The double-headed barley α-amylase/subtilisin inhibitor (BASI)\(^1\) of the Kunitz soybean trypsin inhibitor family acts on proteases of the subtilisin family and the endogenous high pI α-amylase (AMY2) but has no effect on the minor isozyme AMY1 that shares 80% sequence identity with AMY2 (1–8). Under favorable conditions, BASI inhibits the AMY2-catalyzed hydrolysis of starch with a \(K_i\) of \(0.1\) nM (2, 4). This \(K_i\) value is in excellent agreement with the \(K_D\) of 0.07 nM estimated by equilibrium fluorescence titration and stopped-flow kinetics according to a fast 1:1 two-step tight binding reaction (9). Surface plasmon resonance (SPR) analysis gave weaker affinities (\(K_D\) \(>\) 1 nM) presumably due to mass transfer limitations characteristic of the two-phase system and chip surface heterogeneity (4).

In vivo, plausible functions of BASI might be to inhibit AMY2 emerging during premature seed sprouting (1, 10) or the inhibition of proteases from pathogens, e.g. fungi belonging to the genus Fusarium (11). The in vitro demonstration of a ternary complex of AMY2, BASI, and subtilisin (1) is consistent with this latter function. BASI and wheat α-amylase/subtilisin inhibitor (WASI) are highly homologous (92% sequence identity), whereas rice α-amylase/subtilisin inhibitor (RASI), acting on insect α-amylase, shares only 58% sequence identity with BASI (12, 13). BASI is assigned to the soybean trypsin inhibitor-like superfamily of β-trefoil fold proteins implicated in various protein-protein interaction roles and shares 20–30% sequence identity with Kunitz-type trypsin inhibitors (6, 14). Six different loops and three β-stands on one side of BASI present residues that interact with both the A and B domains of AMY2 via several hydrogen bonds and salt bridges, resulting in a relatively large protein interface of 2355 Å\(^2\) (3) (Fig. 1). Distinct differences in the corresponding AMY1 regions provide a structural rationale for the strict specificity of BASI for AMY2. Most interestingly, a novel feature of the AMY2-BASI complex is the presence of a fully hydrated Ca\(^{2+}\) (Ca\(^{2+}\)) embedded at the complex interface (3). This ion seems to mediate binding between inhibitor residues and the catalytic groups in the enzyme via an extended hydrogen bonding network (Figs. 1 and 2). In addition to Ca\(^{2+}\), AMY2 contains three calcium ions bound to the active site isozyme 2; RASI, rice α-amylase/subtilisin inhibitor; SPR, surface plasmon resonance; TMA, T. molitor α-amylase; WASI, wheat α-amylase/subtilisin inhibitor; Mes, 4-morpholineethanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; PPA, porcine pancreatic α-amylase.

\(^{1}\) The abbreviations used are: BASI, barley α-amylase/subtilisin inhibitor; CBD, chitin binding domain; AMY1, barley α-amylase isozyme 1; AMY2, barley α-amylase isozyme 2; RASI, rice α-amylase/subtilisin inhibitor; SPR, surface plasmon resonance; TMA, T. molitor α-amylase; WASI, wheat α-amylase/subtilisin inhibitor; Mes, 4-morpholineethanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; PPA, porcine pancreatic α-amylase.

\(^{2}\) B. Henriassit, amfb.cnrs-mrs.fr/-pedroCAZY/.
catalytic groups (21–23) in the target enzyme or by docking on key aromatic groups in proximal subsites (24). In this context, the AMY2-BASI system presents a different example of how inhibition can be attained even though no direct bonds between any of the catalytic groups of the enzyme and the inhibitor are present. Although some binding kinetics and mutagenesis were reported for lectin-like (25–27), cereal-type (28, 29), and ten-damistat inhibitors (30), thorough analysis is lacking for most systems. An exception is AMY2-BASI, which has been the subject of extensive studies with respect to binding mechanisms and structure/function relationships (2, 5, 6, 9). Recently data from mutants of AMY2 residues binding BASI (7), SPR, and isothermal titration calorimetry of AMY2-BASI wild-type protein interactions have demonstrated the effects of ionic strength, pH, and Ca\(^{2+}\) on kinetics and thermodynamics of the complex formation (5, 8), but many important questions are still unanswered. The newly established heterologous expression system in Escherichia coli (4) prompted dissection of the contribution of individual regions and side chains to the overall inhibitory activity of BASI. Another key question addressed is whether the encoded Ca\(^{2+}\) and its solvent coordination sphere, as well as other buried solvent molecules, play a significant role in the complex formation and dissociation. This first elaborate mutational analysis of a bifunctional Kunitz-type inhibitor offers insight into the determinants of affinity and the driving forces for complex formation with target enzymes, which paves the way for rational design of other \(\beta\)-trefoil fold family members.

**EXPERIMENTAL PROCEDURES**

**Materials**—NdeI, BamHI, T4 ligase, and factor Xa were from Promega (Madison, WI); SapI was from New England Biolabs (Beverly, MA), and Expand High Fidelity Polymerase was from Roche Applied Science. Oligonucleotides were from DNA Technologies (Århus, Denmark). Apcilin, isopropyl \(\beta\)-D-thiogalactopyranoside, diithreitol, and azoalbumin, and savinase were from Sigma. Insoluble Blue Starch was (gift from S. O. Andersen), respectively. (100 \(\mu\)g) was generated using standard PCR methods and the cDNA was cloned into the pTYB1 vector (intein-system) or the pET11a vector. The primers encoded mutations at positions 168 and 170: E168Q, 5\'-TGCAG-3\'; E168T, 5\'-TGCAGACACGCCGTGTAGGCGCGG-3\'; Y170F, 5\'-GG-CTGGTAGGAGCAGGACTCTCAGTGG-3\'; and E168Q/Y170P, 5\'-TGCAGACACGCCGTGTAGGCGCGG-3\'.

