Site-Directed Mutagenesis of the Catalytic Base Glutamic Acid 400 in Glucoamylase from *Aspergillus niger* and of Tyrosine 48 and Glutamine 401, Both Hydrogen-Bonded to the γ-Carboxylate Group of Glutamic Acid 400†

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ABSTRACT: Replacement of the catalytic base Glu400 by glutamine in glucoamylase from *Aspergillus niger* affects both substrate ground-state binding and transition-state stabilization. Compared to those of the wild-type enzyme, K_m values for maltose and maltoheptaose are 12- and 3-fold higher for the Glu400→Gln mutant, with k_{cat} values 35- and 60-fold lower, respectively, for the same substrates. This unusually high residual activity for a glycosylase mutant at a putative catalytic group is tentatively explained by a reorganization of the hydrogen bond network, using the crystal structure of the related *Aspergillus awamori* var. *X100* glucoamylase in complex with 1-deoxynojirimycin [Harris, E. M. S., Aleshin, A. E., Firsov, L. M., & Honzatko, R. B. (1993) *Biochemistry 32,* 1618−1626]. Supposedly Gln400 in the mutant hydrogen bonds to the invariant Tyr48, as does Glu400 in the wild-type enzyme. For Tyr48→Trp *A. niger* glucoamylase k_{cat} is reduced 80−100-fold, while K_m is increased only 2−3-fold. Gln401 also hydrogen bonds to Glu400, but its mutation to glutamic acid has only a minor effect on activity. The Tyr48→Trp and Glu400→Gln glucoamylases share particular features in displaying unusually high activity below pH 4.0—which reflects lack of the wild-type catalytic base function—and unusually low binding affinity at subsite 2. Both mutants have lost 13−16 kJ mol^{-1} in transition-state stabilization energy. The Glu400→Gln mutant confirms the role of Glu400 in catalysis, and mutation of Tyr48 suggests that this side chain is functionally linked to Glu400 and is important for maintaining the active site geometry and for stabilization of an oxocarbonium ion substrate intermediate. The properties of the glucoamylase mutants are compared with results of mutational analysis in other carbohydrases.

Mutational analysis in conjunction with crystallography is a valuable approach to understanding the relation of active site structure and specific catalytic mechanism in carbohydrases [for reviews, see Sinnott (1990), Svensson and Søgaard (1993), and Svensson (1994)]. Structure/function investigations of the starch hydrolase glucoamylase (GA)[1] (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) from *Aspergillus niger* (identical to *Aspergillus awamori* GA; Boel et al., 1984; Nunberg et al., 1984) have involved site-directed mutagenesis (Sierks et al., 1989, 1990, 1993; Sierks & Svensson, 1993) of the recombinant enzyme produced in either *Saccharomyces cerevisiae* (Innis et al., 1985) or *A. niger* (Christensen et al., 1988). The three-dimensional structure of a 471-residue C-terminally truncated highly homologous GA from *A. awamori* var. *X100* (Aleshin et al., 1992, 1994; Harris et al., 1993; B. Stoffer, A. E. Aleshin, L. M. Firsov, B. Svensson, and R. B. Honzatko, unpublished) has served as an excellent model for rationalizing the properties of the GA mutants.

GA catalyzes the release of β-D-glucose from nonreducing ends of starch and related oligo- and polysaccharides and is widely used in the manufacture of glucose syrups. Since information is available on (i) substrate binding kinetics (Hiromi et al., 1983; Olsen et al., 1992, 1993), (ii) crystal structures of free GA and of several inhibitor complexes (Aleshin et al., 1992, 1994; Harris et al., 1993; B. Stoffer, A. E. Aleshin, L. M. Firsov, B. Svensson, and R. B. Honzatko, unpublished), (iii) effects on activity of chemical modification (Clarke & Svensson, 1984a,b; Svensson et al., 1990), and (iv) mutation of functionally important groups (Sierks et al., 1989, 1990, 1993; Sierks & Svensson, 1993), GA is an attractive choice for studies of substrate binding and catalysis in carbohydrases. GA primarily cleaves the α-1,4-glucosidic linkages, α-1,6-linked substrates being hydrolyzed at the same catalytic site with around 500-fold lower specificity (Hiromi et al., 1966; Sierks et al., 1989). Seven consecutive subsites accommodate substrate glucosyl residues with hydrolysis taking place between subsites 1 and 2 (Hiromi et al., 1983). The mechanism of GA involves

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[1] Abbreviations: bp, base pair; GA, glucoamylase.

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two carboxyl groups (Hiromi et al., 1966). One functions as a general acid catalyst in protonation of the glycosidic oxygen of the scissile bond, and the other functions as a base catalyst, activating water for nucleophilic attack at carbon C-1. These acids and additional anionic groups are proposed to stabilize an oxocarbonium ion intermediate (Matsui et al., 1989; Konstantinidis & Sinnott, 1991). Glu179 has been identified as the general acid catalyst in *A. niger* GA by differential labeling and site-directed mutagenesis (Sierks et al., 1990; Svensson et al., 1990). The crystal structure of the related GA with the bound inhibitor 1-deoxynojirimycin has confirmed this finding and suggested that Glu400 is the catalytic base (Harris et al., 1993). In GA the active site is formed from six highly conserved peptide segments that connect the C-termini of the outer helices to the N-termini of six antiparallel inner helices of an $(\alpha/\alpha)_6$-barrel (Aleshin et al., 1992, 1994; Harris et al., 1993; B. Stoffer, A. E. Aleshin, L. M. Firsov, B. Svensson, and R. B. Honzatko, unpublished). The first 30 residues of a 70 residue long *O*-glycosylated C-terminal linker region (Svensson et al., 1983) wrap around the $(\alpha/\alpha)_6$-barrel (Aleshin et al., 1992). Site-directed mutation has been performed at five of the conserved stretches in *Saccharomycopsis fibuligera* GA (Itoh et al., 1989), *A. awamori*, or the identical *A. niger* GA (Sierks et al., 1989, 1990, 1993; Sierks & Svensson, 1993) and a very distantly related GA from a thermophile bacterium (Ohnishi et al., 1994). This includes mutants at Asp55 (*A. niger* numbering) from the first conserved region, which hydrogen bonds with OH-4 and OH-6 of the substrate glucose ring in subsite 1 (Harris et al., 1993; Sierks & Svensson, 1993), and Trp120 from the second region, critical for transition-state stabilization (Clarke & Svensson, 1984b; Sierks et al., 1989; Olsen et al., 1993), which hydrogen bonds to the catalytic Glu179 and stacks with the substrate ring in subsite 3 (Aleshin et al., 1994). Mutations in the third region include Glu179 and Glu180, which is important for substrate recognition at subsite 2 (Sierks et al., 1990; Sierks & Svensson, 1992).

