Improved Activity and Modulated Action Pattern Obtained by Random Mutagenesis at the Fourth β-α Loop Involved in Substrate Binding to the Catalytic (β/α)₈-Barrel Domain of Barley α-Amylase 1

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The functionality of the sequence Arg₁⁸³-Gly₁⁸₄-Tyr₁⁸₅ of the substrate binding fourth β-α loop in the (β/α)₈-barrel of barley α-amylase isoyme 1 (AMY1) was studied by random mutagenesis. A motif of polar Gly₁⁸₄ hydrophobized was present in active mutants selected by starch plate screening of yeast transformants. Gly₁⁸₄ was important, probably due to the carbonyl group binding to Ca²⁺ and the spatial proximity of Phe₁⁸₁. Mutation of both flanking residues as in Ser₁⁸₃, Gly₁⁸₄,Met₁⁸₅ (SGM-) and TGL-AMY1 decreased the Ca²⁺ affinity. SGM-AMY1 has 2-fold increased activity for amylase but reduced activity on maltoligosaccharides, whereas KGY-AMY1 has up to 3-fold elevated activity toward the oligosaccharides. TGL-AMY1 has modest activity on all substrates. Shifted action pattern on maltoligosaccharides for NGY-, SGM-, and TGL-AMY1 support that Arg₁⁸₃ in wild type is located at subsites +1 and +2, accommodating two sugar rings toward the reducing end from the site of cleavage. In the crystal structure of barley α-amylase 2 (AMY2), Lys₁⁸₂ (equivalent to AMY1 Arg₁⁸₃) is hydrogen-bonded with sugar OH-3 in subsite +2. Higher Kᵢₛₐₜ for acarbose inhibition of KGY-AMY1 and parent AMY1 compared with the other mutants suggests favorable substrate interactions for Arg/Lys₁⁸₂. KGY-AMY1 was not inhibited by the AMY2-specific proteinaceous barley α-amylase/subtilisin inhibitor, although Lys₁⁸₂ of AMY2 is salt-linked to the inhibitor.

α-Amylase (α-1,4-d-glucan glucanohydrolase, EC 3.2.1.1) hydrolyzes internal α-1,4-glucosidic bonds in starch and related oligo- and polysaccharides. High resolution x-ray structures are known for Taka-amylose A (TAA) from Aspergillus oryzae (1), acid α-amylase from Aspergillus niger (2), isoyme I of porcine pancreatic α-amylase (PPA; Ref. 3), the AMY2–2 isoyme from barley malt (4), and an inactive, protease-cleaved form of α-amylase from Bacillus licheniformis (5). These enzymes are organized in three domains, an N-terminal catalytic (β/α)₈-barrel fold (domain A) having a long β-α loop (domain B) protruding at the third β-strand and a C-terminal either five- or eight-stranded β-sheet (domain C). Oligosaccharide inhibitor complexes are determined of TAA (1), PPA (6), and AMY2–2 (71). The structure of Saccharomyces fibuligera α-amylase (Sfamy) was modelled on that of TAA (7).

Barley α-amylases occur in two isoym families (8), a low pI AMY1 and a high pI AMY2, of 80% sequence identity (9) but significantly different substrate affinity, turnover rate (10–12), Ca²⁺ dependence of activity (13–15), and stability (13, 15, 16). Only AMY2 is inhibited by the endogenous α-amylase/subtilisin inhibitor (BASI) present in barley seeds (17, 18). The subsite maps of binding affinities for substrate glucosyl residues are very similar for AMY1 and AMY2 and comprise ten subsites, six toward the nonreducing and four toward the reducing end relative to the cleavage point in linear maltoligosaccharides (19). They moreover possess, like subsite maps from Bacillus subtilis (20) and Bacillus amylyoquaei faciens α-amylases (21), TAA (22), and Sfamy (23), a large negative affinity, indicating distortion of the glucose residue at the catalytic site, flanked by two subsites of large positive affinity.

Sequence alignment and prediction of secondary structures indicated that different starch hydrolases and related enzymes contain a catalytic (β/α)₈-barrel similar to the α-amylases (24, 25). In a recent structure-based classification of glycosylases, these amylolytic enzymes belong to family 13 (26), currently including 18 EC classes, representing variations in substrate/product specificity (27). Seven short conserved sequences characterize family 13 members, but only seven amino acid residues are invariant (28, 29). Although α-amylases strictly hydrolyze the α-1,4, related enzymes can act on either α-1,4 or α-1,6 glucosidic bonds or have dual bond-type specificity. A conserved region in the β-α loop extending at the C terminus of the fourth β-strand of the (β/α)₈-fold is interpreted to play an important role in the specificity (25, 27, 30–32). Remarkably, Arg₁⁸₃-Gly₁⁸₄,Tyr₁⁸₅ at this loop in AMY1 is distinctive to plant α-amylases; others have Lys-His-Z, where Z is hydrophobic (9, 25, 27, 33–35). Arg₁⁸₃ in AMY1 aligns with Lys₁⁸₂ in AMY2 (9), which in the crystal structure of AMY2-acarbose (71) forms a hydrogen bond to OH-3 of the sugar ring at subsite +2 and in the crystal structure of AMY2–BASI (36) interacts with a glucose