**Generation of Mutants**—S77A, K140L/K140N, E168Q/E168T, and Y170F/Y170P BASI or savinase was determined by using a BIAcore 3000 (Biosensor, Homburg, Germany) for 100 \(\mu\)M AMY2 and 200 \(\mu\)M savinase (125 nM; 200 \(\mu\)l), respectively, and 1.6 \(\mu\)l was measured at 620 nm (MRX-TC Revelation microtiter plate reader, Dynex Technologies) (2). The \(K_{\text{d}}\) values for AMY2 were 1.0 \(\times\) 10\(^{-3}\) and 1.3 \(\times\) 10\(^{-3}\) g \(\text{ml}^{-1}\) under optimal and suboptimal conditions, respectively, and 1.6 \(\times\) 10\(^{-3}\), 1.1 \(\times\) 10\(^{-3}\), and 2.5 \(\times\) 10\(^{-3}\) g \(\text{ml}^{-1}\) at \(1 \mu\)M Ca\(^{2+}\) (present in the buffer without CaCl\(_2\) addition), 1.0 \(\mu\)M, and 20 \(\mu\)M CaCl\(_2\) (pH 8.0), as determined by fitting rates at seven insoluble Blue Starch concentrations (0.2–5.0 \(\mu\)g ml\(^{-1}\)) to the Michaelis-Menten equation.

**Test for Inhibition of Yellow Meal Worm (T. molitor) \(\alpha\)-Amylase**—Up to 42 \(\mu\)l was E168Q, Y170F, E168Q/Y170P, or wild-type BASI and TMA (21 nm) were preincubated at 37 °C (30 min) in 50 mM sodium acetate, pH 5.5, 5 mM CaCl\(_2\), 0.1 M NaCl, 0.05% bovine serum albumin (400 \(\mu\)l); and insoluble Blue Starch was added (6.25 mg in 600 \(\mu\)l) and incubated at 37 °C. After 30 min, the reaction was stopped by addition of 100-fold centrifuged, and the absorbance at 400 nm was measured.

**Surface Plasma Resonance**—Binding kinetics of BASI and either AMY2 or savinase was determined by using a BIAcore 3000 (Biosensor, Amersham Biosciences) (4, 8). The sensor chip were charged by 300–1000 resonance units of either biotinylated AMY2 (streptavidin-sensor chips), BASI, or azoalbumin by using the amine coupling procedure (15) (1000 sensor chips) (8). Sensorgrams (response units versus time) in duplicate were recorded at a flow rate of 30 \(\mu\)l min\(^{-1}\) at 25 °C, using five analyte concentrations (20–300 nM) in 10 mM Hepes, pH 8.0, 5 mM CaCl\(_2\), and 0.005% surfactant P20. The effect of Ca\(^{2+}\) was measured in 10 mM Mes, pH 6.5, 0.005% surfactant P20. The association and dissociation were monitored for 4 and 5 min, respectively, and the chip was regenerated by 10 mM sodium acetate, pH 5.0 (AMY2), or by 5 min of prolonged dissociation (savinase). Sensorgrams were analyzed using

**Protein Characterization**—SDS-PAGE (4–12%) and isoelectric focusing, pH 3–10 (NOVEX and Invtrogen), were performed as recommended by the manufacturers. Protein concentrations were calculated from amino acid contents of hydrolyzates (2–11 nmol) (1), and finally purified on Sephacryl S-100 HR (Amersham, England; 60 \(\times\) 1.6 cm). Optional factor Xa cleavage was performed as described (4).
Mutational Analysis of the AMY2-BASI Complex Formation

FIG. 1. Stereo view of AMY2/BASI (3). AMY2 domains A in gray, B in turquoise, and C in purple; BASI is in red. The fully hydrated Ca\(^{503}\) (red) is located at the protein-protein interface. The structural Ca\(^{500}\), Ca\(^{501}\), and Ca\(^{502}\) in AMY2 are shown as yellow spheres. Ser\(^{77}\), Lys\(^{140}\), Tyr\(^{131}\), and Asp\(^{150}\) are dark blue; Glu\(^{168}\) and Tyr\(^{170}\) are green; Asp\(^{156}\) is turquoise; and Tyr\(^{87}\), Thr\(^{89}\), Ser\(^{93}\), and Glu\(^{95}\) are yellow. The catalytic acids of AMY2 are shown in orange.

BLAevaluation version 3.1 software applying a single site 1:1 (Langmuir) binding model: \(A + B \leftrightarrow AB\) and deriving \(k_{on}\), \(k_{off}\), and \(K_D\) values (8). Binding energy differences were calculated using \(\Delta G = -RT \ln(K_D)\) (37).

Illustrations—Molecular graphics were made using AMY2-BASI accession IAVA (3) in the Protein Data Bank (38), Swiss-PdbViewer (39) (us.expasy.org/spdbv/), and POV-Ray (www.povray.org/). Multiple sequence alignments were made using the GenBank\(^{TM}\) entries and the ClustalW program at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw/).