The present study addresses Glu400—the putative catalytic base (Harris et al., 1993)—and Gln401, both located in the sixth conserved region, and Tyr48 from the first. The conserved Tyr48 and Gln401 both hydrogen bond to Glu400 (Harris et al., 1993). Since Glu400→Gln and Tyr48→Trp GAs displayed similar unusual enzymatic properties, while the Gln401→Glu mutation caused minor changes, the question arose whether Glu400 and Tyr48 have a particular functional connection. Aromatic side chains are widely recognized to play important roles in protein–carbohydrate complexes via stacking (Vyas, 1991); we propose, however, that nonbonding electrons of Tyr48 OH contribute to stabilization of an oxocarbonium ion substrate intermediate. Catalytic site mutants in GA and other carbohydrases, representing different specificities and mechanisms, will be discussed.

MATERIALS AND METHODS

Materials. Acarbose–Sepharose was made (Clarke & Svensson, 1984b) by coupling acarbose (gift of Drs. D. Schmidt and E. Truscheit, Bayer, Germany) to EAH-Sepharose (Pharmacia). Isomaltose, maltotriose through maltoheptaose, and the glucose oxidase kit were from Sigma. Maltose monohydrate was from Merck. 4-Nitrophenyl α-D-glucopyranoside was from Boehringer Mannheim. Chemicals for Tricine–SDS–polyacrylamide gels were of electrophoresis purity from Bio-Rad. Rabbit antibodies against *A. niger* GA were a gift of Dr. P. Schneider (Novo Nordisk, Denmark). Water was drawn from a Milli-Q system (Millipore). Other chemicals used were of analytical grade.

Construction of Mutant Genes. The *A. niger* GA gene (Boel et al., 1984) was mutated by a modified PCR method (Nelson et al., 1989) using the following primers: Glu400→Gln, 5′-GCAACGG*A*TC*G*ATGTCC**C**AGCAATAC-CACAAG-3′; Gln401→Glu, 5′-GCAACGG*A*TC*G*ATGTC-CGAG**G**A**G**TACGACAAG-3′. The nucleotides replaced for GA mutation are in bold; silent mutations, facilitating control of the construct by restriction enzyme analysis, are in italics. A reverse hybrid primer (A: 5′-GTATGATCAGGATGC-TAGACCCCATCCTTTAACTATAGCG-3′) was used downstream of the GA C-terminus, composed of a 3′ 21-nucleotide sequence complementary to the genomic DNA inverse strand and a 5′ 19-nucleotide unique sequence; a forward primer (B: 5′-CCGTTCACAAGCTGAAGAGC-3′) upstream of the GA N-terminus and a reverse primer (C: 5′-GTATGAT-CAGGATGCTAGAC-3′) of identical sequence to the unique 5′ 20-nucleotide half of primer A were also used. PCR amplification was performed using GeneAmp (Perkin-Elmer Cetus) in 100 μL with 2.5 units of Taq polymerase for each reaction. The repeated PCR cycles consisted of 2 min at 95 °C to denature the template DNA (pJaL 37), 2 min at 45 °C to allow primers to anneal, and 3 min at 72 °C for DNA extension. The reaction mixture for the first step contained 50 pmol of each of the mutation primers and primer A and 20 pmol of circular plasmid DNA (pJaL 37) and was cycled 10 times. The expected product of 720 bp was extracted from a 1% agarose gel and used as a template in the second PCR. The second reaction mixture contained about 0.6 pmol of the step 1 product and 20 pmol of the circular plasmid DNA (pJaL 37) and was run as a single cycle of 5 min at 95 °C, 2 min at 45 °C, and 10 min at 72 °C. Primers B and C were then added (50 pmol of each), and 20 additional cycles were completed. The final mutagenic 2476-bp PCR fragment was digested with the restriction endonucleases *Xba*I and *Dra*III. The resulting 272 bp *Xba*I/*Dra*III fragment was extracted from a 1% agarose gel following electrophoresis and cloned into the parent vector, pJaL 37, in place of the corresponding wild-type 272-bp *Xba*I/*Dra*III fragment. After transformation of *Escherichia coli*, clones were selected by the predicted appearance of an extra *Sau*3A restriction endonuclease site. Tyr48→Trp was obtained by PCR with the mutation primer 5′-GAGTCTCGAGTCCAGGTGTA-GAA**C**C**A**GTCCGGGTTATC-3′ and the above forward primer. Together with the GA mutation (bold), a 75-bp intron is removed between the underlined nucleotides. PCR was carried out as above for step 1, using 15 instead of 10 cycles. The final mutagenic 263-bp PCR fragment was digested with restriction endonucleases *Hin*dIII and *Xho*I; the resulting 253-bp *Hin*dIII/*Xho*I fragment was extracted from a 1% agarose gel following electrophoresis and cloned into the parent vector, pJaL 37, in place of the corresponding wild-type 337-bp *Hin*dIII/*Xho*I fragment. After transformation of *E. coli*, clones were selected by the predicted disappearance of a *Sac*I site located in the intron. To confirm the mutation and that no other mutations happened during PCR, the entire cloned fragments were sequenced.

The parent vector pJaL 37 is a modification of pCAMG91 (Boel et al., 1984) where three unique restriction endonu-

clease sites (*Hin*dIII, *Xho*I, and *Xba*I) have been created by introducing three silent mutations (Jan Lehmbeck, unpublished).

Transformation and Expression. A. niger TSA-1 is a GA-negative strain lacking part of the GA-encoding *glaA* gene. Protoplasts of this strain were cotransformed by a mixture of the mutant GA plasmid and pToC90 as described (Christensen et al., 1988). pToC90 carries the *Aspergillus nidulans amdS* gene encoding an acetamidase (Corrick et al., 1987). Transformants are selected by the ability to use acetamide as sole nitrogen source. Four independent transformants were grown in 10 mL of YPD (10 g/L Bacto yeast extract, 20 g/L Bacto peptone, and 2% maltose) for 4 days at 30 °C, followed by estimation of the GA content in the medium by SDS−PAGE and rocket immunoelectrophoresis. Activity was measured using TES strips (Lilly): 10 μL of medium is mixed with 10 μL of 2% maltose; the mixture is incubated for 30 min at 45 °C, and 1 μL is spotted onto the glucose test strip. The transformant showing the highest level of GA was propagated in a fermentor (8 L), after reconfirmation of the mutation by Southern blotting using the mutant primer and direct sequencing of the genomic DNA.

