Arg-27, Arg-127 and Arg-155 in the β -trefoil protein barley α -amylase/subtilisin inhibitor are interface residues in the complex with barley α -amylase 2

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Arginine residues in barley α -amylase/subtilisin inhibitor (BASI) involved in binding to barley α -amylase 2 (AMY2) were differentially labelled using AMY2 as protectant and phenylglyoxal (PGO) and [14 C]PGO as modifying agents. Chymotryptic fragments of labelled BASI were purified by reverse-phase HPLC, and we concluded that the radiolabelled Arg-27, Arg-155 and most likely Arg-127, identified by amino acid, sequence and 14 C analyses, are protected by AMY2. While Arg-106 and Arg-107 showed intermediate reactivity and apparently were only partly accessible, Arg-15, Arg-41 and Arg-61 reacted with PGO and were thus exposed in the BASI–AMY2 complex. Patterns of arginine modification by [14 C]PGO in free or in AMY2-complexed BASI were consistent with the results of differential

labelling. The AMY2-protected arginines in BASI are at a distance from each other, as deduced from crystal structures of different β -trefoil proteins (*Erythrina caffra* and soybean trypsin inhibitors, interleukin-1 α and -1 β and WASI, the wheat homologue), suggesting that the BASI–AMY2 complex has multiple contacts at a larger interface. Accordingly, 11–16-residue-long BASI oligopeptides synthesized to include Arg-27, Arg-106/Arg-107 or Arg-127 were unable to suppress the formation of BASI–AMY2 or the effect of an inhibitory monoclonal antibody to BASI. Since Arg-27 is not conserved in rice and wheat ASIs, we further propose that Arg-155 in BASI is the kinetically identified PGO-sensitive group that is essential for inhibition [Abe, Sidenius and Svensson (1993) *Biochem. J.* 293, 151–155].

INTRODUCTION

α -Amylase inhibitors belong to distinct families defined by sequence similarities [1]. The barley α -amylase/subtilisin inhibitor (BASI), a member of the soybean trypsin inhibitor (Kunitz) family, is double-headed and specific for cereal α -amylases of the high-pI type and serine proteinases of the subtilisin family [1–5]. The physiological role of BASI has not been identified, but BASI mRNA is expressed in developing and germinating seeds [6] and an important *in vivo* function may be to inhibit α -amylase 2 (AMY2) which is synthesized *de novo* in aleurone cells during germination [7]. The three-dimensional structure of the wheat homologue, WASI, has a β -trefoil fold [8,9] found also for trypsin inhibitors from *Erythrina caffra* [10] and soybean [11,12], various proteins involved in recognition [9], e.g. the mammalian growth factors fibroblast growth factor [13], interleukin (IL)-1 α and IL-1 β [14–17], and in histactophilin, a unique actin-binding protein [18]. Since WASI and BASI have 90 % sequence identity, the three-dimensional structures of WASI [8] and BASI and of WASI–proteinase K [19] and BASI–subtilisin complexes respectively are very similar. A structural model, however, of the inhibitor– α -amylase complex and the mechanism of α -amylase inhibition remain to be reported.

BASI and AMY2 bind tightly at pH 8, with a K_i of 2.2×10^{-10} M [5]. The affinity is weakened with decreasing pH or increasing ionic strength [4,20]. Electrostatic forces dependent on groups titrating at neutral pH thus seem critical for the stability of BASI–AMY2. This is reminiscent of the arginine–carboxylate salt bridge postulated to be buried in a hydrophobic pocket by

the binding of pig pancreas α -amylase to tendamistat, a *Streptomyces* inhibitor with no sequence similarity to BASI [21,22]. Recently, barley AMY2 was shown to protect four arginine residues in BASI against inactivating modification by phenylglyoxal (PGO), and kinetic analysis suggested that a single arginine is essential for the inhibitory activity [5]. The present work aims at identification of arginine residues in BASI that are located at the BASI–AMY2 interface. Using a differential labelling approach we found that AMY2 protects Arg-27, Arg-155 and possibly Arg-127, while other arginines are either reactive and thus exposed in BASI–AMY2, or did not react at all with PGO. The overall reactivity pattern is in excellent agreement with the structural model of WASI [8,19]. Furthermore, the identified AMY2-protected arginines in BASI, in conjunction with the recently published crystal structure of WASI–proteinase K [19], support the proposal that a previously reported ternary BASI–AMY2–subtilisin complex [2] can be formed.