RESULTS

Choice and Production of BASI Mutants—The structure of AMY2-BASI (3), sequence alignment of BASI, WASI, and RASI (Fig. 3), and the structure of proteinase K/WASI (40) guided mutations in four different regions of BASI (Figs. 1 and 2). Three of the four regions were located at the AMY2-BASI interface, and the fourth was on the opposite side of the inhibitor, which is suggested to be involved in protease inhibition. In the case of the AMY2-BASI interface, mutations were focused on two regions of BASI, the first of which is in contact with AMY2 domain A, and the other overlooks the active site of the enzyme (Fig. 2, A and B). In addition, one charge-reversed mutation was introduced in a third region of BASI in contact with AMY2 domain A but fairly distant from the active site (Fig. 1 and 2C). In the first region of BASI, Ser\(^{77}\), Tyr\(^{131}\), Lys\(^{140}\), and Asp\(^{150}\), which form various hydrogen bonds to the two previously mutated AMY2 residues Arg\(^{128}\) and Asp\(^{142}\) (Table I; Figs. 2, A and B and 3) and also to Gly\(^{144}\), were replaced mostly by sterically similar side chains (41). Glu\(^{168}\) and Tyr\(^{170}\) were the targets of mutations in the second binding region located at the center of the binding interface. These residues are involved in an extended hydrogen bonding network comprising the hydration shell of the embedded Ca\(^{503}\) and the three AMY2 catalytic groups Asp\(^{179}\), Glu\(^{204}\), and Asp\(^{289}\) (Table I; Fig. 2B). The only AMY2-BASI direct bond involving these residues is between Glu\(^{168}\) in BASI and Lys\(^{182}\) in AMY2 that in turn forms a hydrogen bond to a substrate glucosyl residue at subsite +2 (15, 19). Moreover, Glu\(^{168}\) and Tyr\(^{170}\) correspond to glutamine and proline in the rice homologue RASI (Fig. 3) that inhibits insect \(\alpha\)-amylase (13), and the RASI mimics E168Q, Y170P, and E168Q/Y170P were designed to assess if they could confer inhibitory activity on insect \(\alpha\)-amylase. In the third region Asp\(^{156}\) (Fig. 2C), located at the periphery of the complex and having only a water-mediated bond to AMY2, was charge-reversed to probe how changes in charge density in that region would affect the binding kinetics of BASI to AMY2. Another interesting feature of this region is the presence of a network of buried solvent molecules mediating several AMY2-BASI interactions. Finally, although coordinates for the complex of the wheat homologues WASI and proteinase K were not deposited, a molecular graphics representation (40) showed that the segment Ala\(^{86}\)-Thr\(^{89}\) (BASI Ala\(^{86}\)-Thr\(^{89}\)) of the loop connecting \(\beta\)-strands 5 and 6 may participate in protease binding. This was tested by the Y87A and T89A as well as two adjacent BASI mutants S93A and E95Q.

Initially the mutants S77A, K140L/K140N, E168Q/E168T, and Y170F/Y170P were made using the autoaceleavable intein-CBD tag, but poor yields motivated shifting to the His\(^{6}\) tag system. The mutations did not adversely alter the expression levels indicating that proper global folding of the recombinant proteins and SDS-PAGE confirmed the molecular size and the purity (not shown). As expected, neutral mutations caused no change in pI (Fig. 4, lanes 3, 8, 9, 12, and 14–16 compared with lanes 2 and 11), whereas charge elimination mutations decreased or increased the pI depending on the change in net charge (Fig. 4, lanes 4–7, 13, and 17). K140N had lower pI compared with K140L, probably due to an intramolecular hydrogen bond (3.3 Å) formed between Asn\(^{140}\)N82 and Asp\(^{150}\)O81, which stabilizes the negative charge of Asp\(^{150}\). The differences in activity of the various recombinant forms of wild-type BASI were negligible (Table III). The inhibitory activity of factor Xa-cleaved mutants was indistinguishable from those with a His\(^{6}\) tag (4); therefore, the cumbersome and inefficient factor Xa cleavage step was omitted. The CD spectra of selected mutants exhibiting varied affinities and located on different parts of BASI (S77A, Y87A, S93A, Y131F, D150N, E168T, and E168Q) were identical to that of wild-type BASI (not shown), indicating correct folding. The unaffected \(K_D\) value for savinase of BASI mutants confirmed maintenance of global structural integrity (Table II).
with the exception of E168T, for which decreased $k_{on}$ caused slightly increased $K_i$ values.

**AMY2 Inhibition by BASI Mutants**—The effect of mutations in BASI on the inhibitory activity of on AMY2 was assayed on Blue Starch under optimal and suboptimal conditions with respect to pH and ionic strength. The $K_i$ values as a result of the mutations in the three AMY2 binding regions increased from a few to several hundred times or were out of range (at 400-fold BASI molar excess) (Fig. 5; Table III). The four mutants, Y87A, T89A, S93A, and E95Q, located on the opposite site of the AMY2-BASI interface and wild-type BASI showed identical $K_i$ values. Thus, these mutants served as positive controls, and the effects on inhibition observed for the rest of the mutants could be considered as the result of local changes and not due to unspecific long range structural perturbation.

The mutant S77A showed only 3-fold reduced affinity. This mutation, however, was accompanied by remarkably high sensitivity to electrostatic screening of the inhibition of AMY2. The $K_i$ value of S77A BASI thus increased 160-fold at pH 6.8 and 0.2 M NaCl relative to the optimal binding at pH 8.0, whereas the $K_i$ value of wild-type increased 11-fold (Table III). Apart from S77A, the rest of the mutants in this region were characterized by a substantial loss of inhibition especially in the case

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**Table I**

**BASI side chains subject to mutation in this study**

| BASI mutant | Wild type side chain interactions$^b$ | Distance$^a$ |
|-------------|---------------------------------------|-------------|
| S77A        | S77Oγ-R128Ne                           | 2.83        |
| K140L/K140N | K140Nγ-D142O                           | 2.82        |
| Y131F       | Y131Oγ-D142O                           | 2.97        |
| D150N       | D150Oγ-G144N                           | 2.91        |
| E168Q/E168T | E168Oγ1-K182Nγ                        | 2.98        |
| Y170F/Y170P | Y170Oγ-Wat286                          | 2.64        |
| D156K       | D156Oγ2-Wat286                         | 2.62        |

*See Ref. 3.