Purification. Recombinant wild-type and mutant GAs were purified from culture filtrates on individual acarbose−Sepharose columns (Clarke & Svensson, 1984b) and by separation of the molecular forms on HiLoad Q-Sepharose; purity and activity were evaluated by Tricine−SDS−PAGE, amino acid and N-terminal sequence analyses, and hydrolysis of 4-nitrophenyl α-D-glucopyranoside (Stoffer et al., 1993). The 616-residue G1 form containing the N-terminal catalytic domain, the glycosylated linker region, and the starch binding domain (Svensson et al., 1983) was used throughout the present study.

Analytical Techniques. Protein concentrations were determined spectrophotometrically at 280 nm using $\epsilon = 1.37 \times 10^5$ M^{-1} cm^{-1} for GA G1 (Clarke & Svensson, 1984a) and/or by amino acid analysis, performed on 0.2−0.5 nmol of protein hydrolysate (24 h, 6 M HCl, 110 °C), using a Pharmacia LKB Alpha Plus amino acid analyzer. N-Terminal sequence analysis was carried out on an Applied Biosystems Model 477A or 470A sequenator equipped with a Model 120 PTH analyzer.

Enzyme Assays. The initial rates of GA-catalyzed hydrolysis were determined for the series of maltooligodextrins from maltose to maltoheptaose and for isomaltose at 45 °C and pH 4.5 in 0.05 M sodium acetate using 12 substrate concentrations ranging from 0.125 x K_m to 8 x K_m. The released glucose was measured by the glucose oxidase method adapted to be monitored in microtiter plates (Fox & Robyt, 1991; Palcic et al., 1993). Enzyme concentrations in the incubation mixtures ranged from 0.0335 to 0.98 μM for Tyr48→Trp and Glu400→Gln GA and from 0.00117 to 0.834 μM for Gln401→Glu and wild-type GA, respectively. k_{cat} and K_m values were obtained by fitting initial rates, v, as a function of v/[S] to the Eadie−Hofstee equation using the program ENZFITTER (Leatherbarrow, 1987).

The pH activity dependence was determined using 50 mM citrate/phosphate buffers (McIlvaine, 1921) at 16 different pH values ranging from pH 2.4 to 7.2. Maltose was used as substrate at concentrations approximately 6 x K_m and 0.17 x K_m, to estimate pK_a of the catalytic groups in the GA−substrate complex and the free GA, respectively (Sierks et al., 1990, 1993; Sierks & Svensson, 1993). Enzyme concentrations were 0.65−1.9 μM for Tyr48→Trp and Glu400→Gln GA and 0.0338−0.19 μM for Gln401→Glu and wild-type GA.

The change in activation energy for substrate hydrolysis caused by the mutation, $\Delta(\Delta G)$, was calculated from the equation $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$ (Wilkinson et al., 1983).

The subsite map, i.e., the affinities of individual substrate glucosyl binding subsites, was generated from values of k_{cat} and K_m determined with maltose through maltoheptaose as substrates at 45 °C and pH 4.5 according to a previously described model (Hiromi et al., 1983).

RESULTS

Production, Chemical Properties, and Stability of Mutant GAs. Tyr48→Trp, Glu400→Gln, and Gln401→Glu GAs were produced in amounts of approximately 0.25, 0.04, and 0.03 g/liter of culture liquid, respectively. The GA was purified in quantitative yield by acarbose−Sepharose affinity chromatography, and the individual forms were separated by ion-exchange chromatography (Stoffer et al., 1993). The molecular size, amino acid composition, and N-terminal sequence were confirmed (data not shown). The G1 form (Svensson et al., 1983), which was used in the present work, typically constituted 75−90% of the total GA protein. The stability of Gln401→Glu at elevated temperatures was measured as earlier described (Stoffer et al., 1993), and a found T_m of 69 °C was similar to the 71 °C value of wild-type GA, while Tyr48→Trp and Glu400→Gln GAs had lower stabilies with T_m values of 59 and 67 °C, respectively (data not shown). Tyr48→Trp and Glu400→Gln were 50% unfolded at 4.7 and 4.0 M guanidinium chloride, respectively, compared to 5.6 M guanidinium chloride for wild-type GA (data not shown). These decreases in stability did not significantly influence the analysis of enzymatic properties.

Kinetic Parameters. k_{cat} and K_m values were determined using maltose through maltoheptaose and the α-1,6-linked isomaltose as substrates (Table 1). In the hydrolysis of α-1,4-linked substrates, Tyr48→Trp and Glu400→Gln GA had 80−100- and 35−60-fold reduced k_{cat} values, respectively. K_m for hydrolysis of maltose by Tyr48→Trp and Glu400→Gln GA increased 3- and 12-fold, respectively, and less so for longer substrates (Table 1). Both Tyr48 and Glu400 mutants had extremely low activity on isomaltose; at high concentrations of enzyme (8.5 μM) and isomaltose (200 mM), turnover rates of 0.0027 and 0.0014 s^{-1}, respectively, were found. Such low activity precluded accurate analysis of K_m and k_{cat}, but we assume both parameters were adversely affected. Gln401→Glu GA, in contrast, retained good activity; K_m increased slightly in the case of maltotriose, maltotetraose, and maltopentaose, while k_{cat} was only reduced 2−3-fold with all substrates (Table 1).

The increase in activation energy, $\Delta(\Delta G)$, was calculated to be 13−16 kJ mol^{-1} for hydrolysis of the maltooligodextrin series by Tyr48→Trp and Glu400→Gln compared to wild-type GA (Table 2). $\Delta(\Delta G)$ values of this magnitude are indicative of loss of a charged hydrogen bond (Fersht et al., 1985) present in the transition-state complex of wild-type GA. The Gln401→Glu mutation gave small $\Delta(\Delta G)$ values of 1.8−3.1 kJ mol^{-1} and showed no change from wild-type

Table 1: Kinetic Parameters for Hydrolysis of Isomaltose and Maltooligodextrins by Wild-Type and Mutant Glucoamylases from *Aspergillus niger*[a]