MATERIALS AND METHODS

Materials

Barley and malt were obtained from Carlsberg Maltings. BASI was purified [5] from barley seeds (cv. Piggy) and AMY2 from malt (cvs. Triumph or Menuet) essentially as described [23]; the AMY2-2 form [24] was used here. PGO was obtained from Aldrich; [14 C]PGO was from Amersham (specific radioactivity 1.1 GBq/mmol); insoluble blue starch was from Pharmacia; TPCK-treated α -chymotrypsin, L-arginine, 2-vinylpyridine and

Abbreviations used: AMY2, barley α -amylase isoenzyme 2 (high pI); BASI, barley α -amylase/subtilisin inhibitor; IL, interleukin; MAb, monoclonal antibody; 2-pe, 2-pyridylethyl; PGO, phenylglyoxal; PTH, phenylthiohydantoin; RASI, rice ASI; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; WASI, wheat ASI.

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BSA were from Sigma; ULTIMA GOLD scintillation liquid was from Canberra Packard; HPLC-grade acetonitrile, trichloroacetic acid and trifluoroacetic acid were from Lab-Scan; SP-Fractogel 650 M was from Merck; and dialysis membrane (cut-off 3500 Da) was from Spectrapor.

Chemical modification of arginine residues

Treatment of BASI-AMY2 with PGO or [^{14}C]PGO

BASI (100 nmol) and an excess of AMY2 (200 nmol) were preincubated for 30 min in 5 mM Hepes, 5 mM CaCl_2 , pH 8 (2.7 ml), at 37 °C to ensure that BASI was present in the complexed form, i.e. BASI-AMY2 [5]. PGO (1 M in 50 % ethanol) was added to 10 mM in a final volume of 5 ml and the Hepes concentration adjusted to 50 mM. The mixture reacted for 2 h at 37 °C, followed by addition of L-arginine (28 mM in a final volume of 7 ml) to quench the remaining PGO. After 30 min at 37 °C sodium acetate was added to a final concentration of 100 mM, adjusted to pH 4.8, to obtain dissociation of BASI-AMY2 under conditions where PGO-arginine is fairly stable [5,25]. The resulting mixture was dialysed against 20 mM sodium acetate, 1 mM CaCl_2 , pH 4.8 (3 $\times$ 3 litres), and the derivatized BASI was recovered by chromatography on an SP-Fractogel 650 M column (1.6 cm $\times$ 25 cm) eluted by a linear gradient (2 $\times$ 190 ml) of 0–0.5 M NaCl in the same buffer at a flow rate of 16 ml $\cdot$ h $^{-1}$ [5]. The activities of AMY2 and BASI were determined and fractions containing inhibitor only (75 nmol) were pooled and concentrated (as above) to around 10 ml.

For direct labelling [^{14}C]PGO, dissolved in a PGO solution (as above), was added (0.925 MBq) to BASI-AMY2 as for the PGO treatment. The resulting [^{14}C]PGO-BASI_{complex} was purified on SP-Fractogel.

Treatment of PGO-BASI or BASI with [^{14}C]PGO

PGO-BASI (75 nmol; see above) was dialysed against 5 mM Hepes, 5 mM CaCl_2 , pH 8, immediately prior to labelling with [^{14}C]PGO (0.925 MBq) to minimize exposure of the PGO-derivatized arginines at pH values above neutrality. Uncomplexed BASI (BASI_{free}) (0.4 mg $\cdot$ ml $^{-1}$) was modified in the same way. The reactions were quenched after 2 h (see above), and the resulting [^{14}C]PGO/PGO-BASI or [^{14}C]PGO-BASI_{free} was precipitated with 10 % (w/v) trichloroacetic acid, centrifuged and washed twice with 5 % (w/v) trichloroacetic acid and once with deionized water.

