likely associated with O-5, C-1, C-2, or OH-2, assumed to have a slightly different orientation in the two states (Sinnott, 1990). According to the three-dimensional structure of GA−inhibitor complexes (Harris et al., 1993; Aleshin et al., 1994; Stoffer et al., 1995), Arg305 and Trp178 interact with one or more of the preceding groups. In strong support of Arg305 being a likely candidate for stabilizing the ground state over the transition state, the mutant Arg305→Lys GA has K_M > 400 mM (Frandsen et al., 1995). Arg54 and also Asp55, however, clearly play roles in transition-state stabilization, since mutation of these residues resulted in a 300−1000-fold decrease in k_{cat} and only a minor change in K_M (Sierks & Svensson, 1993; Frandsen et al., 1995). The correspondingly large $\Delta\Delta G^{\ddagger}$ suggests that the Arg54 and Asp55 side chains form charged hydrogen bonds with OH-4 and OH-6 in subsite 1, in accordance with the crystal structures (Wilkinson et al., 1983; Aleshin et al., 1994; Svensson et al., 1994; Stoffer et al., 1995). The decrease in transition-state stabilization without an effect on the ground-state is illustrated in Figure 3 by the dashed line, where both ΔG^* and $\Delta G^{\ddagger}$ increase by $\Delta\Delta G^*$.

Role of Base Catalyst. The similar pH dependencies of wild-type and Glu400→Gln GAs suggest that the same catalytic mechanism is valid in both GAs. The role of the general base catalyst in GA, therefore, is not straightforward. When GF is bound, Glu400 is not strictly required for assisting the hydrolytic water in nucleophilic attack. Since GF may not occupy subsite 2, water can have freer access to the catalytic site than when complexed with maltose or longer oligosaccharides. Moreover, the 10-fold higher K_M for maltose hydrolysis with Glu400→Gln GA (Frandsen et al., 1994) implicates Glu400 in substrate recognition, probably via interactions at subsite 1, a role not expected of a base catalyst. It is likely that stabilization of the partial charge developed in the carbonium ion intermediate is not secured by Glu400 alone, but depends on several carboxylic acid residues, possibly including Asp55, Asp176, Glu179, and Glu180 situated at subsites 1 and 2 (Harris et al., 1993; Aleshin et al., 1994).

Implications for the Mechanism. The reaction of GA with substrate can be summarized as consisting of four steps, as depicted in Figure 4. The first step represents very fast, but weak binding of substrate to enzyme, most likely involving association with subsite 2 and influenced strongly by Glu180 (Hiromi et al., 1983; Sierks et al., 1990; Sierks & Svensson, 1992; Olsen, 1994) and Arg305 (Frandsen et al., 1995). The second step is a unimolecular rearrangement of the initial substrate complex to form a more tightly bound intermediate. This step is slower although still quite rapid, with a forward rate constant greater than 1100 s^{-1} at 8 °C for maltose (Olsen et al., 1993). The Trp120 residue is implicated in this step as conservative substitution by Phe decreases the rate constant for this rearrangement by up to 20 times, depending on substrate length (Olsen et al., 1993). The third step in the GA mechanism is bond hydrolysis, wherein Glu179

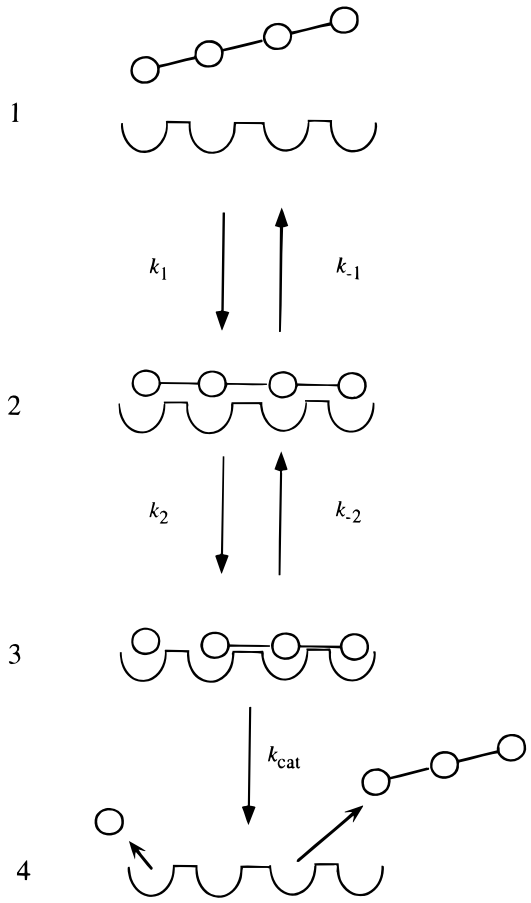

FIGURE 4: Proposed mechanism for the hydrolysis of maltooligosaccharides by glucomylase. Step 1 represents the initial loose complex formed between enzyme and substrate, step 2 represents formation of the tight enzyme−substrate complex, step 3 is the hydrolysis of the glycosidic bond, and step 4 represents the proposed rate-limiting step, release of the reducing-end product from the active site. The k_i and k_{-i} values represent the rate constants for the forward and reverse reactions for each step, and k_{cat} is the constant for the rate-limiting step.