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
	wild type		
isomaltose	0.41 ± 0.04[b]	19.8 ± 2.8	0.021 ± 0.004
maltose	10.7 ± 0.6	1.21 ± 0.14	8.84 ± 1.14
maltotriose	33.5 ± 0.7	0.28 ± 0.04	120 ± 17
maltotetraose	41.3 ± 1.4	0.12 ± 0.01	344 ± 31
maltopentaose	48.0 ± 3.1	0.11 ± 0.01	436 ± 49
maltohexaose	56.2 ± 5.0	0.11 ± 0.01	511 ± 65
maltoheptaose	59.7 ± 1.6	0.12 ± 0.01	498 ± 44
	Tyr48→Trp		
isomaltose	n.d.[c]		
maltose	0.120 ± 0.002	3.92 ± 0.45	0.031 ± 0.004
maltotriose	0.458 ± 0.012	0.512 ± 0.015	0.895 ± 0.035
maltotetraose	0.821 ± 0.049	0.291 ± 0.007	2.82 ± 0.18
maltopentaose	0.681 ± 0.002	0.205 ± 0.007	3.32 ± 0.11
maltohexaose	0.587 ± 0.029	0.159 ± 0.018	3.69 ± 0.46
maltoheptaose	0.762 ± 0.086	0.168 ± 0.026	4.54 ± 0.87
	Glu400→Gln		
isomaltose	n.d.[c]		
maltose	0.299 ± 0.011	14.8 ± 0.9	0.020 ± 0.001
maltotriose	0.561 ± 0.001	1.27 ± 0.015	0.513 ± 0.006
maltotetraose	0.923 ± 0.021	0.512 ± 0.032	1.80 ± 0.12
maltopentaose	1.01 ± 0.06	0.443 ± 0.028	2.28 ± 0.20
maltohexaose	1.15 ± 0.03	0.445 ± 0.021	2.58 ± 0.14
maltoheptaose	1.05 ± 0.31	0.380 ± 0.043	2.76 ± 0.87
	Gln401→Glu		
isomaltose	0.146 ± 0.008	20.4 ± 1.8	0.0072 ± 0.0007
maltose	3.29 ± 0.37	1.09 ± 0.12	3.21± 0.51
maltotriose	14.6 ± 1.8	0.357 ± 0.078	41.0 ± 10.3
maltotetraose	26.0 ± 1.5	0.231 ± 0.013	112 ± 9
maltopentaose	25.9 ± 1.6	0.164 ± 0.021	158 ± 22
maltohexaose	23.8 ± 5.0	0.112 ± 0.016	213 ± 54
maltoheptaose	20.9 ± 0.1	0.084 ± 0.004	250 ± 12

[a] Determined at 45 °C in 0.05 M sodium acetate, pH 4.5. [b] Standard deviation. [c] Not determined.

Table 2: Increase in Transition-State Binding Energies $\Delta(\Delta G)^a$ (kJ mol^{-1}) between Wild-Type and Mutant Glucoamylase from *Aspergillus niger*[b]

substrate	enzyme: Tyr48→Trp	Glu400→Gln	Gln401→Glu
isomaltose	*c*	*c*	2.8 ± 0.5
maltose	15.0 ± 0.5[d]	16.1 ± 0.4	2.7 ± 0.5
maltotriose	13.0 ± 0.4	14.4 ± 0.4	2.8 ± 0.8
maltotetraose	12.8 ± 0.3	14.0 ± 0.3	3.1 ± 0.3
maltopentaose	12.9 ± 0.3	13.9 ± 0.4	2.7 ± 0.5
maltohexaose	13.0 ± 0.5	14.0 ± 0.4	2.3 ± 0.8
maltoheptaose	12.5 ± 0.6	13.8 ± 0.9	1.8 ± 0.3

[a] $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$. [b] Calculated from the data in Table 1. [c] Not determined. [d] Standard deviation.

GA in discrimination of substrate length or bond type for the α-1,4- and α-1,6-linked substrates (Table 2).

Subsite Map. The free energy of binding of substrate glucosyl residues at seven consecutive subsites was calculated for GA (Table 3) from the kinetic parameters determined with maltose through maltoheptaose (Table 1). Association at the tight-binding subsite 2 took place with affinities of −20.4 and −11.8 kJ mol^{-1} for wild-type and Glu400→Gln GA, respectively. The mutant, however, had higher affinity than wild-type GA at subsite 3 (Table 3). The same trend was found for Tyr48→Trp GA of which the affinity at subsite 2 was −15.1 kJ mol^{-1}. The affinity at subsite 1 increased moderately for Tyr48→Trp and Glu400→Gln GAs compared to that of wild-type GA. Free energies calculated from worst

Table 3: Subsite Binding Energies (kJ mol^{-1}) for Hydrolysis of Maltooligosaccharides by Wild-Type and Mutant Glucoamylases from *Aspergillus niger*[a]

subsite		enzyme: wild type	Tyr48→Trp	Glu400→Gln	Gln401→Glu
1		−2.33	−4.23	−6.00	2.49
	min[b]	−1.53	−3.79	−5.90	6.62
	max	−3.13	−4.71	−6.10	0.73
2		−20.45	−15.40	−11.79	−21.39
	min	−19.38	−14.60	−11.50	−19.86
	max	−21.53	−16.20	−12.07	−23.00
3		−6.90	−8.90	−8.58	−6.74
	min	−6.17	−8.47	−8.42	−5.58
	max	−7.61	−9.36	−8.75	−7.79
4		−2.79	−3.04	−3.32	−2.66
	min	−2.19	−2.76	−3.11	−1.84
	max	−3.42	−3.30	−3.52	−3.63
5		−0.63	−0.43	−0.63	−0.91
	min	−0.08	−0.18	−0.21	−0.31
	max	−1.16	−0.69	−1.03	−1.48
6		−0.42	−0.28	−0.33	−0.79
	min	0.22	0.16	0.04	0.33
	max	−1.06	−0.68	−0.71	−1.78
7		0.07	−0.55	−0.18	−0.42
	min	0.63	0.33	0.96	0.30
	max	−0.52	−1.36	−1.05	−1.32

[a] Calculated from the data in Table 1. [b] The minimum and maximum estimates given show the effect of worst case combinations of errors on k_{cat}/K_m.

case combinations of errors on k_{cat}/K_m did not alter the above conclusions, although a large variation is inherent to the method used to obtain the affinity of subsite 1 (Hiromi et al., 1983; Fagerström, 1991). Subsites 4−7 possessed weak affinities for wild-type GA and these mutant GAs (Table 3), and the entire subsite map of Gln401→Glu closely resembled that of wild-type GA.

