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Stopped-flow kinetic studies of the reaction of barley α-amylase/subtilisin inhibitor and the high pI barley α-amylase

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Abstract The interaction of α-amylase/subtilisin inhibitor (BASI) from barley seeds and the high pI barley α-amylase (AMY2) de novo synthesized during seed germination, has been studied at pH 8.0, 25°C, using stopped-flow fluorescence spectroscopy, equilibrium fluorescence titration and kinetic analysis of the displacement of BASI from the BASI–AMY2 complex by the substrate blue starch. The results are in accordance with a two-step reaction model:

$$\text{BASI} + \text{AMY2} \overset{K_1}{\rightleftharpoons} \text{BASI} - \text{AMY2} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{BASI} - \text{AMY2}^*$$

The resulting values of the kinetic parameters were: $k_2/K_1 = (1.0 \pm 0.2) \times 10^6\ M^{-1} \cdot s^{-1}$, $K_1 = 0.4 \pm 0.21$ mM, $k_2 = 320 \pm 150\ s^{-1}$, $k_{-2} = (7.2 \pm 0.6) \times 10^{-5} s^{-1}$, and the overall dissociation constant $K_d = (0.7 \pm 0.1) \times 10^{-10}$ M. BASI thus is best characterized as a fast reacting, tight-binding inhibitor of AMY2.

Key words: Reaction mechanism; Bifunctional α-amylase/serine protease inhibitor; Stopped-flow fluorescence kinetics; Substrate-mediated inhibitor displacement; Barley; High pI α-amylase

1. Introduction

Barley seeds contain a bifunctional α-amylase/subtilisin inhibitor, (BASI) of M_r 19,865 [1,2]. Based on sequence similarities it belongs to the soybean trypsin inhibitor (Kunitz) family [1–3]. A preliminary three-dimensional model has been constructed for the closely related wheat α-amylase/subtilisin inhibitor [4]. The strict isoenzyme specificity of BASI in its reactions with barley α-amylases has previously been established, thus BASI reacts with α-amylase 2 or high pI α-amylase (AMY2), but not with the 80% identical isozyme 1 or low pI α-amylase [5–7]. The BASI–AMY2 complex is tightly bound at pH 8 and shows a pH optimum more alkaline than that of the AMY2 substrate reaction [8–11]. The overall K_i of the BASI–AMY2 complex has been reported to be 2.2×10^{-10} M at pH 8, 37°C, in the presence of 0.05% BSA [7]. Compared with protein proteinase inhibitor reactions [12,13] little is known about those of proteinaceous α-amylase inhibitors. The stoichiometry of some reactions has been reported: α-amylase from *Tenebrio molitor* L. larvae binds two molecules of the 0.28 α-amylase inhibitor from wheat, and one molecule of the dimeric 0.19 α-amylase inhibitor from wheat [14]. No α-amylase inhibitor reaction has been thoroughly, kinetically investigated, but those of the red kidney bean α-amylase inhibitor and porcine pancreatic α-amylase have been partially characterized by Wilcox and Whitaker [15], who reported the formation of a 1:1 complex in a two-step reaction path.

In the present study the kinetics of the formation of complexes between BASI and AMY2 have been investigated in order to expand our knowledge of the inhibition mechanism of proteinaceous α-amylase inhibitors. The results were in accordance with a two-step reaction model, the equilibrium and rate constants of which were determined from a combination of stopped-flow and substrate displacement kinetic data. The BASI–AMY2 and the red kidney bean α-amylase inhibitor–α-amylase reactions apparently differ markedly in their second reaction step, so that, in spite of similar overall dissociation constants, they seem to belong to different classes. In contrast to the red kidney bean inhibitor, which is slow reacting, BASI was found to be a fast reacting, tight-binding inhibitor.