Preparation and purification of peptide fragments

Trichloroacetic acid-precipitated derivatized BASI was dissolved in 8 M guanidinium chloride (25 μ l) and the volume was increased to 1 ml by addition of 7 M guanidinium chloride, 0.1 M Tris and 10 mM EDTA, pH 7.5. The solution was flushed with N_2 (5 min) and after dithiothreitol (40 mM) reduction for 2 h at room temperature, 2-vinylpyridine (33 μ l) was added, followed by another portion (33 μ l) after 1 h [26]. After 40 min the mixture was dialysed against 10 % (v/v) ethanol. Aliquots were removed for ^{14}C and amino acid analyses and the mixture was lyophilized. Derivatized 2-pyridylethyl (2-pe)-BASI (1 mg $\cdot$ ml $^{-1}$) was digested by α -chymotrypsin in 1 M urea, 50 mM Hepes, pH 8, for 2 h at 37 °C and a proteinase/substrate ratio of 1:25 (w/w). The mixture of BASI fragments (3–8 nmol) obtained was made 0.1 % in trifluoroacetic acid (TFA), centrifuged, and the supernatant subjected to HPLC (Waters) using a Vydac C-18 column (0.45 cm $\times$ 25 cm) pre-equilibrated in 0.1 % TFA (solvent A) and eluted by a linear gradient of 0–100 % solvent B (90 % acetonitrile/0.1 % TFA) over 180 min at a flow-rate of

1 ml $\cdot$ min $^{-1}$. The eluate was monitored at 216 nm and fractions were collected manually. Of each fraction, 10 % was used for ^{14}C analysis and 50 % typically for amino acid analysis; the remaining portion was available for sequencing. Prior to the latter two analyses the samples were dried (Speed-Vac centrifuge) and redissolved in 200 μ l of 6 M HCl or 30 % (v/v) acetic acid respectively. Essentially all applied radioactivity was recovered in the HPLC eluate. Assuming a PGO/arginine ratio of 1:1 (mol/mol) in the product [25], the extent of [^{14}C]PGO incorporation was calculated from the specific radioactivity of [^{14}C]PGO in the reaction mixture of 4×10^3 d.p.m. $\cdot$ nmol $^{-1}$, the content of BASI or peptide being determined by amino acid analysis.

Analyses

N-terminal sequencing of peptides (approx. 0.1 nmol) was done using an Applied Biosystems Model 470A sequencer equipped with an on-line Model 120A phenylthiohydantoin (PTH) analyser. The derivative of PGO-arginine eluted approx. 30 s later than the PTH of arginine. Amino acid analysis was performed on hydrolysates (6 M HCl, 110 °C, 24 h) of protein or peptide (0.2–1.0 nmol) using a Pharmacia/LKB Alpha Plus amino acid analyser. The content of modified arginine was deduced from the loss of arginine [5] in BASI or fragments [3] thereof. The concentrations of AMY2 and BASI were determined either by amino acid analysis or spectrophotometrically at 280 nm using $A_{1\text{cm}}^{1\%} = 24$ [23] and $A_{1\text{cm}}^{1\%} = 13$ [5] respectively.

BASI inhibition assay

The activity of BASI was measured [5] by preincubation of AMY2 (30 nM) with the inhibitor (30 nM) in 40 mM Tris, 5 mM CaCl_2 , pH 8.0 (50 μ l), for 15 min at 37 °C, and analysis of residual α -amylase activity in an aliquot (25 μ l) using insoluble blue starch as substrate (6.25 mg $\cdot$ ml $^{-1}$) in the same buffer (4 ml) containing 0.5 mg $\cdot$ ml $^{-1}$ BSA. The reaction was stopped by addition of 0.5 M NaOH (1 ml) and insoluble starch was removed by centrifugation. The percentage inhibition was calculated as $100 \times [1 - (\text{Act}_i/\text{Act}_o)]$, where Act_i and Act_o represent α -amylase activity in the presence and the absence of inhibitor respectively, expressed as absorbance at 620 nm. One unit of α -amylase activity gives an increase of 1×10^{-3} in $A_{620} \cdot \text{ml}^{-1}$ over 15 min (for 5 ml) [27].

Synthetic BASI fragments

BASI oligopeptides Y 20 YVLSANRAHGGGLT 34 (1), CD 99 SE-LAAGRRHV 109 CG (2) and S 118 PSGRENAFRIEKYSG 133 (3), made by automated solid-phase peptide synthesis (generously provided by Dr. Morten Meldal, this laboratory), were dissolved in 30 % (v/v) dimethyl formamide and purified by reverse-phase (RP)-HPLC. The sequence was confirmed by amino acid and sequence analyses. Compared with the BASI sequence, 2 is N- and C-terminally extended by Cys and Cys-Gly respectively, to mediate disulphide loop stabilization. Oligopeptides encompassing Arg-155 were synthesized, but had too low solubility for testing in the different inhibition assays.