electrophoresis; Sfamy, S. fibuligera α-amylase; HPLC, high pressure liquid chromatography.
tamic acid side chain of BASL. In TAA and PPA, NZ, the side chain nitrogen, of the corresponding Lys forms the same hydrogen bond with substrate (1, 6). The K209F TAA mutant was inactive, whereas K209R showed 50% and 200% activity toward starch and 4-nitrophenoxy α-maltoside, respectively (37). Similarly, Lys210 of Sfamy is found to be close to the catalytic site (38, 39).

Using local random mutagenesis we have been able to engineer barley α-amylase to have improved activity even toward amylose. This approach moreover provided a powerful method to both extract information on the tolerance of individual positions to substitutions and to define the role of the target residues. Thus replacements obtained in the sequence Arg183, Glu184,Tyr185 of AMY1 allowed detailed structure/function relationship investigations and resulted in enzyme variants with significantly improved activity and altered substrate specificity. This is the first report on random mutagenesis applied to a specific substrate-binding β-α loop of the catalytic (β/α)8-barrel of a member of glycosylase family 13.

**EXPERIMENTAL PROCEDURES**

**Materials**—Soluble starch and the 2-chloro-4-nitrophenyl β-maltoside to prepare substrates (3) were from Merck; G1-PNP and G2-PNP were purchased from Sigma; and G7-PNP was from Boehringer Mannheim. Rabbit antibodies against barley α-amylase were raised using AMY2 as antigen (custom immunization by Dako A/S, Denmark).

**Strains and Plasmids**—*Escherichia coli* NM522 (42) was used as the host of pBAL7 C95A (43), carrying the mutations in the parent AMY1 insert. *Saccharomyces cerevisiae* DBY746 (α, his3 Δ1, leu2–3, leu2–11, ura3–52, trp1–299) was used for the production of parent and mutant AMY1, pUC18 (44) and pBAL7 C95A were from house collections.

**Site-directed Mutagenesis and Random Mutagenesis**—Standard cloning techniques (45) were used throughout. Because two SacI and two SfII sites were present in the AMY1 cDNA (46), silent mutations were introduced in the SacI (at 465–461) and SacII (at 471–476) sites of the AMY1 gene (43) to make the outer SacI (at 376–381) and SacII (at 1388–1393) sites unique. This involved using polymerase chain reaction (PCR) (Perkin-Elmer Instrument DNA Thermal Cycler) with Vent DNA polymerase (New England BioLabs), two outside primers (SAC01BAM and SAC02ECO) (see above), with a different set of inside primers (SAC03DEL, 5′-GGCCGACTCCTGACATTTCC-3′, and SAC04DEL, 5′-GAGCAGGTAGTGCTCCTGATCTCCTGCGG-3′), the PCR product was subcloned into pUC18, and the recombinant plasmid pUCBAL C95A ΔS I, II was purified as described above.

Random mutants of AMY1 at Arg183-Gly184-Tyr185 were made in pUCBAL C95A ΔS I, II by PCR using the outside primers described above, the inside primers described above, 5′-CTGCCGGAGATGGCCAAGGT-3′, plus PL05RV, 5′-AGCGAGTTCAAGGCGCCACCGTCGAGAGCGGAG-3′; random mutants at Gly184 were made using the same outside primers and the inside primers: PL11, 5′-CGTGGGCCCCCTGATCGTGAGGNTNTCTC-3′, plus PL05RV, and Gly184 random mutations, 10% (100 μl) of the transformation mixture was used as above. The transformant colonies were counted, each of seven clones was propagated in 5 ml of LB + Ampi medium, and the plasmids were purified and sequenced. The size of the pool of random mutants was estimated from the total number of transformants and the mutation efficiency as determined by DNA sequencing of the isolated plasmids. Each of the remaining portions of the transformation mixtures (900 μl) was propagated in 1 liter of fresh LB + Ampi medium at 25 or 30 °C for 2 days (MBR BioReactor, AG Switzerland). The recombinant AMY1 was purified by affinity chromatography on a specific barley barrel of a member of glycosylase family 13.

**Materials**—Soluble starch and the 2-chloro-4-nitrophenyl β-maltoside to prepare substrates (3) were from Merck; G1-PNP and G2-PNP were purchased from Sigma; G3-PNP, G4-PNP, G5-PNP, and G6-PNP were from Calbiochem; and G7-PNP was from Boehringer Mannheim.