2. Materials and methods

2.1. Materials

The proteins were purified essentially as previously described: BASI [7] from barley seeds (cv. Piggy) and AMY2 [16] from barley kilned malt (cv. Triumf), each from Carlsberg Maltings. The protein concentrations were determined by amino acid analysis of acid hydrolysates (6 N HCl, 110°C, 24 h) using an LKB model Alpha Plus amino acid analyzer, or spectrophotometrically at 280 nm using $\varepsilon = 2.6 \times 10^4\ M^{-1} \cdot cm^{-1}$ for BASI [1,7] and $\varepsilon = 1.13 \times 10^5\ M^{-1} \cdot cm^{-1}$ for AMY2 [16]. Insoluble blue starch was from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade from either Merck, Darmstadt, Germany, or Sigma, St. Louis, MO.

2.2. Fluorescence titration

Intrinsic fluorescence emission spectra of BASI, AMY2 and the BASI–AMY2 complex were obtained using a Perkin-Elmer LS-50 fluorometer equipped with FLDM software. Excitation was at 280 nm with a slit width of 2.5 nm, the emission spectra were recorded in the range 300–500 nm with a slit width of 7.5 nm and a cut-off filter at 290 nm. The excitation slit width was kept at 2.5 nm in order to avoid marked decreases in the intrinsic protein fluorescence probably due to photochemical destruction of tryptophan residues, which was observed at values greater than 2.5 nm when samples were repeatedly excited. Titration experiments were performed at 25°C in 20 mM HEPES, 1 mM $CaCl_2$ at pH 8.0 by adding appropriate amounts of BASI (200 μl of 0–0.2 μM concentrations) to the sample and reference cuvettes containing, respectively, AMY2 (2.3 ml, 80 nM) and buffer (2.3 ml). After mixing and 5 min of preincubation, each emission spectrum was recorded in the range 300–500 nm, three times. The fluorescence intensities were each taken as the integrated signal and then averaged. The relative fluorescence change (ΔF) was calculated as $(F-F_0) \times 100\%/F_0$, where F is the averaged fluorescence intensity of the sample solution minus that of the reference solution, and F_0 is F of AMY2 in the absence of BASI.

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Abbreviations: BASI, barley α-amylase/subtilisin inhibitor; AMY2, barley high pI α-amylase; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; pI, isoelectric point.

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2.3. Stopped-flow fluorescence kinetics

The fast kinetics of the binding of BASI to AMY2 was studied using the stopped-flow technique. The changes in intrinsic protein fluorescence intensity indicative of complex formation were measured as a function of time after rapid mixing (dead time approx. 10^{-3} s) of solutions of BASI and of AMY2 in a HI-Tech Scientific PQ/SF-53 spectrofluorometer equipped with a high-intensity xenon arc lamp. The reactions were followed for various lengths of time (from 0.2 to 20 s), within which only insignificant decreases of intensity due to Trp destruction occurred. The excitation wavelength was 280 nm, and the slit width was 5 mm. Light emitted from the sample was monitored after passage of a cut-off filter (WG 320; 80% transmittance at 320 nm) to yield an integrated emission signal. In each experiment 400 pairs of data points were collected, and those of a series of 2–3 experiments were averaged. Each averaged set of data was then fitted to several non-linear equations using the HI-Tech HS-1 Data pro software. Experiments were carried out under second order reaction conditions by mixing equimolar concentrations of AMY2 and BASI in the range 0.09–0.7 μM. Similar series of experiments designed to obey pseudo first order conditions were performed using BASI in the range 0.8–100 μM and AMY2 in the range 0.3–6.5 μM. All experiments were performed at 25°C in 20 mM HEPES, 1 mM $CaCl_2$ at pH 8.0. The concentration dependence of the observed rate constant was analyzed using the non-linear least-squares fitting program GraFit [17].