Peptides 1–3 were dissolved in 5 mM Hepes, 5 mM CaCl_2 , pH 8; the solution of 2 was flushed with air for disulphide formation. Peptides 1–3 were tested at concentrations ranging from 3 nM to 0.3 mM both as BASI mimetics and in competition with BASI. Following preincubation of 1–3 and AMY2 (30 nM) for 30 min at 37 °C, BASI (30 nM) was added and the residual AMY2 activity was measured as above.

Table 1 Properties of BASI derivatives

Unmodified arginine was determined by amino acid analysis. The activity gives the capacity to inhibit AMY2 relative to that of unmodified BASI. n.d., not determined.

Protein	Arg (mol/mol)	Activity (%)
BASI	12.0*	100
1. PGO-BASI†	8.8 $\pm$ 0.1	97
2. [14 C]PGO-BASI _{complex} ‡	9.0 $\pm$ 0.2	n.d.
3. [14 C]PGO-BASI _{free} ‡	7.0 $\pm$ 0.5	n.d.
4. [14 C]PGO/PGO-BASI§	4.0 $\pm$ 0.8	0

* see [3].
† Product from the first step of differential labelling.
‡ Arginine determination after 2-pyridylethylation.
§ Prepared by [14 C]PGO treatment of PGO-BASI (derivative 1).

Monoclonal antibodies (MAbs) to BASI

Mice were immunized with BASI and MAbs were prepared using a standard protocol [28]. Hybridomas producing MAbs to BASI were subcloned three times. MAbI and MAbII were purified from culture supernatants using Protein G (Bio-Rad) affinity chromatography according to the supplier's recommendation and tested by immunoblotting after SDS/PAGE according to a standard protocol [28] using BASI at ≥ 1 ng. They were tested for inhibition of BASI in the assay as above by preincubation for

15 min at 37 °C with BASI (six MAb concentrations in the range 30 nM–3 μ M). The α -amylase activity was measured towards blue starch and given in units/ μ g of AMY2. The effect of BASI oligopeptides 1–3 on the inhibitory MAbI was tested by incubation at peptide concentrations of 30 nM–300 μ M for 30 min at 37 °C prior to addition of BASI.

RESULTS

Chemical modification of BASI–AMY2 or BASI

Differential labelling

BASI–AMY2 was treated with PGO and PGO-BASI was purified [5] from the derivatized complex. This PGO-BASI, lacking 3.2 arginines (Table 1, derivative 1) but apparently no other residues, was fully active. Subsequent [14 C]PGO treatment of the PGO-BASI caused the loss of 4.8 additional arginines and of the inhibitory activity, leaving this BASI derivative with four unmodified arginines (Table 1, derivative 4). To identify the AMY2-protected arginines [14 C]PGO/PGO-BASI was reduced and 2-pyridylethylated to yield 2-pe-[14 C]PGO/PGO-BASI containing 2.5 [14 C]PGO/BASI molecules, which was cleaved by chymotrypsin and analysed using an HPLC peptide mapping approach.

Direct [14 C]PGO labelling

The accessibility of arginines in BASI was also described by [14 C]PGO modification of free BASI and of BASI–AMY2 (BASI_{complex}) without prior PGO treatment. The obtained 2-pe-[14 C]PGO-BASI_{free} lacked five arginines and 2-pe-[14 C]PGO-

Figure 1 Separation of chymotryptic fragments of derivatives of BASI

(a) RP-HPLC chromatogram for 2-pe-[14 C]PGO/PGO-BASI (only the first 80 min of the elution are shown). Numbered fractions refer to Table 2 (and Table 3). 14 C-contents are given for (b) fractions collected in (a); (c) 2-pe-[14 C]PGO-BASI_{free}; (d) 2-pe-[14 C]PGO-BASI_{complex}. Fractions 2 and 3 in (b) contain urea and no detectable peptides or amino acids.