$^a$ Solvent molecules makes a hydrogen bond to Pro298OAMY2.

$^b$ See Ref. 40, the coordinates were not deposited and distances cannot be obtained.

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**Fig. 2.** Stereo view of close-up of two important BASI contact areas (B). A, Ser77, Tyr131, Lys140, and Asp150 interacting with domain B of AMY2; B, Tyr131, Glu168, and Tyr170 participation in the hydrogen bond network involving water molecules surrounding Ca503 (purple) at the protein interface and the three catalytic acids; C, hydrogen bond network involving Asp156, Arg155, Glu168, Tyr170, and water molecules captured between BASI and AMY2. Ca503 is shown in purple. AMY2 is represented by the molecular surface model in yellow, the catalytic groups in blue, and Pro298 in orange.
of charge-altered mutants. This is evident by the total apparent loss of inhibition observed for K140L and D150N (Table III). In the second binding region, all the mutants showed a large decrease in inhibitory activity varying from 10-fold for Y170F to a total loss of inhibition in the case of E168T. The double mutant E168Q/Y170P resulted in an additive loss of affinity as compared with the single mutants E168Q and Y170P as judged by the $K_i$ values (Table IV). Finally, the charge-reversed mutant D156K led to a 30-fold increase in $K_i$ values.

**SPR Analysis of BASI Mutants Binding with AMY2**—The $K_i$ values increased 2.5–200-fold for the BASI mutants. The real time kinetics (Table IV) showed that changes in affinity were predominantly the result of increased $k_{off}$ which is manifested by a relatively more rapid decline in the response signal during the dissociation phase (Fig. 6). The $k_{on}$ values showed only marginal variation, especially in the case of mutations involving charge invariant and neutral side chains. The charge-reversed mutant D156K, however, showed a 6.5-fold decrease in $k_{on}$ and 4.3-fold increase in $k_{off}$, which contributed almost equally to the resulting loss in affinity (Table IV). The Y131F, K140N, E168Q, and Y170F/Y170P mutations affected $K_i$ values somewhat less than $K_i$ values (Tables III and IV), and generally charge-altering replacements had the largest effect (Table IV). The mutants Y87A, T89A, S93A, and E95Q BASI at...
The rate constants represent the mean of values determined using five BASI concentrations (61–245 nM) at 25 °C in 50 mM Bicine, 2 mM CaCl₂, 1 M KCl, 0.005% surfactant P20 (see "Experimental Procedures"). Ki values were calculated from inhibition of enzyme activity (see "Experimental Procedures").

**Table II**

| Inhibitor | $k_{	ext{on}} \times 10^6$ | $k_{	ext{off}} \times 10^{-3}$ | $K_D$ | $K_i$ |
|-----------|----------------|----------------|-------|-------|
| Wild type$^a$ | 1.17 (0.33) | 5.47 (0.12) | 46.8 (7.0) | nt |
| Wild type$^b$ | 2.10 (0.40) | 5.30 (0.11) | 25.9 (3.9) | 77 (7.8) |
| S77A$^a$ | 1.62 (0.24) | 5.61 (0.21) | 34.6 (2.4) | nt |
| Y170P$^a$ | 2.10 (0.48) | 6.00 (0.17) | 28.0 (4.0) | nt |
| K140L$^b$ | 2.40 (0.31) | 6.60 (0.24) | 27.0 (3.0) | nt |
| K140N$^a$ | 1.47 (0.16) | 5.38 (0.40) | 36.7 (4.7) | nt |
| K140L$^b$ | 1.20 (0.16) | 5.40 (0.58) | 46.0 (7.3) | nt |
| E168Q$^a$ | 0.36 (0.17) | 5.31 (0.38) | 146.6 (32) | nt |
| E168Q$^b$ | 3.50 (0.70) | 5.70 (0.10) | 17.0 (3.5) | nt |
| Y170F$^a$ | 1.50 (0.08) | 4.80 (0.18) | 33.0 (1.8) | nt |
| Y170P$^b$ | 2.20 (0.15) | 4.90 (0.30) | 22.0 (2.0) | nt |
| Y87A$^a$ | 0.44 (0.40) | 12.0 (1.8) | 268.0 (53) | 270 (47) |
| T99A$^a$ | 1.60 (0.23) | 5.50 (0.12) | 30.0 (2.9) | 135 (15) |
| S93A$^a$ | 2.30 (0.50) | 5.10 (0.13) | 48.0 (4.3) | 96 (11) |
| E95Q$^b$ | 2.50 (0.37) | 6.30 (0.11) | 26.0 (3.3) | 98 (12) |

$^a$Produced using the intein-CBD tag system.

$^b$Produced using the His₆ tag system.

**FIG. 5.** Inhibition of AMY2 by wild-type BASI and BASI mutants. A, wild-type BASI from barley (x), His₆-BASI (□), S77A (○), E168Q (■), and Y170F (▲). B, the low affinity BASI mutants K140N (○) and Y131F (▲). Inhibition assay was carried out at pH 8.0 (see "Experimental Procedures"). Curves are drawn through the experimental points.

the putative protease-binding site were bound to AMY2 with essentially the same kinetics as wild type (Table IV), confirming the global conformational integrity of the mutants.