2.4. Displacement kinetics

In each experiment two blue starch suspensions (50 ml: 12.5 g/l, 20 mM HEPES, 1 mM $CaCl_2$, pH 8.0), and two protein solutions (in the example shown in Fig. 4: 280 μl of 0.2 μM BASI + 280 μl of 0.2 μM AMY2 and 280 μl of buffer + 280 μl of 0.2 μM AMY2) were preincubated for 25 min at 25°C. Then at time, $t = 0$, protein and blue starch were mixed and kept at 25°C. The enzyme activity as a function of time was evaluated by A_{620} measurements of the supernatants of 1 ml samples, mixed with 0.5 ml NaOH (0.5 M), at various chosen times, t, to stop the reaction, and kept at 0°C until centrifugation. The blue starch assay is described by Česká et al. [18,19]. The time dependence of starch hydrolysis was analysed using the non-linear least-squares fitting program GraFit [17].

3. Results

3.1. Fluorescence titration

Fig. 1 shows a fluorescence titration curve of 74 nM (final concentration) of AMY2 with BASI. A 1:1 stoichiometry of the BASI–AMY2 complex is obvious. ΔF is proportional to the amount of complex formed, is positive, and amounts to a change of 25% of the intrinsic fluorescence of the complex relative to the sum of that of the same concentrations of BASI and AMY2. Fig. 1 shows equilibrium data obtained at nM concentrations of the reactants, which was found to be the lower limit of concentrations to give reproducable results. Clearly there is no sign of free, uncomplexed proteins at the equivalence point, which means that the K_d value of the complex is much less than 74 nM and cannot be determined from equilibrium measurements of any kind in this concentration range.

Table 1
Second order rate constants from stopped-flow experiments

[BASI] (μM)	k_{on} (μM^{-1}·s^{-1})
0.70	1.17 ± 0.03
0.53	1.20 ± 0.05
0.35	1.16 ± 0.06
0.27	1.20 ± 0.07
0.18	1.25 ± 0.08
0.13	1.43 ± 0.12
0.09	1.17 ± 0.15

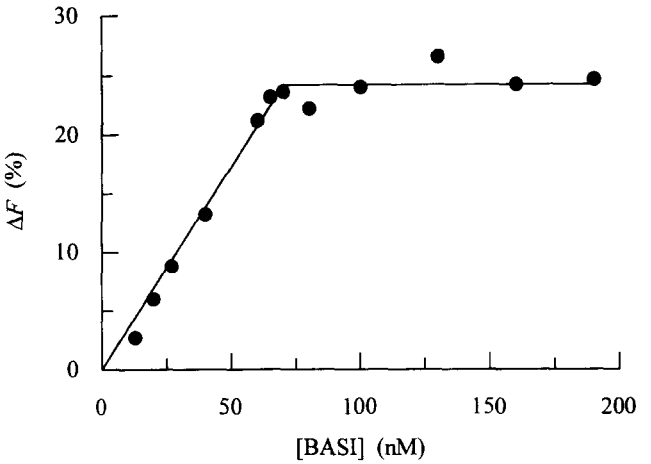

Fig. 1. Equilibrium fluorescence titration of AMY2 with BASI. Samples (pH 8.0, 25°C) containing 74 nM (final concentration) of AMY2 was mixed with BASI and their fluorescence spectra were obtained after preincubation for 5 min, which was sufficient for equilibrium to prevail. The relative fluorescence increase is plotted as a function of the BASI concentration.

3.2. Second order stopped-flow fluorescence experiment

Because of the large ΔF values resulting from the formation BASI–AMY2 complexes, it was possible to use low protein concentrations (i.e. in the nM range) in these experiments. Second order conditions were obtained using equal concentrations of BASI and AMY2, based on titrations as shown above. An example of the averaged stopped-flow traces obtained is shown in Fig. 2. Also shown in Fig. 2 is the trace resulting from the fit of a second order rate equation to the data. No other rate equation showed a good fit. The second order rate constants of the association (k_{on}) determined from this series of experiments are listed in Table 1, the resultant average value of this overall association rate constant is $1.23 \pm 0.08 \times 10^6$ M^{-1}·s^{-1}.