Table 2 Chemical analysis of peptide fragments from different BASI derivatives

The numbering of BASI is as published [3]. N-terminal sequences and ^{14}C contents were determined of peptide fragments from RP-HPLC fractions (Figure 1a). The amino acid compositions matched the indicated sequences. ^{14}C PGO incorporation was calculated based on ^{14}C PGO = 4000 d.p.m./nmol (= 100%) and assumes a 1:1 molar ratio of PGO/Arg. The yield is the percentage of peptide recovered out of the total amount of BASI fragments applied (totals: **a**, 3.2 nmol; **b**, 8 nmol; **c**, 4 nmol). r = PGO-Arg; $r^{\dagger}$ = PGO-Arg content of around 50%; n.i. = not identified. Only the stretch up to the underlined residue (included) has been determined by sequencing. In most cases, the state of arginine modification was also deduced by amino acid analysis.

Arg residue	HPLC fraction	^{14}C (d.p.m.)	N-terminal sequence	Amount (nmol)	^{14}C PGO content (%)	Yield (%)
(a) 2-pe- ^{14}C PGO/PGO-BASI						
127	13	0	R ¹²⁷ IEKY-COOH	0.9	0	28
27	21	1 663	S ²⁴ ANrAHGGGLTM-COOH	0.4	104	13
27	23	830	S ²⁴ ANrAHGGGL-COOH	0.3	69	9
155			R ¹⁵⁵ DLKGGG-COOH	1.2	0	38
27	24	2 230	S ²⁴ ANrAHGGGL-COOH	0.5	21*	16
127			r ¹²⁷ IEKY-COOH	1.0	45*	31
15	27	346	r ¹⁵ ADANY-COOH	0.5	17	16
27	32	394	V ²² LSANrAHGGGL-COOH	0.2	0	6
41			T ³⁴ MAPGHGRHCPL-COOH	0.2	0	6
27	34	1 522	V ²² LSANrAHGGGL-COOH	1.0	38	31
41			T ³⁴ MAPGHGRHCPL-COOH	0.1	0	3
41	35	773	T ³⁴ MAPGHGRHCPL-COOH	0.4	0	13
155			r ¹⁵⁵ DLKGGG-COOH	0.3	72	9
15	36	1 199	P ³ PPVHDTAGHELrADANY-COOH	0.4	0	13
155			r ¹⁵⁵ DLKGGG-COOH	0.4	75†	13
75	39	3 848	G ⁶⁶ VAPSDKIIRL-COOH	0.5	0	16
155			r ¹⁵⁵ DLKGGG-COOH	1.0	96	31
27	44	1 298	S ²⁴ ANrAHGGGL-COOH	0.4	81	13
41			T ³⁴ MAPGHGRHCPL-COOH	0.1	0	3
41	46	1 604	T ³⁴ MAPGHGRHCPL-COOH	1.0	($\leq 40\ddagger$)	31
41	48	1 967	T ³⁴ MAPGHGRHCPL-COOH	0.4	0	13
106–107			H ⁹⁷ IDSELAAG ^{RH} HVITGPVKDPSPSGRENA-COOH	0.4	100	13
61	50	1 832	V ⁴⁷ SQDPNGQHDGFPVrITPY-COOH	1.4	11§	44
155			K ¹³⁹ LMSCGDWCQDLGVrDLKGGG-COOH	0.3	100§	9
61	52	2 679	F ⁴⁶ VSQDPNGQHDGFPVrITPY-COOH	1.5	($\leq 45§$)	47
(b) 2-pe- ^{14}C PGO-BASI _{free}						
15	34	4 925	A ¹ DPPPVHDTDGHELrADANY-COOH	1.5	82	19
—			C ¹⁴⁸ QDLGVF-COOH	0.7	0	9
15	35	8 861	A ¹ DPPPVHDTDGHELrADANY-COOH	1.6	85	20
41			T ³³ MAPGHGRHCPL-COOH	1.0	85	13
15	37	5 813	A ¹ DPPPVHDTDGHELrADANY-COOH	1.4	91	18
27			V ²² LSANrAHGGGL-COOH	0.4	100	5
41			T ³³ MAPGHGRHCPL-COOH	0.4	0	5
75			G ⁶⁵ VAPSDKIIRL-COOH	0.4	0	5
61	38	7 056	V ⁴⁷ SNDPNGQHDGFPVrITPY-COOH	0.5	99	6
155			r ¹⁵⁵ DLKGGG-COOH	1.2	99	15
41	42	4 901	T ³³ MAPGHGRHCPL-COOH	1.2	75	15
61			V ⁴⁷ SQDPNGQHDGFPVrITPY-COOH	1.2	100	15
41	45	13 862	T ³⁴ NAPGHGRHCPL-COOH	1.2	96	15
61			V ⁴⁷ SQDPNGQHDGFPVrITPY-COOH	2.4	96	30
61	47	9 144	F ⁴⁶ VSQDPNGQHDGFPVrITPY-COOH	2.5	91	31
41	54	6 197	M ³⁵ APGHGRHCPL-COOH	1.2	130	15
—			S ⁴⁸ QDPNGQHDGFCOOH	0.6	0	8
(c) 2-pe- ^{14}C PGO-BASI _{complex}						
15	37	3 527	A ¹ DPPPVHDTDGHELrADANY-COOH	0.6	98	19
41			T ³⁴ MAPGHGRHCPL-COOH	0.3	98	9
81			S ⁷⁷ TDVRISF-COOH	0.7	0	22
41	39	1 541	T ³⁴ MAPGHGRHCPL-COOH	0.5	0	16
75			G ⁶⁶ VAPSDKIIRL-COOH	0.7	0	22
106–107			A ¹⁰³ AG ^{RH} HVITGPVKDPSPSGRENA-COOH	0.7	100	22
—	47	3 840	T ³⁴ MAPGHGRHCPL-COOH	0.3	0	9
61			V ⁴⁷ SQDPNGQHDGFPVrITPY-COOH	1.1	86	34
—			G ¹⁴⁵ DWCQDLGVF-COOH	0.7	0	22
61	49	3 526	F ⁴⁶ VSQDPNGQHDGFPVrITPY-COOH	1.2	71	38