pH Activity Dependence. The activity of GA depended on a base and an acid catalyst with pK_a values that were found to be 2.7 and 5.9, respectively (Hiromi et al., 1966; Sierks et al., 1990). The mutant of the proposed catalytic base (Harris et al., 1993), Glu400→Gln GA, showed increased activity below pH 4 in both free and substrate-complexed form (Figure 1). This behavior is in accordance with the lack of the catalytic group with low pK_a in Glu400→Gln GA. In contrast, the characteristic decrease in activity above pH 5 of the wild-type GA was maintained, indicating that the acid catalyst Glu179 was functional in Glu400→Gln GA (Figure 1). Interestingly, a depressed pK_a of the catalytic base was revealed in the pH profile of free Tyr48→Trp GA (Figure 1b), although complexation with substrate seemed to restore a protonation important in the activity at low pH (Figure 1a)—presumably of Glu400. Thus in addition to a low activity, Tyr48→Trp GA had an altered pH-activity profile. Since this behavior was not observed in other active site mutants, except Glu400→Gln GA, we interpreted it to result from loss of the hydrogen bond between Tyr48 OH and Glu400 OE1, but that substrate binding induced structural features crucial for protonation of Glu400 in the Tyr48→Trp GA. In contrast, the behaviors of Gln401→Glu and wild-type GAs were very similar, although activity also of this mutant decreased proportionally less at low pH (Figure 1). Probably the glutamic acid substituting for Gln401 in the mutant suppressed the pK_a of the nearby catalytic base. k_{cat} and K_m values (data not shown) for hydrolysis of maltose by wild-type and Gln401→Glu GAs

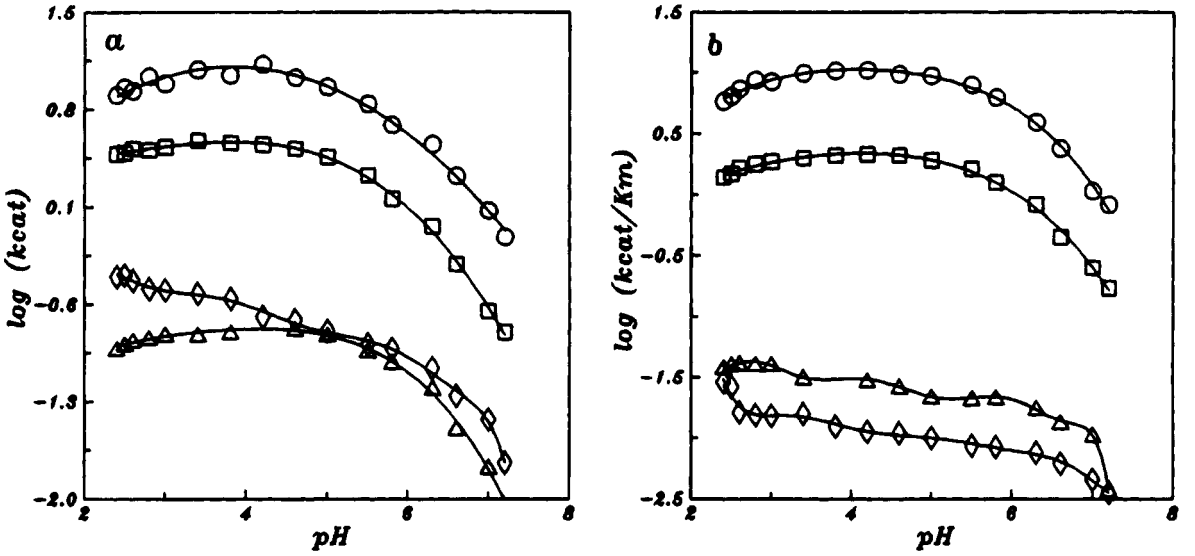

FIGURE 1: pH activity profiles for maltose-complexed (a) and uncomplexed (b) wild-type (○), Tyr48→Trp (△), Glu400→Gln (◇), and Gln401→Glu (□) glucoamylases from *Aspergillus niger* at 45 °C. Substrate concentrations were 6.0, 50.0, 90.0, and 6.0 mM for (a) and 0.2, 0.5, 2.5, and 0.2 mM for (b) for wild-type, Tyr48→Trp, Glu400→Gln, and Gln401→Glu GA, respectively. Lines drawn through the points were fitted using a fourth-degree polynomial and are intended to illustrate the shape of the plots.

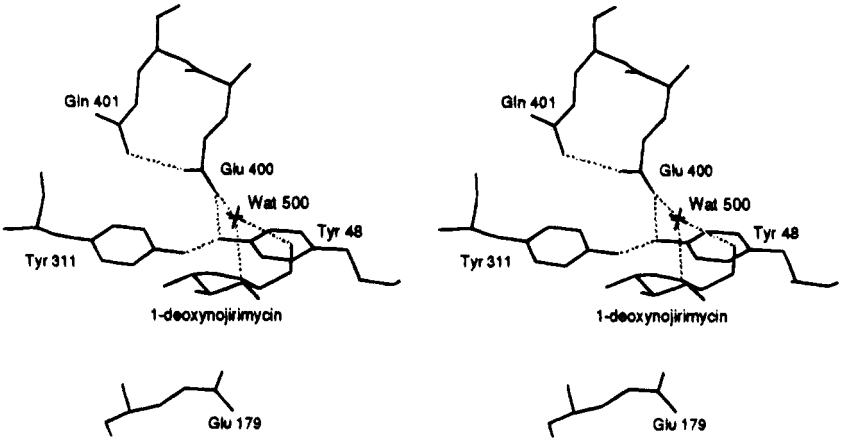

FIGURE 2: Stereoview of the active site of glucoamylase from *Aspergillus awamori* var. *X100* with bound 1-deoxynojirimycin (Harris et al., 1993). Atoms within the hydrogen bond donor−acceptor distance of 3.2 Å are represented by dashed lines.

at different pH values indicated that the decrease in activity at pH 2.4 relative to pH 4.5 was essentially an effect in K_m (2-fold increase), possibly related to the conspicuous K_m increase of the Glu400→Gln GA mutant. At the higher pH (pH 7.2) both Gln401→Glu and wild-type GA showed reduced k_{cat} and increased K_m values compared to the values determined at pH 2.4 and 4.5, supporting that the acid catalyst in Gln401→Glu and wild-type GA behaved similarly.