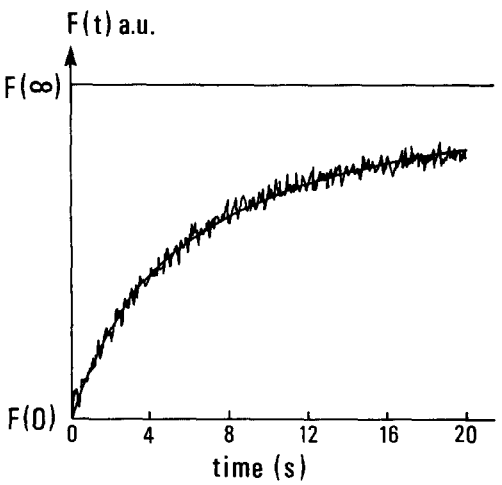

Fig. 2. Typical stopped-flow time-course of the intrinsic protein fluorescence intensity increase obtained after mixing of BASI and AMY2 at pH 8.0, 25°C. In the actual experiment the final protein concentrations were 0.13 μM BASI and 0.13 μM AMY2. The fitted curve corresponds to an overall second order progress: $F(t)-F(0) = [F(\infty) - F(0)]\,[1-((0.13 \times 10^{-6})\, k_{on}\, t + 1)^{-1}]$. The k_{on} values obtained from such fits are listed in Table 1.

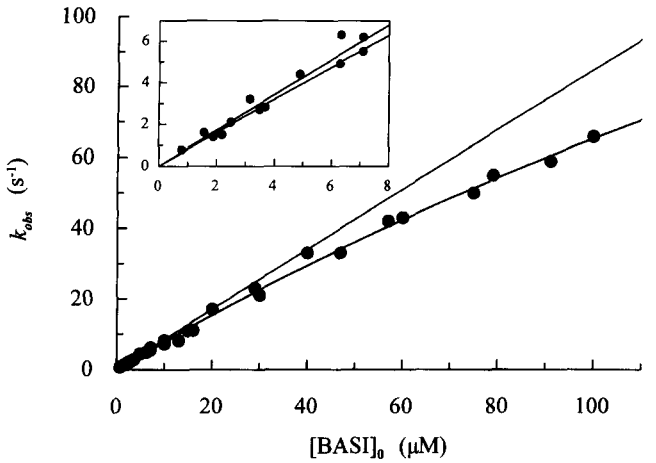

Fig. 3. Results of the stopped-flow kinetics of the BASI–AMY2 interaction in pseudo first order conditions using excess of BASI. The observed rate constant, k_{obs} is plotted against the concentration of BASI. The two curves shown are the one obtained by fitting of Eq. 2 to the data and a line obtained by a linear fit to the data in the concentration range 0.8–10 μM BASI; within experimental error the extrapolated k_{obs} value at 0 μM is zero. (Insert) An expanded view of the data at low concentrations, also the curves fitted are shown. Experimental conditions: pH 8.0, 25°C.

3.3. Pseudo first order stopped-flow fluorescence experiment

In these experiments excess of BASI was mixed with AMY2 and the resulting stopped-flow traces each showed a single exponential increase of the fluorescence corresponding to the expected (pseudo) first order reaction. A linear dependence of the observed first order rate constant, k_{obs}, on the concentration of BASI would indicate that BASI and AMY2 form a single complex, which gives rise to the intrinsic protein fluorescence change. As is seen in Fig. 3 the results, however, are more complicated than that, since with increasing concentration of BASI significant deviation from linearity is observed. This behaviour is in accordance with the two-step reaction model shown in Eq. 1:

$$\text{BASI} + \text{AMY2} \overset{K_1}{\rightleftharpoons} \text{BASI} - \text{AMY2} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{BASI} - \text{AMY2}^* \quad (1)$$

where K_1 is the equilibrium constant for a first step, k_2 and k_{-2} are the forward and reverse rate constants, respectively, of the second step, in which the observed change of the fluorescence, designated with *, takes place.