donates the hydrogen, and a water molecule that is bound partly by Glu400 during maltooligosaccharide hydrolysis provides the nucleophilic hydroxyl group attack at C-1 of the nonreducing-end glucosyl ring. For GF hydrolysis, the net turnover rate for these three steps is reported to be at least 730 s^{-1} (Konstantinidis & Sinnott, 1991). The rate for oligosaccharide hydrolysis is probably lower as F^- is a better leaving group than glucose.

The final step in the GA mechanism, and the postulated rate-limiting step for oligosaccharide hydrolysis (Kitahata et al., 1981), is release of the reducing-end glucosyl chain. It is in this step that the interacting Trp120 region again plays a key role, as seen by the results reported here with the Tyr116→Ala and Trp120→Phe GAs. With these mutants, hydrolysis of maltose occurs 20−100-fold more slowly than the wild-type rate of 10 s^{-1}, which is significantly slower than the rate of around 730 s^{-1} found for GF. Attempts directed toward improving the efficiency of GA catalysis therefore should address increasing the rate of product release. It is interesting to consider the evolutionary advantage for GA in keeping the reducing-end product in the active site.

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REFERENCES

Aleshin, A. E., Golubev, A., Firsov, L. M., & Honzatko, R. B. (1992) *J. Biol. Chem. 267*, 19291−19298.
Aleshin, A. E., Firsov, L. M., & Honzatko, R. B. (1994) *J. Biol. Chem. 269*, 15631−15639.
Clarke, A. J., & Svensson, B. (1984a) *Carlsberg Res. Commun. 49*, 111−121.
Clarke, A. J., & Svensson, B. (1984b) *Carlsberg Res. Commun. 49*, 559−566.
Cornish-Bowden, A., & Wharton, C. W. (1988) *Enzyme Kinetics* (Rickwood, D., Ed.) IRL Press, Washington D.C.
Coutinho, P. M., & Reilly, P. J. (1994) *Protein Eng. 7*, 749−760.
Dixon, M., & Webb, E. C. (1979) *Enzymes*, 3rd ed., pp 148−164, Longman, London.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman & Co., New York.
Fersht, A. R., Shi, J., Knill-Jones, J., Lowek, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature 314*, 235−238.
Firsov, L. (1978) *Biokhimiya 43*, 2222−2232.
Frandsen, T. P., Lehmbeck, J., Dupont, C., Stoffer, B., Sierks, M. R., Hansen, M. T., & Svensson, B. (1993) *Proceedings of the 6th European Congress on Biotechnology*, Firenze, June 13−17, 1993.
Frandsen, T. P., Dupont, C., Lehmbeck, J., Stoffer, B., Sierks, M. R., Honzatko, R. B., & Svensson, B. (1994) *Biochemistry 33*, 13808−13816.
Frandsen, T. P., Christensen, T., Stoffer, B., Lehmbeck, J., Dupont, C., Honzatko, R. B., & Svensson, B. (1995) *Biochemistry 34*, 10162−10169.
Harris, E. M. S., Aleshin, A. E., Firsov, L. M., & Honzatko, R. B. (1993) *Biochemistry 32*, 1618−1626.
Hehre, E. J., Sawai, T., Brewer, C. F., Nakano, M., & Kanda, T. (1982) *Biochemistry 21*, 3090−3097.
Henrissat, B., Coutinho, P. M., & Reilly, P. J. (1994) *Protein Eng. 7*, 1281−1282.
Hiromi, K. (1970) *Biochem. Biophys. Res. Commun. 40*, 1−6.
Hiromi, K., Takahashi, K., Hamauzu, Z., & Ono, S. (1966a) *J. Biochem. 59*, 469−475.
Hiromi, K., Kawai, M., & Ono, S. (1966b) *J. Biochem. 59*, 476−480.
Hiromi, K., Ohnishi, M., & Tanaka, A. (1983) *Mol. Cell. Biochem. 51*, 79−95.
Itoh, T., Ohtsuki, I., Yamashita, I., & Fukui, S. (1987) *J. Bacteriol. 169*, 4171−4176.
Jespersen, H. M., MacGregor, E. A., Henrissat, B., Sierks, M. R., & Svensson, B. (1993) *J. Protein Chem. 12*, 791−805.
Kitahata, S., Brewer, C. F., Genghof, D. S., Sawai, T., & Hehre, E. J. (1981) *J. Biol. Chem. 256*, 6017−6026.
Konstantinidis, A., & Sinnott, M. L. (1991) *Biochem. J. 279*, 587−593.
Koshland, D. E. (1953) *Biol. Rev. Cambridge Philos. Soc. 28*, 416−436.
Laszlo, E., Hollo, J., Hoschke, A., & Sarosi, G. (1978) *Carbohydr. Res. 61*, 387−394.
Liu, W., Madsen, N. B., Brown, C., & Withers, S. G. (1991) *Biochemistry 30*, 1419−1424.
MacGregor, E. A., & Svensson, B. (1989) *Biochem. J. 259*, 145−152.
Matsui, H., Blanchard, J. S., Brewer, C. F., & Hehre, E. J. (1989) *J. Biol. Chem. 264*, 8714−8716.
Matsui, H., Tanaka,Y., Brewer, C. F., Blanchard, J. S., & Hehre, E. J. (1993) *Carbohydr. Res. 250*, 45−56.
Matsuura, Y., Kusunoki, M., Harada, W., & Kakudo, M. (1984) *J. Biochem. 95*, 697−702.