DISCUSSION

A. niger Glucoamylase Tyr48→Trp, Glu400→Gln, and Gln401→Glu. The enzymatic properties of these three single mutants at residues playing the role of general base catalyst or being hydrogen bonded to this group (Figure 2) were interpreted on the basis of the crystal structure of the closely related *A. awamori* var. *X100* GA complexed with the inhibitor 1-deoxynojirimycin (Harris et al., 1993). The side chain of Tyr48 is located near C-1, and the endocyclic nitrogen of 1-deoxynojirimycin and NE2 of Gln401 hydrogen bonds to OE2 of Glu400 (Figure 2). Tyr48, Glu400, and Gln401 are conserved (Coutinho & Reilly, 1994), and the significance of the hydrogen bond between OE1 of the proposed catalytic base, Glu400, and Tyr48 OH ought to be examined by replacing Tyr48 with phenylalanine, but attempts to construct the corresponding mutant gene have failed. Contrary to the published alignment (Coutinho & Reilly, 1994), we aligned 16 GA sequences (not shown), revealing six rather that five conserved regions and showing Glu400 and Tyr48 as invariant. Thus the result of the present mutations presumably applies to GAs in general. Preliminary studies, however, on Glu632→Gln GA from the very distantly related *Clostridium* sp. G0005 GA—proposed to match Glu400 in *A. niger* GA—showed a 500−1000-fold decrease in k_{cat} (Ohnishi et al., 1994) compared to 35-fold for Glu400→Gln GA. In addition to a modest decrease in k_{cat}, Glu400→Gln GA showed remarkable changes in K_m, activity at acidic pH values, and subsite map compared to wild-type GA. The general acid catalyst in *A. niger* GA was even more sensitive to mutation: the k_{cat} of Glu179→Gln GA for hydrolysis of maltoheptaose was thus reduced 2000-fold, while K_m remained essentially unchanged (Sierks et al., 1990). In this respect *A. niger* GA conformed with the pattern typical for carbohydrases, where the acid catalyst seemed most critical for catalysis (Malcolm et al., 1989; Chauvaux et al., 1992; for reviews see Svensson & Søgaard, 1993; Svensson, 1994).

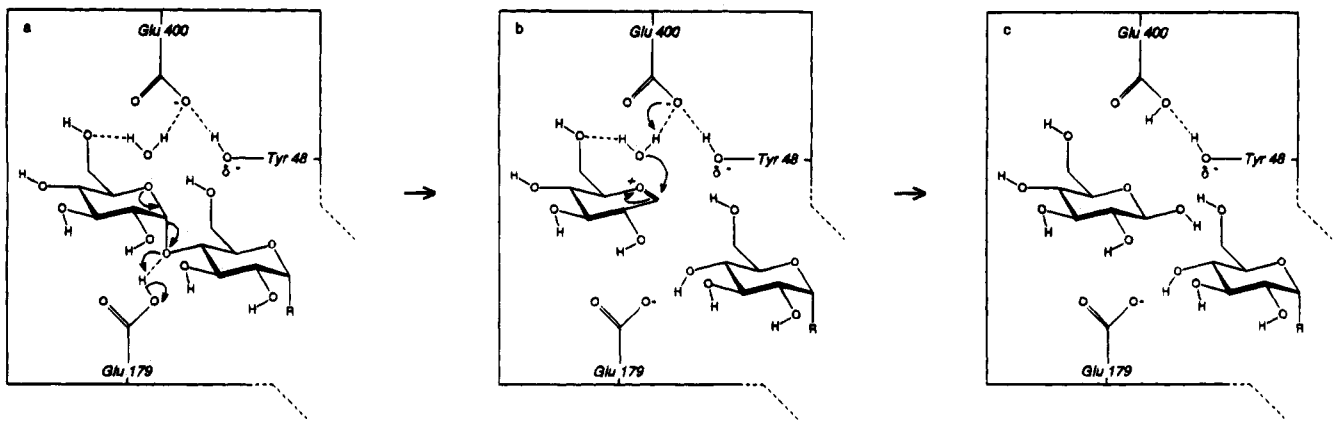

FIGURE 3: Schematic representation of three steps in the glucoamylase-catalyzed hydrolysis of an oligosaccharide, illustrating the reaction mechanism. Movements of electrons are indicated by arrows, and hydrogen bond interactions are shown by dashed lines: (a) initial attack on the glucosidic oxygen by the general acid Glu179, resulting in a Tyr48 assisted formation of an oxocarbonium ion intermediate; (b) addition of a catalytic water molecule to the oxocarbonium ion intermediate guided by the general base Glu400; and (c) the newly formed β-D-glucose product.

Among several GA mutants at the active site (Sierks et al., 1990; Sierks & Svensson, 1993) only Glu400→Gln and Tyr48→Trp GA showed relatively high activity at low pH values, implying that the loss of an important link between Glu400 and Tyr48 or the introduction of the bulky tryptophan, or both, perturbed protonation of Glu400. Such elevated activity in the acidic pH range may reflect the lack of the base catalyst. The catalytic water in Glu400→Gln GA presumably acts as explained schematically in Figure 3, but has decreased efficiency, because of a weaker polarization afforded by Gln400 compared to Glu400. Since Tyr48→Trp GA displayed similarly elevated activity at pH values below 4.0, it was not likely a result of partial reversion to wild-type GA by deamidation of Gln400. In light of the unusually large decrease in affinity for subsite 2 in both Tyr48→Trp and Glu400→Gln GAs, the importance of the proximity of Tyr48 and Glu400 (Figure 2) for the function of wild-type GA was further emphasized. Glu180→Gln and Asp176→Asn GAs also had reduced affinities at subsite 2 of -13.4 and -17.4 kJ mol^{-1} (Sierks et al., 1990; Sierks & Svensson, 1993) compared to -15.1 and -11.8 kJ mol^{-1} for Tyr48→Trp and Glu400→Gln GA, respectively; wild-type GA had an affinity of -20.4 kJ mol^{-1}. Whereas OD2 of Asp176 was within hydrogen-bonding distance of the backbone amides of residues 179 and 180 and OE2 of Glu180 was hydrogen-bonding with OH-2 in maltose (Sierks & Svensson, 1992; Harris et al., 1993; Aleshin et al., 1994; B. Stoffer, A. E. Aleshin, L. M. Firsov, B. Svensson, and R. B. Honzatko, unpublished), neither Tyr48 nor Glu400 (Harris et al., 1993) was engaged in a substrate hydrogen bond. Tyr48 and Glu400 were suggested therefore to influence the integrity of the binding site through interactions, e.g., with Tyr311 (Figure 2), recently shown to stack with the glucosyl ring bound at subsite 2 (Aleshin et al., 1994). For these four mutants, the ground-state affinity improved with increasing length of the substrate. The adverse effect of mutation was best compensated via the enhanced enzyme–substrate interactions in the case of Glu400→Gln and Tyr48→Trp GA having K_m values for maltoheptaose of 0.38 and 0.17 mM, respectively, while K_m was 0.64 mM for Asp176→Asn and 10.5 mM for Glu180→Gln; the wild-type K_m was 0.12 mM. Tyr48→Trp GA, however, suffered only a modest loss, the K_m for maltose being increased 3 times; the 12-fold increase in K_m of Glu400→Gln GA thus appeared only partly mediated via contacts controlled by Tyr48. In comparison K_m was increased 35-fold for Glu180→Gln GA (Sierks et al., 1990) which lost an important charged hydrogen bond to OH-2 of the second substrate ring (Sierks & Svensson, 1992; Aleshin et al., 1994; B. Stoffer, A. E. Aleshin, L. M. Firsov, B. Svensson, and R. B. Honzatko, unpublished). Again, since Glu400 does not bind directly to ligand in GA complexes, we speculated on possible reasons for the large loss in affinity of Glu400→Gln GA, perhaps a destabilization due to disruption of the hydrogen bond network involving Tyr48 OH, the γ-carboxyl group of Glu400, and the OH of Tyr 311 (Aleshin et al., 1994; Stoffer, A. E. Aleshin, L. H. Firsov, B. Svensson, and R. B. Honzatko, unpublished); another possibility was a weakening of the indirect interaction of Glu400 with substrate OH-6 via water 500 (Figure 2). Modulation of binding energy by mutation could be attributed to modification in hydrogen bonding and/or aromatic stacking (Vyas, 1991), as has already been described for invariant GA residues from the binding pocket: Asp55 in the first conserved region (Sierks & Svensson, 1993), Trp120 in the second (Sierks et al., 1989; Olsen et al., 1993) and Asp176 and Glu180 in the third region (Sierks et al., 1990). These residues may in turn be influenced by the catalytic base via the hydrogen bond network involving also the bound ligand, which moreover contributes important van der Waals contacts with the protein (Aleshin et al., 1994; B. Stoffer, A. E. Aleshin, L. M. Firsov, B. Svensson, and R. B. Honzatko, unpublished). According to Fersht et al. (1985) $\Delta(\Delta G)$ values of 13–16 kJ mol^{-1}, as found for maltooligodextrin hydrolysis by Tyr48→Trp and Glu400→Gln GAs, would suggest removal of a charged hydrogen bond in the transition state. But in the GA crystal structure no candidates appeared at or near Tyr48 or Glu400 for formation of a charged hydrogen bond to substrate. Maybe conformational changes accompanied these mutations which altered protein–ligand interactions in the transition state, a question to be addressed by future crystal structure determination of selected GA mutants.