* Partially differentially labelled (see fraction 34, Table 3 and text).

† Assuming Arg-15 is not differentially labelled.

‡ Assuming Arg-41 is partially labelled.

§ The fraction contains 0.5–1.0 nmol of unidentified peptide.

|| Possibly partially labelled with PGO/Arg = 2:1 [25].

Table 3 Elevated content of unmodified Arg-127 and Arg-155 in peptide fragments due to AMY2 protection

The recovery (%) of unmodified BASI fragment was based on sequence* and amino acid† analyses. Fraction numbers for fragments of [^{14}C]PGO/PGO-BASI are shown in Figure 1(a). The C-terminal BASI fragment, KKAPPA, is recovered in essentially 100% yield from all three experiments shown in Figures 1(a)–1(d).

	Arg-127	Arg-155	
Fragment...	RIEKY	RDLKGGGA	KKAPPA
Fraction no....	13	23	7
Recovery (%) in...			
[^{14}C]PGO/PGO-BASI	28	38	100
[^{14}C]PGO-BASI _{free}	25*	30*	100†
[^{14}C]PGO-BASI _{complex}	50†	70†	100†

BASI_{complex} three arginines (Table 1, derivatives 3 and 2 respectively). Some loss of [^{14}C]PGO from the different BASI derivatives obviously occurred in the course of reduction and 2-pyridylethylation.

Identification of modified arginines in the different BASI derivatives

Chymotryptic fragments of 2-pe-[^{14}C]PGO/PGO-BASI (3.2 nmol) were separated by RP-HPLC (Figures 1a and 1b), and the [^{14}C]PGO contents were determined for individual peptides (Table 2a). Although the resulting elution profile was complex (Figure 1a), chymotrypsin was preferred for cleavage because mainly soluble fragments were generated. Since PGO and arginine can form different reaction products [25] and a given arginine residue may be found in fragments of varying length and appear in different states [1, unmodified; 2, differentially labelled (100% [^{14}C]PGO incorporation); 3, partially differentially labelled ([^{14}C]PGO < 100% and some PGO incorporation); 4, labelled but not protected by AMY2 (PGO incorporation)], a complex elution pattern and distribution of radioactivity are expected, making it very difficult to account completely for the fate of all arginine residues.