McCarter, J. D., & Withers, S. G. (1994) *Curr. Opin. Struct. Biol. 4*, 885−892.
Meagher, M. M., & Reilly, P. J. (1989) *Biotechnol. Bioeng. 34*, 689−693.
Olsen, K. (1994) Ph.D. Thesis, University of Copenhagen, Copenhagen, Denmark.
Olsen, K., Christensen, U., Sierks, M. R., & Svensson, B. (1993) *Biochemistry 32*, 9686−9693.
Savel'ev, A. N., & Firsov, L. M. (1982) *Biokhimiya 47*, 1618−1620.
Sierks, M. R., & Svensson, B. (1992) *Protein Eng. 5*, 185−188.
Sierks, M. R., & Svensson, B. (1993) *Biochemistry 32*, 1113−1117.
Sierks, M. R., Ford, C., Reilly, P. J., & Svensson, B. (1989) *Protein Eng. 2*, 621−625.
Sierks, M. R., Ford, C., Reilly, P. J., & Svensson, B. (1990) *Protein Eng. 3*, 193−198.
Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171−1202.
Stoffer, B., Frandsen, T. P., Busk, P. K., Schneider, P., Svendsen, I., & Svensson, B. (1993) *Biochem J. 292*, 197−202.
Stoffer, B., Aleshin, A. E., Firsov, L. M., Svensson, B., & Honzatko, R. B. (1995) *FEBS Lett. 358*, 57−61.
Street, I. P., Armstrong, C. R., & Withers, S. G. (1986) *Biochemistry 25*, 6021−6027.
Svensson, B. (1988) *FEBS Lett. 230*, 72−76.
Svensson, B. (1994) *Plant Mol. Biol. 25*, 141−157.
Svensson, B., & Sierks, M. R. (1992) *Carbohydr. Res. 227*, 29−44.
Svensson, B., & Søgaard, M. (1993) *J. Biotechnol. 29*, 1−37.
Svensson, B., Pedersen, T. G., Svendsen, I., Sakai, T., & Ottesen, M. (1982) *Carlsberg Res. Commun. 47*,
Svensson, B., Clarke, A. J., Svendsen, I., & Møller, H. (1990) *Eur. J. Biochem. 188*, 29−38.
Svensson, B., Stoffer, B., Frandsen, T. P., Søgaard, M., Sierks, M. R., Rodenburg, K. W., Sigurskjold, B. W., & Dupont, C. (1994) *Proceedings of 36th Alfred Benzon Symposium* (Bock, K., & Clausen, H., Eds.) pp 202−213, Munksgaard, Copenhagen.
Tanaka, Y., Tao, W., Blanchard, J. S., & Hehre, E. J. (1994) *J. Biol. Chem. 269*, 32306−32312.
Terwisscha van Scheltinga, A. C., Kalk, K. H., Beintema, J. J., & Dijkstra, B. W. (1994) *Structure 2*, 1181−1189.
Weill, C. E., Burch, R. J., & Van Dyk, J. W. (1954) *Cereal Chem. 31*, 150−158.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581−3586.
Withers, S. G., Rupitz, K., Trimbut, D., & Warren, R. A. J. (1992) *Biochemistry 31*, 9979−9985.

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