Using the crystal structure of wild-type GA in consideration of the possible hydrogen-bonding networks in the Gln400 mutant offers one possible explanation for its unusually high residual activity. In wild type, Glu400 hydrogen bonds to the putative catalytic water (water 500 of Figure 4A) and is most likely responsible for abstracting

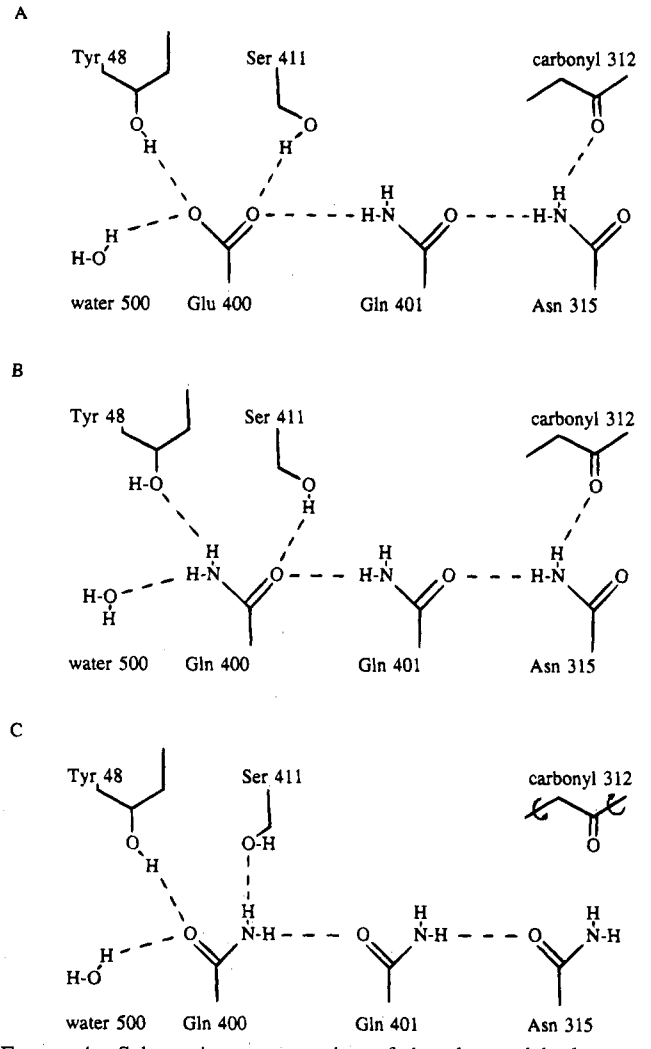

FIGURE 4: Schematic representation of the observed hydrogen-bonding network in wild-type glucoamylase (A) from *A. awamori* var. *X100* and two possible hydrogen-bonding networks (B and C) for the mutant having Gln400.

a proton from this water, as well as maintaining it in a catalytically productive orientation. In the wild-type GA an unambigous hydrogen-bonding network exists (Harris et al., 1993; Aleshin et al., 1994). This network not only fixes water 500 and the side chains of Tyr48, Asn315, Glu400, Gln401, and Ser411 in specific orientations but also is responsible for unfavorable ϕ and ψ angles of Ser411 and Asn313 (Aleshin et al., 1992). Mutation of Glu400 to glutamine will perturb the hydrogen-bonding network of wild-type GA, resulting in the formation of two possible hydrogen-bonding networks. One such possible network (B of Figure 4) misorients the catalytic water, whereas the other disrupts the hydrogen bond between Asn315 and carbonyl 312 (C of Figure 4). The loss of this hydrogen bond could trigger a small conformational change in the enzyme, with the release of dihedral strain in the main chain of Asn313. Network C of Figure 4, however, would retain the catalytically productive orientation of water 500, and as a consequence, the mutant enzyme should still exhibit appreciable activity.

Alternatively, the high residual activity of the Gln400 mutant may reflect that Glu400 in the wild-type GA is a poor base catalyst of which the efficiency may depend on the degree of cation character of the transition state as well as the position and the polarization of the catalytic water 500. Kinetic studies (Matsui et al., 1989; Konstantinidis & Sinnott, 1991) suggested significant oxocarbonium ion character for the transition state, inconsistent with a general base catalyzed S_N2 reaction. Therefore Glu400, the deprotonated catalytic acid Glu179, and the phenol of Tyr48 (Figure 3) likely stabilize an oxocarbonium ion transition state. An intermediate stabilized by the active site to some degree would react rapidly, however, with water 500 even without assistance of a base catalyst, and the decrease in electrostatic stabilization in the Gln400 GA may be the major factor in the decreased activity.