**Calcium Effect on AMY2-BASI**—The effect of the mutations over-looking the active site of AMY2 on the calcium dependence of inhibition was explored by SPR and by Blue Starch inhibition assay. Identical SPR conditions were chosen to reproduce the calcium dependence pattern reported earlier for wild-type BASI (8). Regarding the inhibition of catalysis, optimal conditions were chosen to perform the assay. Increasing [Ca²⁺] from the low micromolar range (when no CaCl₂ was added) to 20 mM resulted in a 29- and 26-fold decrease in $K_i$ and $K_D$ values, respectively, for wild-type BASI (Table V). In contrast, the calcium dependence of binding was significantly reduced for the Y170F mutant, as the same addition resulted in 13- and 15-fold decreases of $K_i$ and $K_D$ values, respectively. Finally, E168Q BASI was rendered virtually insensitive to calcium as judged by the modest change in both its $K_D$ and $K_i$ values in the tested [Ca²⁺] range (Table V).

**Inhibition of TMA**—The RASI mimics E168Q, Y170P, and E168Q/Y170P BASI (Fig. 3) failed to inhibit TMA at a 2000-fold molar excess (data not shown), suggesting that additional elements besides these two residues are required for the molecular recognition of TMA by RASI. However, a marked decrease in affinity and inhibition potency of these mutants was observed on AMY2 (Tables III and IV). The affinity of these three RASI mimics to savinase, however, was unaffected (Table II), thus ruling out that the lack of TMA inhibition stemmed from misfolding of the mutants.

**Mutants in the Putative Savinase-binding Site**—Y87A BASI increased $K_i$ and $K_D$ 3.5- and 11-fold, respectively, whereas T99A, S93A, and E95Q, located in the same region of BASI (Fig. 1), showed little change in affinity as judged from both the $K_i$ and $K_D$ values (Table II). The residue Tyr⁸⁷ thus may be involved, albeit not critically, in inhibition of savinase. These four mutants had similar affinity for AMY2 as wild-type BASI (Tables III and IV), discounting the possibility of major structural changes as a result of the mutations.

**DISCUSSION**

Proteinaceous inhibitors of α-amylases play a role in human and animal nutrition (42, 43), plant defense against pests and pathogens (6, 44), and in control of endogenous α-amylase activity (1, 10, 45). BASI, the sole example of an α-amylase inhibitor being active on an endogenous enzyme (AMY2), is a relevant target for counteraction of pre-harvest sprouting and for enhancing cereal defense systems against pathogens. Currently, BASI is one of the best characterized α-amylase inhibitors, as a complex structure, and kinetic and thermodynamic data on the interaction of BASI to AMY2 have been reported. Yet it remains unclear how individual regions at the AMY2-BASI interface contribute to the observed high affinity binding. This mutational study was undertaken to identify pivotal sites along this large interface. The effects of the different mutations were evaluated by SPR and inhibition of enzyme activity assays. Each of these techniques presents a number of advantages and drawbacks, but together, they offer a powerful tool for the evaluation of the designed mutations. The enzyme activity inhibition assay is based on steady state binding kinetics in solution, whereas SPR monitors real time association and dissociation of an analyte in solution (e.g. BASI) to its ligand.
**Mutational Analysis of the AMY2-BASI Complex Formation**

$K_i$ values were calculated from inhibition of enzyme activity (see “Experimental Procedures”). Standard deviations are shown in parentheses. Optimal assay conditions are as follows: 40 mM Tris, pH 8.0, 5 mM CaCl$_2$, 0.05% bovine serum albumin. Suboptimal assay conditions are as follows: 20 mM Hepes, pH 6.8, 5 mM CaCl$_2$, 0.2 mM NaCl, 0.05% bovine serum albumin. $\Delta \Delta G = RT \ln(K_{i,\text{optimal}}/K_{i,\text{suboptimal}})$. ND indicates not detected within the experimental condition of the assay.

| Inhibitor       | $K_{i,\text{optimal}}$ | $K_{i,\text{suboptimal}}$ | $K_{i,\text{suboptimal}}/K_{i,\text{optimal}}$ | $\Delta \Delta G_{\text{optimal}}$ |
|-----------------|------------------------|-----------------------------|----------------------------------------------|-----------------------------------|
| Wild type$^a$   | 0.10 (±0.03)           | 1.10 (±0.31)                | 11.0                                         |                                   |
| Wild type$^b$   | 0.06 (±0.01)           | 0.73 (±0.18)                | 12.0                                         |                                   |
| Wild type$^c$   | 0.09 (±0.02)           | 0.58 (±0.15)                | 6.4                                          |                                   |
| S77A$^a$        | 0.35 (±0.06)           | 55.0 (±13.6)                | 160.0                                        | 0.67                              |
| Y131F$^b$       | 11.0 (±2.10)           | 225.0 (±9.28)               | 20.0                                         | 2.82                              |
| K140L$^a$       | ND                     |                             |                                              |                                   |
| K140N$^a$       | 21.0 (±4.85)           | 250.0 (±43.40)              | 12.0                                         | 2.89                              |
| D150N$^b$       | ND                     |                             |                                              |                                   |
| E168T$^a$       | 4.27 (±0.08)           | 44.0 (±5.38)                | 16.0                                         | 1.75                              |
| E170F$^a$       | 0.90 (±0.12)           | 30.0 (±4.94)                | 34.0                                         | 1.20                              |
| E170P$^a$       | 22.0 (±1.36)           | ND                          |                                              | 2.92                              |
| E168Q/E170P$^b$ | >100                   |                             |                                              |                                   |
| D156K$^b$       | 3.0                    | 33.3                        | 11.1                                         | 2.32                              |
| Y87A$^a$        | 0.07 (±0.03)           |                             | 0.09                                         |                                   |
| T89A$^a$        | 0.05 (±0.02)           |                             | -0.1                                         |                                   |
| S93A$^a$        | 0.05 (±0.01)           |                             | -0.1                                         |                                   |
| E95Q$^a$        | 0.06 (±0.01)           |                             | 0.0                                           |                                   |

$^a$ Produced using the intein-CBD tag system.