When the initial concentration of BASI ($[BASI]_0$) is much higher than the initial concentration of AMY2, as is the case when pseudo first order conditions prevail, k_{obs} is given by (Eq. 2) [20]:

$$k_{obs} = \frac{k_2[BASI]_0}{[BASI]_0 + K_1} + k_{-2} \quad (2)$$

The fitted curve (Fig. 3) corresponds to Eq. 2 (not taking the obviously vanishing value of k_{-2} into consideration). The values of k_2 and of K_1 obtained from this fit are given in Table 2. When $[BASI]_0 \ll K_1$, Eq. 2 reduces to:

$$k_{obs} = \frac{k_2}{K_1}[BASI]_0 + k_{-2} \quad (3)$$

According to Eq. 3 the initial slope of the plot of k_{obs} vs. $[BASI]_0$ (Fig. 3) is $k_2/K_1 = (0.79 \pm 0.04) \times 10^6\ M^{-1} \cdot s^{-1}$, which is in reasonable agreement with the value of $k_{on} = (1.23 \pm 0.08) \times 10^6\ M^{-1} \cdot s^{-1}$ from the second order experiments, as is in agreement with the reaction model suggested (Eq. 1).

3.4. Displacement experiments

Blue starch was found to be suitable as a substrate for displacement of BASI from AMY2 in the BASI–AMY2 complex. In this perturbation method a BASI–AMY2 system in equilibrium is perturbed by addition of substrate, and the resulting equilibration process is observed indirectly by measurements of hydrolysed starch as a function of time, the rate of which is proportional to AMY2 not complexed with BASI. Eq. 4 shows the scheme that describes the substrate displacement experiment:

$$\begin{gathered}\text{BASI} - \text{AMY2}^* \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{BASI} - \text{AMY2} \overset{K_1}{\rightleftharpoons} \text{BASI} + \text{AMY2} \\ \text{AMY2} + \text{S} \overset{K_m}{\rightleftharpoons} \text{AMY2} - \text{S} \overset{k_{cat}}{\longrightarrow} \text{AMY2} + \text{P}\end{gathered} \quad (4)$$

The rate limiting step of the total process is the dissociation of the BASI–AMY2 complex determined by k_{-2}, and the rate of substrate hydrolysis is proportional to the concentration of AMY2 free to react with the substrate at time, t, $E_F(t)$: $v_t = E_F(t)k_{cat}/(1 + K_m/[S])$, so that the product formation expressed as a function of time is (Eq. 5):

$$P = v_{eq}\, t - \frac{v_{eq}}{k_{-2}}(1 - \exp(-k_{-2}\, t)) \quad (5)$$

where P is the amount of product formed at time t after the pertubation of the initial equilibrium and v_{eq} the rate at final equilibrium. Fig. 4 shows the increase in P measured as A_{620} resulting from the hydrolysis of blue starch in the absence and presence of BASI. In the presence of BASI the slow release of AMY2 from the enzyme–inhibitor complex is demonstrated.