**Expression Vector Construction and Yeast Transformation**—pBAL7 C95A was thoroughly digested with SacI and SacII, and the 0.8-kilobase fragment was replaced by the corresponding mutated fragment of pUCBAL C95A ΔS I, II. DNA sequencing confirmed that the two silent mutations but no additional mutation had occurred in the AMY1 C95A coding region. The 10-kilobase fragment of pBAL7 C95A ΔS I, II was ligated with the 0.85-kilobase Apat-SacII fragment of pUCBAL C95A X(183–185) ΔS I, II, and pUCBAL C95A X(184) ΔS I, II, respectively. Site-directed mutagenesis of Arg183 to Lys in pUCBAL C95A ΔS I, II was done by PCR as above using two different inside primers (PL13, 5′-CGTGGGCCCCCTGATCGTGAGGNTNTCTC-3′, and PL05RV). The BamHI-EcoRI fragment was subcloned into pUC18 to yield pUCBAL C95A R183K ΔS I, II, and the DNA sequence was confirmed.

**Expression**—The wild type ApaI-SacII fragment was replaced to give pUCBAL C95A X(184) ΔS I, II. The expression vector pBAL7 C95A R183K ΔS I, II was constructed as above and used for yeast transformation. The transformants were grown on SD–Leu medium at 25 or 30 °C for 2 days (MBR BioReactor, AG Switzerland). The recombinant AMY1 was purified by affinity chromatography on β-cyclodextrin-Sepharose (49) and analyzed by isoelectric focusing (IEF), SDS-polyacrylamide gel electrophoresis (PAGE) (50), and Western blotting using PhastSystem (Pharmacia). For IEF (PhastGel, pl 4–6.5) 30 ng of protein was applied (15 ng of RGC-AMY1) and visualized by silver staining according to the manufacturer’s recommendation. A starch/λ-zymogram was prepared as earlier described (49). Native PAGE was carried out on PhastGel (10–15%) using the same amount of protein for zymogram development as in IEF. For SDS-PAGE (PhastGel, 10–15%) enzyme (6 μl, 10 μg/ml; RGC-AMY1, 5 μg/ml) was mixed with sample buffer (6 μl) and boiled for 5 min, marker dye (1 μl) was added, and 3 μl was applied to the gel; protein was detected by silver staining. Western blotting was performed on Phast Western blot AP system (Promega) using rabbit anti-AMY2 serum at 105-fold dilution. The concentration of AMY1, parent and mutants, was determined by amino acid analysis (15, 49). The secretion level of recombinant AMY1 in fermentor cultures was estimated from the amount of purified enzyme.

**HPLC Analysis of the Hydrolysis Products of 4-Nitrophenyl α-D-
Mutants at the Fourth (β/α)₈-Barrel Loop of α-Amylase

TABLE I
Sequences at the fourth β-α loop of AMY1 mutants evaluated by analysis of enzymatic activity on starch plate and Western blotting

| Clone number | DNA sequence (amino acid sequence) | Activity on starch plate | Western blotting |
|--------------|-----------------------------------|--------------------------|-----------------|
| Wild type    | AGG GGC TAC R¹⁻³ G Y              | ++++                     | +               |
| 31           | AGA GGT TGC R G C                 | +                        | +               |
| 73           | AAT GGG TAT N G Y                 | ++++                     | +               |
| 89           | ACC GGG CTG T G L                 | ++                       | +               |
| 117          | AGT GGT ATG S G M                 | ++++                     | +               |
| R183K-1⁺⁻    | AAA GGC TAC K G Y                 | ++++                     | +               |
| 6            | TTT CAT CAT F H H                 | –                        | –               |
| 22           | ATT ATG ATT I M I                 | –                        | –               |
| 24           | CGC AAG TAT R K Y                 | –                        | –               |
| 25           | TAT AAA GCA Y K A                 | –                        | –               |
| 32           | CTT AGG AGT L R S                 | –                        | –               |
| 58           | TTT TAT AAT L S N                 | –                        | –               |
| 59           | TGG GAT CTG W D L                 | –                        | –               |
| 66           | CGG TGG GGT R W G                 | –                        | –               |
| 72           | GAT ACT GGG D T G                 | –                        | –               |
| 77           | GGA AAC CAA G N Q                 | –                        | –               |
| 98           | GTG GTG TTG V V L                 | –                        | –               |
| 119          | ATA CTC AGT I L S                 | –                        | –               |

a R183K-AMY1 was obtained by site-directed mutagenesis. Other mutants resulted from random mutagenesis. The full length of the AMY1 insert was sequenced for transformant clones listed.

b 800-fold concentrated culture filtrates were used. Italic letters and bold letters are polar and hydrophobic residues, respectively, as classified according to Ref. 45.