$^b$ Produced using the His$_8$ tag system.

$^c$ Purified from barley seeds.

**TABLE IV**

SPR-derived binding parameters for AMY2 and BASI mutants

The rate constants represent means of determinations using five BASI concentrations (61–245 nM). Standard deviations are shown in parentheses. Measurements were performed at 25 °C in 10 mM Hepes, pH 8.0, 5 mM CaCl$_2$, 0.005% surfactant P20.

| Inhibitor       | $k_{on} \times 10^3$ | $k_{off} \times 10^{-4}$ | $K_D$ | $\Delta \Delta G$ |
|-----------------|----------------------|--------------------------|-------|-------------------|
|                 | $\text{s}^{-1}$       | $\text{s}^{-1}$          | \(\text{mol} \times \text{L}^{-1} \times \text{s}^{-1}\) | \(\text{cal} \times \text{mol}^{-1}\) |
| Wild type$^a$   | 1.34 (±0.46)         | 1.6 (±0.2)               | 11.0  | 0.75              |
| Wild type$^b$   | 1.63 (±0.64)         | 1.1 (±0.1)               | 0.7   | 0.36              |
| Wild type$^c$   | 1.37 (±0.31)         | 1.8 (±0.1)               | 1.3   | 1.37              |
| S77A$^a$        | 0.86 (±0.46)         | 3.3 (±0.1)               | 3.9   | 0.57              |
| Y131F$^b$       | 1.60 (±0.56)         | 6.7 (±0.3)               | 4.2   | 1.06              |
| K140L$^a$       | 1.35 (±0.97)         | 37.6 (±1.7)              | 28.0  | 3.0              |
| K140N$^a$       | 0.51 (±0.13)         | 26.0 (±2.8)              | 50.0  | 2.26              |
| D150N$^b$       | 0.55 (±0.29)         | 74.0 (±34)               | 130.0 | 3.12              |
| E168T$^a$       | 0.63 (±0.34)         | 55.0 (±7.5)              | 87.0  | 2.59              |
| E170F$^a$       | 1.66 (±0.31)         | 13.6 (±1.7)              | 8.2   | 1.19              |
| Y170F$^a$       | 0.65 (±0.36)         | 1.8 (±0.1)               | 2.8   | 0.55              |
| Y170P$^a$       | 2.25 (±0.43)         | 10.0 (±0.6)              | 7.6   | 1.14              |
| E168Q/Y170P$^b$ | 1.30 (±0.45)         | 28.0 (±3.1)              | 22.0  | 2.04              |
| D156K$^b$       | 0.25 (±0.02)         | 6.90 (±0.30)             | 28.5  | 2.19              |
| Y87A$^a$        | 1.80 (±0.65)         | 1.5 (±0.1)               | 1.8   | 0.56              |
| T89A$^a$        | 1.60 (±0.69)         | 1.1 (±0.1)               | 0.7   | 0.06              |
| S93A$^a$        | 1.50 (±0.56)         | 2.2 (±0.4)               | 1.5   | 0.45              |
| E95Q$^a$        | 2.40 (±0.75)         | 1.2 (±0.1)               | 0.5   | -0.20             |

$^a$ Produced using the intein-CBD tag system.

$^b$ Produced using the His$_8$ tag system.

$^c$ Purified from barley seeds.

**Fig. 6.** Real time binding sensorgrams for AMY2 interacting with BASI mutants measured by SPR. Five concentrations (20–300 nM) of BASI were injected over immobilized AMY2 to determined $K_D$ values. The shown sensorgrams represent a BASI concentration of 153 nM.
Wild-type BASI was purified from barley seeds. The mutants were made using the intein-CBD tag expression system. The rate constants represent mean of determinations using five AMY2 concentrations (61–245 nM). Measurements were performed at 25 °C in 10 mM Mes, pH 6.5, 0.005% surfactant P20, and the indicated [CaCl2]. $K_d$ values were calculated as described under “Experimental Procedures.” Assay conditions are as follows: 40 mM Tris, pH 8.0, 0.05% bovine serum albumin including the indicated [Ca$^{2+}$]. Standard deviations are shown in parentheses.

| Ca$^{2+}$ (mM) | $K_d$ (nM) | $k_{on} \times 10^5$ (s$^{-1}$) | $k_{off} \times 10^{-4}$ (s$^{-1}$) | $K_d$ (nM) |
|----------------|------------|-------------------------------|----------------------------------|------------|
| BASI           |            |                               |                                  |            |
| 2–6 × 10$^{-3}$| 4.04 (+1.1)| 0.62 (+0.09)                  | 18.5 (+3.1)                      | 31.3 (+9.1) |
| 0.1            | 1.10 (+0.18)| 7.2 (+0.7)                    | 6.7 (+1.6)                       |            |
| 1              | 1.64 (+0.24)| 4.1 (+0.4)                    | 2.6 (+0.6)                       |            |
| 5              | 2.00 (+0.39)| 1.9 (+0.1)                    | 1.0 (+0.2)                       |            |
| 20             | 1.80 (+0.38)| 2.0 (+0.1)                    | 1.2 (+0.3)                       |            |
| Y170F          |            |                               |                                  |            |
| 2–6 × 10$^{-3}$| 2.33 (+0.52)| 0.86 (+0.04)                  | 21.0 (+0.9)                      | 24.3 (+3.8) |
| 0.1            | 0.94 (+0.09)| 12.0 (+0.8)                   | 13.0 (+2.2)                      |            |
| 1              | 1.10 (+0.23)| 4.9 (+0.2)                    | 4.3 (+0.6)                       |            |
| 5              | 1.00 (+0.31)| 2.2 (+0.2)                    | 2.2 (+0.3)                       |            |
| 20             | 1.10 (+0.32)| 1.8 (+0.1)                    | 1.6 (+0.2)                       |            |
| E168Q          |            |                               |                                  |            |
| 2–6 × 10$^{-3}$| 3.33 (+0.15)| 2.16 (+0.15)                  | 12.1 (+1.8)                      | 5.6 (+2.7) |
| 0.1            | 1.20 (+0.19)| 9.0 (+0.7)                    | 7.4 (+1.0)                       |            |
| 1              | 1.10 (+0.33)| 5.5 (+0.2)                    | 5.2 (+0.6)                       |            |
| 5              | 1.80 (+0.53)| 4.0 (+0.5)                    | 2.2 (+0.5)                       |            |
| 20             | 5.15 (+1.57)| 3.3 (+0.3)                    | 4.2 (+0.6)                       |            |