Although inverting exoglucanases use simple binding and catalytic mechanisms (Sinnott, 1990; Konstantinidis & Sinnott, 1991; Svensson & Søgaard, 1993), insight at the atomic level is still lacking for the conversion of substrate. Catalysis by GA is likely to be initiated by proton transfer from Glu179 to the glycosidic oxygen of the substrate (Figure 3a). Next the aglycon leaves and an oxocarbonium ion is developed at carbon C-1 of the glycon (Figure 3b), probably assisted by the phenol of Tyr48, the two catalytic carboxylates, and other carboxylates (Harris et al., 1993). The catalytic base Glu400 polarizes water 500 for attack at C-1 from above the plane of the ring, resulting in inversion of the anomeric configuration (Figure 3c). A number of closely related conformations were proposed to fulfill the energetic requirements for an oxocarbonium ion intermediate, but a preferred one was not uncovered (Andrews et al., 1991). It was suggested on kinetic grounds that the energy gained by hydrogen-bonding substrate OH groups to GA was spent in ring deformation at subsite 1 (Sierks et al., 1992). But there has been no hard crystallographic evidence of a ground-state distortion in GA, whereas in hen egg white lysozyme several protein–substrate interactions were pointed out to promote a distorted substrate conformation facilitating generation of an oxocarbonium ion (Strynadka & James, 1991). Perhaps the key role of Tyr48 in GA is to stabilize the positive charge developed at C-1 (Figure 3). The mutation of Tyr48 to tryptophan clearly demonstrated that Tyr48 is functionally important and sensitive to changes. Interestingly, Tyr8 and Tyr191, respectively, in crystal structures of inverting endo β-glucanases from *Humicola insolens* (Davies et al., 1993) and *Thermomonospora fusca* (Spezio et al., 1993) were placed relative to proposed catalytic aspartic acid residues in a manner reminiscent of the geometry of Tyr48, Glu179, and Glu400 in GA.

Catalytic Carboxyl Group Mutants in Different Carbohydrases. While the catalytic base mutant of GA had unusually high residual activity, amylolytic enzymes of the α-amylase family underwent complete inactivation by mutation of any of the three invariant catalytic site carboxyl groups [for reviews, see Svensson and Søgaard (1993) and Svensson (1994)]. These enzymes catalyze bond cleavage with retention of the anomeric configuration and have a $(\beta/\alpha)_8$-barrel catalytic domain [for a review, see Svensson (1994)], which makes direct comparison with GA difficult. The inverting endoglucanase CelD from *Clostridium thermocellum*—the only other protein found to have an $(\alpha/\alpha)_6$-fold (Juy et al., 1992)—and β-amylases from *Bacillus polymyxa* (Uozumi et al., 1991) and soybean (Totsuka et al., 1994), were also more sensitive to mutation of catalytic residues than the GA from *A. niger*. The CelD Asp201→Ala catalytic base mutant thus has k_{cat} reduced by 3 orders of magnitude, while K_m, as in the case of *A. niger* GA, increased by a factor

of 13 (Chauvaux et al., 1992). The catalytic base mutant Glu163→Gln of bacterial β-amylase had 4 orders of magnitude lower activity than wild-type (Uozumi et al., 1991). Mutation at catalytic groups in soybean β-amylase caused complete inactivation without affecting binding (Totsuka et al., 1994). For a retaining β-glucosidase from *Agrobacterium*, two mutants, Glu358→Gln/Asn, of the proposed catalytic nucleophile had very low activity, while Glu358→Asp retained about 1% (Withers et al., 1992). In hen egg white/human lysozyme, replacement of the catalytic nucleophile Asp 52/Asp 53 by asparagine led to 6%/1% of wild-type activity, while substrate binding was essentially unaffected (Malcolm et al., 1989; Muraki et al., 1991). The Asp20→Glu/Asn catalytic base mutants of phage T4 lysozyme lysed micrococcus cells with 1.2%/0.9% of wild-type activity (Anand et al., 1988). Crystallography of a Thr26→Glu mutant (Kuroki et al., 1993) suggested that wild-type T4 lysozyme is inverting, like GA, whereas Thr26→Glu changed to a double displacement mechanism, resulting in retention of the anomeric configuration in the product.

The apparent pK_a of the general acid catalyst in the GA–substrate complex was barely affected in Glu400→Gln, but was depressed in both Asp176→Asn and Glu180→Gln GAs (Sierks et al., 1990). A few others addressed the effect of pH on the residual activity of mutants at catalytic acids in carbohydrases. For CelC from *Clostridium thermocellum* (Navas & Béguin, 1992), the apparent pK_a of Glu140→Ala/Gln was depressed by about 1 unit, whereas a minor change occurred with Glu140→Asp. The general acid catalyst of free wild-type and Glu358→Asp *Agrobacterium* β-glucosidase showed the same pK_a, while the pK_a differed by 1 unit between the corresponding enzyme–substrate complexes (Withers et al., 1992). Asp53→Asn of human lysozyme showed a slight decrease in the pK_a of the acid catalyst (Muraki et al., 1991). In contrast to GA, these enzymes, however, have neither a low pH optimum nor an inverting mechanism (Hiromi et al., 1966).

Conclusion. Although the present GA mutants have improved insight into the carbohydrase mechanism, further structural, mutational, and kinetic investigations are needed to explain the catalytic events in structural terms and describe the structure of the transition state. The results of mutational analysis in carbohydrases are still too limited to define consensus effects for groups of enzymes with common catalytic mechanisms. In GA the catalytic role of Glu400 was confirmed, and the neighboring Tyr48 was found to be very important, presumably for positioning the base catalyst and the catalytic water, as well as stabilization of an oxycarbonium ion intermediate. We speculate that Tyr48 belongs to a structural motif in active sites of certain carbohydrases. Like other starch hydrolases, GAs are multidomain proteins (Jespersen et al., 1991), and conditions were established for preparation and crystallization of the catalytic domain of *A. niger* GA (Stoffer et al., 1993; B. Stoffer, A. E. Aleshin, R. B. Honzatko, M. Gajhede and B. Svensson, unpublished). We recently crystallized Tyr48→Trp GA, and future structure determination will hopefully enlighten the present work through correlation of functional and structural changes for GA mutants.

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