Maltooligosaccharides—Enzyme and G1-Cl-PNP, G2-Cl-PNP, or G7-Cl-PNP (120 μl) in 50 mM sodium acetate, pH 5.5, 12.5 mM CaCl₂ were incubated at 37 °C. Aliquots (40 μl) were removed at appropriate time intervals, and the reaction was stopped by addition of glacial acetic acid (20 μl). Substrate and products were separated by HPLC on a TSK-GEL oligo-PW column (Toso, 7.8 × 300 mm) and eluted with distilled water at room temperature at a flow rate of 0.7 ml/min (2150 HPLC pump, LKB) (7, 51). 4-Nitrophenyl compounds were monitored at 313 nm (SPD-6A, Shimadzu ultraviolet detector). A mixture containing 1% of each of G1-Cl-PNP, G2-Cl-PNP, G6-Cl-PNP, and G7-Cl-PNP in 50 mM acetic acid buffer, pH 5.5, was used for calibration. The relative activities were calculated using [DC₉₀/DC₅₀] = [t₉₀/5₀] = [E₉₀/E₅₀] = 100, where DC₉₀ and DC₅₀ are degrees of cleavage of the substrate by mutant and wild type enzymes, t₉₀ and t₅₀ are the reaction times, and E₉₀ and E₅₀ are the concentrations of mutant and wild type enzymes, respectively. DC was calculated as [P/(%S; + P);]⁻¹, where %S and P represent residual substrate and total amount of products at reaction time t.

Enzyme Activity Assays—Hydrolysis of DP 17 amylose by mutant and wild type AMY1 (1–5 nM) at pH 5.5 in 20 mM sodium acetate, 5 mM CaCl₂, and 0.5 mg/ml bovine serum albumin was followed by reducing sugar analysis using copper bichinoninate (52, 53). Boiled maltose was used to avoid inactivating glutathionylation of the free thiol group of Cys95 in AMY1 during yeast growth and processing (43). C95A-AMY1 was fully active toward Blue Starch (43) and will here be referred to as wild type or parent AMY1. A pool of 800-fold concentrated culture filtrates was used. Italic letters and bold letters are polar and hydrophobic residues, respectively, as classified according to Ref. 45.

RESULTS

Mutant DNA Sequences at Positions 183–185—In the present study we focused on active mutants of barley AMY1 secreted from the S. cerevisiae host strain. Parent enzyme C95A-AMY1 was used to avoid inactivating glutathionylation of the free thiol group of Cys95 in AMY1 during yeast growth and processing (43). C95A-AMY1 is fully active toward Blue Starch (43) and will here be referred to as wild type or parent AMY1. A pool was followed at 405 nm, and kinetic parameters were calculated as above. α-Amylase (30 nM) was preincubicated with the inhibitor acarbose (0.5–50 μM) for 15 min at pH 5.5 (20 mM sodium acetate, 5 mM CaCl₂, 0.5 mg/ml bovine serum albumin) at 25 °C, the substrate DP 17 amylose (6.25 mg/ml) was added at the given [acarbose], and the activity was measured as above. Kᵣᵢ was calculated as (Aᵣᵢ - A₀) × Lᵢ(Aₚᵢ - A₀), where Aₚᵢ and A₀ are Aₚᵢ values at 0 and Lᵢ μM acarbose, respectively.

Influence of CaCl₂ on Activity—The activity of mutants of AMY1 and wild type AMY1 and AMY2 (2 μM) toward insoluble Blue Starch (6.25 mg/ml) was measured at varying [CaCl₂] (0.0025–100 mM) at pH 5.5 (20 mM sodium acetate, 0.5 mg/ml bovine serum albumin) at 37 °C. Standard deviations were calculated from the data obtained in triplicate experiments performed independently.

BASI Inhibition—AMY1 mutants and wild type AMY2 (30 μM) were preincubicated with BASI (0.02–992 μM) at pH 8.0 (40 mM Tris, 5 mM CaCl₂, 0.5 mg/ml bovine serum albumin) for 15 min at 37 °C. Residual activity was determined by adding an aliquot (25 μl) of this mixture to an insoluble Blue Starch suspension (4 ml, 6.25 mg/ml) in the same buffer. After 15 min the reaction was stopped by the addition of 0.5 M NaOH (1 ml). The absorbance at 620 nm was measured after centrifugation, and the percentage of inhibition by BASI was calculated as described previously (15, 41).
of $10^4$ different random mutants was created on pUC18 and designated as pUCBAL C95A X(183–185) ΔS I, II. 120 yeast transformants harboring pBAL7C95A X(183–185) ΔS I, II were selected and subjected to DNA sequencing, and α-amylase activity was measured. Of these transformants, 83% had a mutant gene, but only 4% secreted active enzyme. DNA sequencing showed that Gly$^{184}$ as well as a polar residue at position 183 and a hydrophobic residue at position 185 were retained in active mutants (Table I). No transformants harboring mutant genes encoding a residue different from glycine at position 184 were found to secrete immunoreactive protein. Although they encoded a polar and a hydrophobic residue at positions 183 and 185, respectively, different residues: Lys (24 and 25), Asp (59), Trp (66), or Thr (72) replaced Gly$^{184}$ (Table I). Transformants producing Asn 183-Gly184-Tyr185 (NGY), KGY, and wild type (RGY) AMY1 gave large starch plate halos, whereas those secreting SGM- and TGL-AMY1 produced smaller halos, and RGC-AMY1 produced a very small halo. The halo size agreed well with the estimated amount of enzyme in fermentor cultures: wild type, 82 μg/l; KGY, 120 μg/l; NGY, 80 μg/l; SGM, 20 μg/l; TGL, 10 μg/l; and RGC mutants, < 10 μg/l.