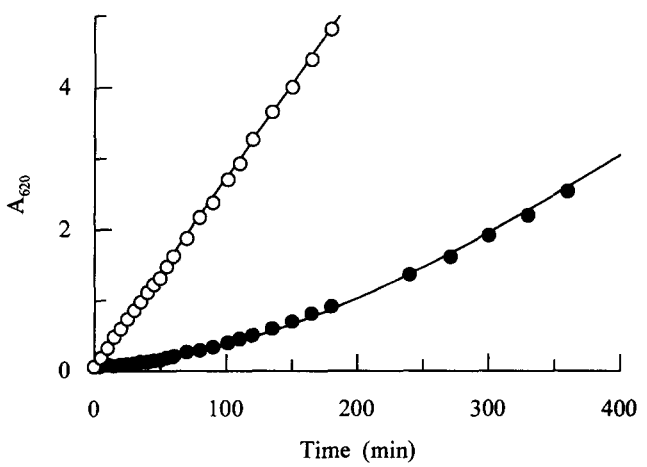

Fig. 4. Displacement kinetics experiment on BASI–AMY2 with blue starch (pH 8.0, 25°C). Typical examples of AMY2 catalysed hydrolysis of blue starch measured as the A_{620} as a function of time in the presence (●) and absence (○) of BASI are shown. Blue starch (12.5 g/l, so that $[S]/K_m = 11.4$) was added after equilibration of BASI (or buffer) and AMY2 and the re-equilibration was investigated. The final, total concentrations of BASI and AMY2 were each 1.1 nM. The rate, v (Table 2), of hydrolysis in the absence of BASI was obtained from a linear fit to the data (shown). A fit of Eq. 5 to the experimental data (curve shown) obtained in the presence of BASI provided v_{eq} and k_{-2} (Table 2).

Table 2
Kinetic parameters of the BASI–AMY2 complex formation in comparison with parameters from [15] of the red kidney bean α-amylase inhibitor–α-amylase reaction

Kinetic parameters and rates	Value	
	BASI-AMY2	Data from [15]
k_n [a] ($M^{-1} \cdot s^{-1}$)	$(1.23 \pm 0.08) \times 10^6$	
k_1/K_i [a] ($M^{-1} \cdot s^{-1}$)	$(0.82 \pm 0.18) \times 10^6$	6×10^3
K_i [a] (mM)	0.39 ± 0.21	(0.031 ± 0.002)
k_{-1} [a] (s^{-1})	320 ± 150	(0.051 ± 0.003)
k_{-2} [b] (s^{-1})	$(7.2 \pm 0.6) \times 10^{-5}$	$\sim 5 \times 10^{-8}$ [c]
k_{-2}/k_2 [a,b]	2.2×10^{-7}	$\sim 10^{-6}$ [c]
v_{eq} [b] (s^{-1})	$(2.35 \pm 0.05) \times 10^{-4}$	
v [b] (s^{-1})	$(4.39 \pm 0.02) \times 10^{-4}$	
K_d [b] (M)	$(0.7 \pm 0.1) \times 10^{-10}$	3×10^{-11}
K_d [a+b] (M)	$(0.9 \pm 0.2) \times 10^{-10}$	

Type of experiment: [a]stopped-flow; [b]displacement. [c]Calculated from values in [15].

A fit of Eq. 5 to the data shows a good correlation. The resultant values of k_{-2} and v_{eq} of this fit are listed in Table 2. In the absence of BASI the expected linear dependence of A_{620} on time with the rate v (the value of the slope) is obtained (Table 2). Since v is obtained at the same concentration of AMY2, as was used in the presence of BASI, v_{eq}/v equals the fraction of AMY2 uninhibited by BASI after re-equilibration.

The overall K_d of the BASI–AMY2 complexes can now be calculated from the known initial concentration, the ratio of the equilibrium rates v_{eq} and v, when the $[S]/K_m$ ratio of the substrate used is known (Eq. 6):

$$K_d = \frac{\left(\frac{v_{eq}}{v}\right)^2 [\mathrm{BASI}]_0}{\left(1 + \frac{[S]}{K_m}\right)\left(1 - \frac{v_{eq}}{v}\right)} \qquad (6)$$

For $v_{eq} = (2.35 \pm 0.05) \times 10^{-4}$ (A_{620}/s), $v = (4.39 \pm 0.02) \times 10^{-4}$ (A_{620}/s) as obtained here (Fig. 4) and $K_m = 1.1 \pm 0.1$ g·l^{-1} [7], K_d becomes $(0.7 \pm 0.1) \times 10^{-10}$ M, which is in good agreement with that calculated from k_{-2} of the displacement results and k_2/K_1 of the stopped-flow results: $K_d = K_1 k_{-2}/(k_{-2} + k_2) = k_{-2}(K_1/k_2) = (0.9 \pm 0.2) \times 10^{-10}$ M, where the low value of k_{-2} validates the assumption $(k_{-2} + k_2) = k_2$. All the kinetic results are listed in Table 2.