BASI Contacts to AMY2 Domain B—In the BASI region in contact with domain B of AMY2, the impact of S77A, Y131F, K140L, R140L, and D150N varied dramatically. The affinity of S77A decreased marginally, whereas mutations of the charged residues Lys$^{140}$ and Asp$^{150}$ severely impaired interaction of AMY2 with Ser$^{77}$. Hydrogen-bonds with Glu$^{129}$O2$_{\text{BASI}}$ and Arg$^{150}$N$_{\text{AMY2}}$ (Figs. 1 and 2A). The side chains of the two latter residues are 5.3 Å apart, making a weak salt bridge consistent with the marginal loss of affinity and the increased sensitivity to electrostatic screening for S77A (Table III). The isosteric Y131F mutation is very unlikely to cause large changes in that region.

Therefore, the intermediate decrease in affinity observed can be best rationalized by the loss of a solvent-mediated network of bonds involving the side chain OH group of Tyr$^{131}_{\text{BASI}}$, Lys$^{140}$N$_{\text{AMY2}}$, and Tyr$^{170}_{\text{BASI}}$ (Table I; Fig. 2C). Abolishing the charged side chain of either Lys$^{140}$ or Asp$^{150}$ resulted in a drastic drop in affinity underscoring the critical role of these residues in complex formation. Lys$^{140}$N$_{\text{AMY2}}$ and Asp$^{150}$O$_1$ in BASI are at a distance of 2.7 Å, making a strong ionic bond and bridging the two β-strands carrying these residues. In addition, both residues are a part of a consortium of bonds involving Lys$^{140}$N$_{\text{BASI}}$ and Asp$^{150}$O$_2$AMY2 2.86 Å apart (resulting in an ionic network between Asp$^{150}_{\text{BASI}}$, Lys$^{140}_{\text{BASI}}$, and Asp$^{150}_{\text{AMY2}}$), as well as Gly$^{144}$AMY2, Tyr$^{170}_{\text{BASI}}$, and Wat$_{\text{BASI}}$ (Fig. 2A, Table I). It is likely that eliminating the charge of either Lys$^{140}$ or Asp$^{150}$ would result in increased local flexibility and perturbation of the interactions with domain B. The enthalpic loss caused by disruption of ionic bonds in addition to the high entropic penalty because of increased flexibility is consistent with the severe loss of inhibitory activity of these mutants (Table III). It has been reported earlier that the BASI-AMY2 interaction is mainly enthalpically driven and that temperature had little effect (<2.9 kcal/mol on the enthalpy of binding in the interval 25 °C–37 °C (8). Assuming little or no change in ΔH, increased conformational flexibility of BASI, equivalent to an increased entropic penalty of binding to AMY2 (ΔΔS < 0), would result in a large loss of binding at 37 °C as compared with 25 °C. This provides a possible explanation for the apparent difference between the SPR data showing less relative decrease in inhibitory activity for these mutants as compared with the inhibition kinetics data (Tables III and IV). Other factors, however, inherent with the SPR analysis such as rebinding on the chip surface cannot be ruled out. Similar arguments apply for the mutant D150N.

BASI Contacts to Domain A—Asp$^{156}$ is situated in a positively charged environment about 5.5 Å from Lys$^{168}$ and making an intramolecular salt bridge with Arg$^{156}$ presented by an adjacent loop. In addition, this residue makes a water-mediated hydrogen bond to AMY2 Pro$^{298}$O (Fig. 2C). The D156K mutant results in the loss of these interactions, and it changes the charge density in that region. Bearing in mind the drastic nature of such a mutation, this residue is unlikely to be critical for inhibition as judged by the limited, albeit significant, decrease in affinity concomitant with this mutation. A previous study has shown that Arg$^{155}_{\text{BASI}}$ is critical for inhibition of AMY2 (7). This residue makes several intramolecular interactions, as well as a direct hydrogen bond to Ser$^{208}$AMY2. Therefore it seems that the interactions conferred by Arg$^{156}_{\text{BASI}}$ grant cardinal importance to this residue as compared with Asp$^{156}$.
AMY2. Tyr\(^{170}\)N hydrogen-bonds with Glu\(^{168}\)Oe1, which makes the only direct bond in that region to AMY2 (Lys\(^{182}\)N\(_2\)), thus fixing this side chain in a favorable orientation to interact with the hydration shell of the embedded Ca\(^{2+}\). In addition, Tyr\(^{170}\)O is involved in an extensive hydrogen bonding network mediated by Wat\(^{336}\), which belongs to the hydration sphere of Ca\(^{2+}\) and also hydrogen-bonds to Glu\(^{168}\)Oe2. This solvent/Ca\(^{2+}\) system mediates contact with the three AMY2 catalytic residues Asp\(^{179}\), Glu\(^{204}\), and Asp\(^{289}\) (Fig. 2B). The Y170F mutant probably maintains the backbone hydrogen bond to Glu\(^{168}\), but it will lose contact with the solvent/Ca\(^{2+}\) system. Because these contacts would be retained by Glu\(^{168}\) the effect of Y170F is modest. By contrast, Y170P caused a 200-fold increase in \(K_I\). The double mutant E168Q/Y170P reiterates the deleterious effect of introducing the proline side chain as it results in a more than 3 orders of magnitude decrease of inhibitory activity as judged by \(K_I\), whereas E168Q shows a modest 25-fold increase of \(K_I\). The conservative mutation E168Q may compensate for the loss of the charged interaction by alternative hydrogen bonds either to Trp\(^{206}\)O or to Lys\(^{182}\)N\(_2\) in AMY2. In contrast, no inhibitory activity was detected for E168T, and this variant showed a substantial increase in \(k_{off}\), manifesting the loss of important short range interactions.