**Mutational Analysis of the Requirement for Glycine at Position 184**—After random mutation of only the codon for Gly$^{184}$, 150 yeast transformants were isolated. Following repeated colony isolation, enzyme secreting transformants (11) were identified by a positive starch plate assay, and the target DNA region was sequenced and found to encode Gly$^{184}$. Codons for Trp, Tyr, Phe, Val, Arg, Ser, or Cys$^{184}$ were identified in genes from transformants not secreting immunoreactive enzyme.

**Electrophoretic Patterns and Western Blot of Mutant and Wild Type AMY1**—In the IEF zymogram only NGY-AMY1 resembled wild type with the expected modest decrease in pl (Fig. 1A, lanes 4 and 5). RGC-AMY1 exhibited much weaker bands (Fig. 1A, lane 1). TGL- and SGM-AMY1 lacked activity in the zymogram and gave faint diffuse bands by silver staining (Fig. 1, A and B, lanes 2 and 3). Clear activity and protein staining, however, were obtained for these mutants in native PAGE (Fig. 1, C and D). Essentially the same molecular size and antigenicity were found for the mutants and wild type AMY1 in SDS-PAGE and Western blotting (Fig. 1, E and F). KGY-AMY1 also showed activity in an IEF zymogram and had the same mobility in SDS-PAGE and native PAGE, and immunoreactivity as the wild type (data not shown).

The mutants and wild type AMY1 migrated as two bands in IEF and native PAGE. Electrospray ionization mass spectrometry of SGM-AMY1, KGY-AMY1, and wild type AMY1 (data not shown) showed a full-length and a truncated form lacking the C-terminal Arg/Ser$^{414}$ dipeptide. The truncation occurs by the known processing of recombinant AMY1 in S. cerevisiae by Kex1p, a carboxypeptidase specific for basic side chains (43, 49). Mass spectrometry also indicated Thr$^{410}$ in a significant fraction of recombinant AMY1 to carry two hexose residues, as identified earlier for recombinant wild type AMY1. Further glycosylation was not observed (data not shown). These modifications did not affect activity (43, 54, 55).

**Action Pattern on 4-Nitrophenyl Maltooligosaccharides**—To investigate the role of Arg$^{183}$ in substrate interaction in detail, the product distribution in hydrolysis of G$_5$-, G$_6$-, and G$_7$-PNP was determined. Drastic changes were seen in the action pattern on G$_5$-PNP, in particular for NGY-, SGM-, and TGL-AMY1, whereas small changes occurred with G$_6$-PNP (Table II). The dominant productive binding mode for hydrolysis of G$_7$-PNP with wild type, KGY-AMY1, and RGC-AMY1 resulted in release of G-PNP in 32–50% yield, whereas NGY-, SGM-, and TGL-AMY1 released PNP in 48–84% yield. Although wild type (RGY) released some PNP (24%), KGY- and RGC-AMY1 formed no or very little PNP (Table II). A similar shift of the preferred bond to be cleaved in G$_7$-PNP was seen for NGY-AMY1 and, to a lesser degree, SGM-AMY1, as a significantly increased hydrolysis of the first bond to release PNP. Wild type, KGY-AMY1, TGL-AMY1, and RGC-AMY1, in contrast, hydrolyzed the second bond in G$_7$-PNP in 63–81% yield (Table II). For the shorter G$_5$-PNP, NGY-, SGM-, and TGL-AMY1 having small side chains at position 183 showed 3–26% of the wild type activity, whereas KGY-AMY1 retained 72% (Table III). In no case, however, did the action pattern shift on G$_6$-PNP, although SGM-AMY1 generated an unusually large amount of glucose (Table II). The results reflect the subsite map of AMY1 (19) with its low binding affinity at subsites +3 and –3 and the high affinity at subsite –6 that plays an important role in binding near the nonreducing end of longer maltooligosaccharides.