4. Discussion

The reaction kinetics and equilibrium of the interaction of α-amylase/subtilisin inhibitor (BASI) from barley seeds and the high pI barley α-amylase (AMY2) has been studied using stopped-flow fluorescence spectroscopy, equilibrium fluorescence titration and equilibrium pertubation with blue starch. A stoichiometry of 1:1 with a 25% increase of the intrinsic protein fluorescence is observed, when the BASI–AMY2 complex forms (Fig. 1). Tryptophanyl residues have previously been reported to be involved in the BASI–AMY2 interaction [9] and to be essential for the AMY2–substrate interaction [16]. The 1:1 stoichiometry is in accordance with inhibition assay data at pH 8 using blue starch as a substrate [7] and with the very recent finding that crystals of the BASI–AMY2 complex show a 1:1 stoichiometry of the two proteins [21]. The fluorescence titration results of Halayko et al. [9], that showed fluorescence intensity decrease and fluorescence quenching plateaus at 1:1 as well as at 2:1 BASI:AMY2 stoichiometry, were obtained on samples where the inhibitor was added successively to the enzyme and the sample after each addition was exposed to UV light. This procedure also in our hands led to fluorescence intensity decreases, as well as loss of protein activity due to photochemical tryptophanyl destruction.

The kinetic results obtained (Figs. 2–4, Table 2) agree with the reaction model described in Eq. 1. The formation of a tightly bound 1:1 BASI–AMY2 complex occurs in two reaction steps. The initial fast association forms an intermediate, which is rather loosely bound, with no apparent change of fluorescence. This is followed by a conformational change that tightens the complex. A concommitant large increase in the protein fluorescence is observed. Also the second step is rather fast, $t^1/_2$ corresponding to k_2 is of the order of magnitude 0.002 s. BASI, thus, at pH 8 is best characterized as a fast reacting tight-binding inhibitor of AMY2, its endogeneous target enzyme. Little is known about the kinetics of the reactions of the numerous other α-amylase inhibitors, except for the red kidney bean α-amylase inhibitor and its reaction with the non-endogeneous, porcine pancreatic α-amylase [15]. To compare these two α-amylase inhibitor systems Table 2 includes data from [15]. The two reactions have almost the same overall free energy change with similar overall dissociation constant, and the dissociation constants of the individual steps, K_1 and (k_{-2}/k_2), each differ by only one order of magnitude; the second reaction steps, however, are markedly different. The values of k_2, which reflect the transition state energies of that step, as well as the lifetimes of the intermediate complexes, differ by four orders of magnitude. Thus the red kidney bean inhibitor appears as a slow, tight-binding inhibitor, whereas BASI is as fast, tight-binding inhibitor.

The red kidney bean inhibitor–α-amylase complexes were reported to possess enzyme activity and to bind starch [15]. The initial slope of blue starch hydrolysis in Fig. 4 of the BASI–AMY2 complex is zero, which demonstrates the lack of amylase activity of its complexes. The reaction resembles numerous protease inhibitor–protease reactions [12,13], showing a two-step reaction scheme, being fast and forming a tight, inactive final complex. Although BASI is not an α-amylase substrate analogue, the interaction presumably involves a close contact of a reactive site of the inhibitor with the active site of the enzyme. It has been proposed that the reactive site of BASI involves at least one of the Arg residues (27, 127 or 155) and recent differential labelling studies using phenylglyoxal as reactant and AMY2 as protectant provides evidence that Arg-155 of BASI is a key group essential for the activity [7,22].

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