A previous study has demonstrated that Ca\(^{2+}\) modulates the AMY2-BASI affinity mainly by lowering \(k_{off}\) (8). It was not clear if Ca\(^{2+}\) was responsible for this effect or if it was caused by Ca\(^{2+}\) bound to domain B. The present data clearly implicate Ca\(^{2+}\) in the dependence of affinity on Ca\(^{2+}\) as judged by comparison of wild type, Y170F, and E168Q (Table V). By assuming that Ca\(^{2+}\) is associated with the relative affinity change (mainly due to altered \(k_{off}\)), this ion is likely to be bound with an affinity in the lower millimolar range. Insight into the affinity of Ca\(^{2+}\) may be instrumental for assessing the biological significance of such a Ca\(^{2+}\)-modulated inhibition, but an accurate affinity estimate is not warranted by current data. Most interestingly, one Ca\(^{2+}\) was recently identified at the same position in the solved crystal structure of AMY1 in complex with thiomethyl maltotetraoside, excluding this substrate from the active site (53).

Relation to Other \(\alpha\)-Amylase/Inhibitor Systems and the Energies of Binding—The determination of several complex structures between \(\alpha\)-amyloses and their inhibitors has unveiled the vast diversity of structural motifs and binding modes characterizing these systems (6). While providing valuable insight, structural data alone fail short of reliably pinpointing energetically important side chains along the interface of a protein complex. It is not uncommon that protein-protein interactions are dominated by a few high energy interactions, so-called “hot spots,” with most crystallographically observed contacts making limited contribution to the binding energy (54, 55). Typically, a hot spot is defined as a residue that when mutated to an alanine gives rise to a distinct drop of binding affinity of 2 kcal/mol or higher (54, 56, 57). In BASI, Asp\(^{150}\), Glu\(^{168}\), and Lys\(^{140}\) match this definition. The limitations of the used inhibition assay and protein availability precluded an estimate of \(K_I\) and thus \(\Delta G\) of the above-mentioned mutants. Nonetheless, it is evident that the affinity of these mutants decreased several orders of magnitude corresponding to \(\Delta G\) values in the 4–9 kcal/mol range attesting to their thermodynamic contribution. Most interestingly, these residues are clustered in one patch at the center of the inhibitor, whereas the peripheral residue Ser\(^{77}\) seems less significant for the binding. This is in conformity with reports suggesting that hot spots of binding energy are surrounded by a shell of energetically insignificant interactions referred to as an O-ring for excluding bulk solvent from hot spots (57). The involvement of a divalent ion and solvent in crucial interactions seems to be a unique feature of BASI, which docks on two regions of AMY2 separated by the deep catalytic groove and another minor solvent-filled cavity. The solvent exclusion shell is much more obvious in the complex structure of the lectin type \(\alpha\)-AI inhibitor with the mammalian PPA \(\alpha\)-amyrase, where the central region of this complex is protected from bulk solvent (22). By contrast to the hydrophilic nature of the BASI surface interacting with AMY2, \(\alpha\)-AI, which inserts two loops in the active site cleft of PPA, makes substantial hydrophobic interactions in its substrate mimetic action. Similarly, tendamistat inhibits PPA by inserting a segment presenting its characteristic Trp-Arg-Tyr. A comparison of the energetic contribution of various contact points of these inhibitors with their targets awaits detailed mutational studies. For instance, it has been reported on the basis of homology modeling that mutating Arg\(^{74}\), Trp\(^{185}\), and Tyr\(^{190}\) abolished the inhibitory activity of \(\alpha\)-AI on PPA (26).

Inspection of the complex structure, however, shows that only Tyr\(^{190}\) is in contact with PPA, implying that the loss of activity observed for the other two residues is probably caused by the perturbation of intramolecular interactions rather than loss of crucial contacts to PPA.

In conclusion, this is the first mutational study that addresses the interactions of a Kunitz-type inhibitor with target enzymes. The data presented illuminate the primary role of charge interaction in the context of BASI inhibitory potency on AMY2. Hence, the hot spots identified were charged residues making a range of intra- and intermolecular interactions. Moreover, the data strongly suggest that the fully hydrated Ca\(^{2+}\) at the AMY2-BASI interface modulates the activity of BASI, revealing a novel facet of \(\alpha\)-amyrase inhibition. The involvement of divalent ions and buried solvent in the steric and electrostatic complementarity of BASI and AMY2 affords additional attention and offers an engineering tool for up- or down-regulation of inhibition of this and related systems.

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