**Kinetic Parameters for Hydrolysis of DP 17 Amylose, G$_7$-Cl-PNP, and the Inhibition by Acarbose**—The activity after mutation was either increased or essentially retained for amylose, which is a substrate that spans the entire active site binding area. $k_{cat}$ thus increased nearly 2-fold for SGM-AMY1; $K_m$ slightly increased, except for TGL-AMY1 (Table III). $k_{cat}/K_m$ after mutation varied as 64–171% of the wild type value. For
the oligosaccharide G₇-Cl-PNP, $k_{\text{cat}}$ was highest for KGY-AMY1 (160%) and lowest for TGL-AMY1 (35%). On this substrate, G₇-Cl-PNP contains a complex (71). In comparison, the shorter Asn, Ser, or Thr 183 corresponds to the equivalent of Lys182 in AMY2 that interacts with the sugar OH-3 at substrate +2 in the crystal structure of the acarbose conformation (71). In contrast, the shorter Asn, Ser, or Thr183 gave decreased $K_m$ around 4 $\mu$M, resulting in $k_{\text{cat}}/K_m$ above the wild type value for NGY- and SGM-AMY1 (Table III). $K_{\text{app}}$ of acarbose inhibition of the hydrolysis of amylose was 2–4 $\mu$M for the NGY-, SGM-, and TGL-AMY1. Because $K_{\text{app}}$ of the parent and KGY-AMY1 was around 16 $\mu$M (Table III), the pseudotetrasaccharide acarbose more effectively inhibited the Asn, Ser, or Thr183 containing mutants.

**Influence of CaCl$_2$ on Activity**—The isozymes AMY1 and AMY2 show distinct variations in activity for insoluble Blue starch as a function of the Ca$^{2+}$ concentration (Fig. 2). Thus, whereas the activity of AMY1 changed little from 0.0025 to 10 mM CaCl$_2$, that of AMY2 increased to reach a maximum around 10 mM CaCl$_2$ (15). Both isozymes are inhibited at [CaCl$_2$] > 20 mM. Whereas SGM- and TGL-AMY1 were clearly less active than the parent enzyme at [Ca$^{2+}$] < 0.01 mM (Fig. 2), resulting in a profile that resembled that of AMY2, the behavior of KGY-AMY1 (data not shown) very much resembled that of the AMY1 parent.

**Inhibition by BASI**—KGY- and NGY-AMY1 were tested for inhibition by up to 3 $\times$ 10$^4$-fold molar excess of BASI. The natural target AMY2, under the conditions used, is inhibited even by stoichiometric amounts of BASI. However, although Lys$^{182}$ of AMY2 forms a salt bridge with a glutamate residue in BASI, replacement of the equivalent Arg$^{183}$ in AMY1 by Lys, or Asn$^{183}$ (in KGY- and NGY-AMY1), did not confer these AMY1 variants detectable sensitivity for BASI.

**DISCUSSION**

**The Sequence Motif at Positions 183–185**—Very few and short sequences are well conserved among $\alpha$-amylases, (ii) Lys-His-Z predominant in animal and microbial $\alpha$-amylases, or (iii) a completely different sequence motif. A classical analysis focused on the fourth $\beta$-barrel of the catalytic ($\beta/\alpha$)$_5$-barrel revealed remarkable sequence variation corresponding to Arg$^{183}$-Gly$^{184}$-Tyr$^{185}$ in AMY1 (boxed in Fig. 3), the present target of random mutagenesis. Family members with different specificity thus contain (i) Arg/Lys-Gly-Ar as in plant $\alpha$-amylases, (ii) Lys-His-Z predominant in animal and microbial $\alpha$-amylases, or (iii) a completely different sequence where Z and Ar signify hydrophobic and aromatic residues,
respectively (9, 34, 35, 46, 56). The carbonyl oxygen of the aligned residues, AMY2 Gly183 (4, 9), TAA His210 (63), and PPA His201 (3), is a ligand to the Ca$^{2+}$ conserved in $\alpha$-amylases, i.e. Ca$^{2+}$ (Ca 500 in Ref. 4) in AMY2 (4). Lys 182 of AMY2 binds to sugar OH-3 at subsite $\varepsilon_{2}$, and Lys200 of PPA shows the same interaction (6). Furthermore, NE2 of His201 from the Lys-His-Z motif in PPA forms a hydrogen bond with sugar OH-2 in subsite $\varepsilon_{1}$, i.e. on the reducing end side of the cleavage (6). Site-directed mutagenesis of His 201 in the 95% identical human pancreatic $\alpha$-amylase revealed its multifunctional role in substrate affinity, pH activity dependence, Cl$^{-}$ activation, and binding of a proteinaceous inhibitor (61, 62).

Site-directed mutagenesis earlier indicated the roles in catalysis and binding of invariant carboxylic acid and histidine residues in barley AMY1 (53). The present goal is to alter substrate specificity and action pattern by random mutagenesis of Arg183-Gly184-Tyr185 involved in substrate binding. All active mutant AMY1 found had conserved the motif: Gly$^{184}$, a polar residue at position 183, and a hydrophobic residue at position 185. Gly$^{184}$ occurs in all known plant $\alpha$-amylase sequences (27, 64). We searched for possible variants by subjecting the corresponding codon to random mutagenesis, but no active mutants were obtained. Attempts to construct G184A failed repeatedly (not described). In accordance with the crystal structure of AMY2 (4), glycine is suggested therefore to be required at that position in plant $\alpha$-amylases.

The amount of mutant protein produced was influenced by the side chains at position 183 or 185. Although KGY- and NGY-AMY1 were produced at the same level as wild type AMY1 (RGY), yields of SGM-, TGL-, and RGC-AMY1 were around 10% of that amount. The segment centered on Gly184 may thus be critical for stability and/or proper folding. In the crystal structure of AMY2, Lys182 (equivalent to Arg 183 of AMY1) is located at a turn in the fourth $\beta$-$\alpha$ loop, in which the

![Figure 2](image1.png)  
Fig. 2. The effect of Ca$^{2+}$ concentration on the enzymatic activity of the mutant and wild type AMY1 and AMY2. 100% was defined as the activity of each enzyme at $1 \text{mM CaCl}_2$. 

![Figure 3](image2.png)  
Fig. 3. Alignment of the sequence in the fourth $\beta$-$\alpha$ loop extending from $\beta$-strand 4 in the $\alpha$-amylase family. $\beta$, L, and $\alpha$ designate residues in $\beta$-strand, loop, and $\alpha$-helix in the three-dimensional structure of AMY2 (4) (modified from Ref. 27). $\alpha$, Z corresponds to a hydrophobic amino acid, b, B is a small side chain (A/S/T). $\varepsilon$–i are cited from Refs. 34, 36, and 56–60.
Although the AMY2 Ca²⁺ different enzymes (Fig. 4) supports that AMY1 Gly 184 is structurally important. TGL-AMY1 both lost activity at Ca²⁺ (4). Replacement of both residues flanking AMY1 Gly184 may served in the different structure after residue 161 was responsible for the high Ca²⁺zymogram activity after IEF. In fact, the behavior of AMY1-carbonyl oxygen of Gly183 is the only Ca²⁺ligand from domain B, that is grafted onto the (5). It is remarkable for Ca²⁺that AMY1-AMY2 isozyme hybrids inhibited by BASI possess AMY1 sensitivity for BASI. This finding agrees with the fact Lys182 probably contributes important stabilization of AMY2-AMY2 forms a salt bridge to a glutamate in BASI. Whereas Lys182 probably contributes important stabilization of AMY2-BASI, the lack of inhibition of the mutant R183K even by a Kd complex with nitrophenyl group and glucose at the reducing end, the relative activities against α-anomeric oligosaccharide derivatives, G7-Cl-PNP, G5-PNP, and G4-PNP, showed drastic decreases from 13 to 3%. For amylose, however, SGM-AMY1 has a high kcat/Km value, which simply reflects an increase in kcat.

The parent (C95A) AMY1 has Kcat of 9.8 cat/mg for G4-Cl-PNP, whereas normal wild type AMY1, both from malt and a recombinant (11, 15) produced in Pichia pastoris, gave Kcat around 1 cat/mg (55). Similarly an approximate 5-fold increase in Kcat to 2.2 mg/ml was obtained for parent AMY1 acting on the large substrate amylose, as compared with Kcat = 0.4 mg/ml for AMY1 from malt and Pichia. This poorer affinity (Table III) may stem from the C95A mutation, which is located near His92 known to be critical for transition state stabilization (53).

Finally, BASI specifically inhibits AMY2 (17, 18) in a 1:1 complex with Kd = 2 × 10⁻¹⁰ M (41). The AMY2-BASI complex was crystallized (36), and in the molecular model Lys⁸⁸² of AMY2 forms a salt bridge to a glutamate in BASI.² Whereas Lys⁸⁸² probably contributes important stabilization of AMY2-BASI, the lack of inhibition of the mutant R183K even by a large excess of BASI showed that Lys⁸⁸² alone does not confer AMY1 sensitivity for BASI. This finding agrees with the fact that AMY1-AMY2 isozyme hybrids inhibited by BASI possess AMY2 sequence from residue 116 (15, 65).

**Conclusion**—In a schematic of the Arg¹⁸³-Gly¹⁸⁴-Tyr¹⁸⁵ region in the fourth β-α loop, based on the crystal structure of AMY2 (4) complexed with acarbose (71), Ca²⁺ stabilizes the contact between domains A and B (Fig. 4). Gly¹⁸⁴ (domain A) binds to this Ca²⁺ together with Asn¹⁸², Asp¹³⁹, Ala¹⁴², and

FIG. 4. Schematic model of the function of the sequence (Arg¹⁸³-Gly¹⁸⁴-Tyr¹⁸⁵) extending from the fourth β-strand of AMY1. A hydrogen bond is proposed between Arg¹⁸³ and OH-2 in glucose ring 1.

**Role of Ca²⁺—**AMY2 has three Ca²⁺, one of which is conserved in the different α-amylase structures (2–4, 63). The carbonyl oxygen of Gly¹⁸³ (Gly¹⁸⁴ in AMY1) come close (4) (Fig. 4). It is remarkable that the α-amylases from higher plants, amyloamylases, and maltotetraohydrolases (27, 64) have a large residue (Phe or His) together with glycine matching Phe¹⁸¹ and Gly¹⁸⁴ in AMY1 (Fig. 4), whereas α-amylases from other sources, maltotetraohydrolase, and cyclodextrin glucanotransferase contain a small residue (Ala, Ser, or Thr) and histidine corresponding to Phe¹⁸¹ and Gly¹⁸⁴, respectively. This complementarity in the different enzymes (Fig. 4) supports that AMY1 Gly¹⁸⁴ is structurally important.

**Enzymatic Properties of AMY1 Mutants—**Arg¹⁸³ in AMY1 most probably binds to substrate. In AMY2 acarbose (an inhibitory pseudotetrasaccharide) (66), NZ of Lys¹⁸² thus hydrogen bonds with OH-3 of the third acarbose ring (71) and is considered a critical determinant of subsite 2. The shifted action pattern on the substrate G4-PNP is compatible with a weakening of this interaction in NGY-, SGM-, and TGL-AMY1 releasing PNP, whereas wild type and mutants having (Arg/Lys)³⁸³ release G5-PNP as major product.

KG-AMY1 shows a 3-fold increase in activity and maintains wild type action pattern for hydrolysis of G6-PNP but has only 72% activity on G7-PNP. NGY-AMY1 shows 91 and 8% activity, respectively, on these substrates. The three-dimensional structures around Lys¹⁸² of AMY2 and Lys⁸⁰⁹ of TAA are similar (4). In case of Sfamy, which is highly homologous to TAA (7, 23, 37, 36, 67), K210R (38) had 20 and 200% (kcat/Km) of wild type activity toward maltopentaose and maltotriose due to decreased affinity at substrate +2 and increased affinity of subsite +1, respectively. The kcat/Km values of K210R Sfamy for G5- and G6-PNP were only 15 and 1% of K210R Sfamy due to reduced affinity at both subsites (38). This behavior is reminiscent of the present AMY1 mutants. The mutant action patterns are consistent with Arg³⁸³ being an important component of subsites +1 and +2, and the strong affinity at subsite +1 required for efficient hydrolysis of short substrates, such as G5-PNP.

The AMY1 mutants have efficient kcat/Km for amylose hydrolysis varying at 64–171% of the wild type value. Noticeably the relative activity of especially SGM-AMY1 increased with substrate length. According to the subsite theory (68, 69) kcat/Km, where kcat and K are the intrinsic rate constant for bond hydrolysis and the binding constant of substrate in productive mode, respectively; kcat = kcat/Km in hydrolysis of long substrates that cover the entire binding site. The strong dependence of SGM-AMY1 on substrate length might be explained by the mutation affecting both parameters resulting in increased kcat and decreased affinity at one or more subsites near the catalytic site. For hydrolysis of short substrates, K decreases are probably more important than kcat increases. This agrees with affinities at subsites +1 and +2 being reduced by the replacement of Arg³⁸³ by Ser, hence kcat/Km is expected to decrease for the mutant compared with the wild type enzyme. Except for an elevated kcat/Km toward G7-Cl-PNP containing a β-anomeric bond between the nitrophenyl group and glucose at the reducing end, the relative activities against α-anomeric oligosaccharide derivatives, G7-Cl-PNP, G5-PNP, and G4-PNP, showed drastic decreases from 13 to 3%. For amylose, however, SGM-AMY1 has a high kcat/Km value, which simply reflects an increase in kcat.
Asp$^{149}$(domain B). Gly$^{184}$ indeed may play an important conformational role involving the steric complementarity with the side chain of Phe$^{181}$, which most probably is obstructed by the long side chain of Arg$^{183}$ contributing to binding at Gly-Tyr at the active site. The introduction of a side chain at Gly$^{184}$. Because the activity for flexibility in this segment, which has the catalytic nucleophile that the long side chain of Arg$^{183}$ contributes to binding at subsites +1 and +2 (Fig. 4) but also confers constraints on substrate processing.

The present results give very promising prospects for activity improvement and modulation of action pattern in plant a-amylases by protein engineering in the distinct sequence (Lys/Arg)-Gly-Tyr at the active site $\beta$-4 segment. This is the first report on an engineered a-amylase with enhanced activity toward the natural substrate. Ultimately a-amylase variants might be useful in transgenic barley for enhancement of malt quality by improving the enzyme activity during germination and mashing (70).

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