Fragments containing Arg-27 and Arg-155 were recovered from 2-pe-[^{14}C]PGO/PGO-BASI in total yields of 88 and 100% respectively (Figures 1a and 1b; Table 2a). Three highly radio-

labelled peptides (in fractions 21, 23 and 44) contained 35% of the recovered Arg-27. For Arg-155, 53% was in a highly radiolabelled form (in fractions 35, 36 and 39), 38% was unmodified (in fraction 23) and 9% had an unspecified degree of radiolabelling (in fraction 50). Arg-127 was found in unmodified and partially radiolabelled states (in fractions 13 and 24 respectively) in a total yield of 59%. A fragment containing Arg-106/Arg-107 (in fraction 48) had a [^{14}C]PGO/Arg ratio of 1:2 (mol/mol), and sequencing showed each arginine to be modified to around 50%. Arg-15, Arg-41 and Arg-61 were fully modified by PGO and contained only modest amounts of radiolabel (Table 2a). As expected, the peptide map of 2-pe-[^{14}C]PGO-BASI_{free} (results not shown) was virtually identical to that of 2-pe-[^{14}C]PGO/PGO-BASI, but with a different distribution of radiolabel (compare Figures 1b and 1c). 2-pe-[^{14}C]PGO-BASI_{complex} gave a different RP-HPLC profile (results not shown), since only arginines accessible in BASI-AMY2 were modified. It contained, as expected, considerably higher amounts of unmodified Arg-127 and Arg-155 than the two other derivatives of BASI (Table 3).

In summary, fully radiolabelled Arg-15, Arg-27, Arg-41, Arg-61 and Arg-155 were found in [^{14}C]PGO-BASI_{free}, whereas only Arg-15, Arg-41 and Arg-61 were identified as being radiolabelled in [^{14}C]PGO-BASI_{complex} (Tables 2b and 2c). In fractions containing the highest amount of radiolabel from [^{14}C]PGO-BASI_{free}, modified Arg-15, Arg-41 and Arg-61 were recovered in total yields of 57%, 58% and 82% respectively (Table 2b). For fraction 52 from [^{14}C]PGO/PGO-BASI (Figures 1a and 1b; Table 2a), containing modified Arg-61, some [^{14}C]PGO was present in unidentified peptides. Since Arg-61 was, however, readily modified in BASI or BASI-AMY2 by [^{14}C]PGO (Table 2c; Figure 1d), it is unlikely that AMY2 protects Arg-61. Arg-106/Arg-107 was partially modified in all three derivatives (not shown for [^{14}C]PGO-BASI_{free}). Arg-75 (fraction 39, Table 2a) and Arg-81 (fraction 37, Figure 1d; Table 2c) were unmodified, while peptides containing Arg-85 or Arg-122 were not recovered. On this basis we assume that these latter four residues are inaccessible to PGO under the conditions used, in agreement with previous quantitative analyses of the extent of arginine reactivity to PGO in BASI [5]. The present results clearly indicate that Arg-27 and Arg-155 are protected by AMY2, but fragments containing [^{14}C]PGO-Arg-127 were not detected in peptide maps of the directly [^{14}C]PGO-derivatized free BASI or BASI-AMY2

Table 4 Summary of arginine reactivity in free and AMY2-complexed BASI

Specific arginine residues were characterized by RP-HPLC peptide mapping of the three BASI derivatives (Figure 1, Table 2). +, ^{14}C -labelled; (+), partially ^{14}C -labelled; —, unmodified; n.i., not identified.

Arg residue	[^{14}C]PGO/PGO-BASI	[^{14}C]PGO-BASI _{free}	[^{14}C]PGO-BASI _{complex}	State of Arg in BASI-AMY2
15	—	+	+	Exposed
27	+	+	n.i.	Protected
41	(+)	+	+	Exposed
61	(+)	+	+	Exposed
75	—	—	—	Non-reactive
81	n.i.	n.i.	—	Non-reactive
85	n.i.	n.i.	n.i.	Non-reactive
106	(+)	n.i.	(+)	Partially exposed
107	(+)	n.i.	(+)	Partially exposed
122	n.i.	n.i.	n.i.	Non-reactive
127	(+)	n.i.	n.i.	Protected
155	+	+	n.i.	Protected

Figure 2 Reaction of MAbI with BASI

The inhibition of AMY2 by BASI is decreased with increasing [MAbI]/[BASI] (mol/mol). See the text for further details.

(Tables 2b and 2c; Figures 1c and 1d). However, the amounts of peptides recovered with unmodified Arg-127 (or for comparison Arg-155) in HPLC fractions of [14 C]PGO-BASI_{free} and [14 C]PGO-BASI_{complex}, equivalent to fractions 13 (Arg-127) and 23 (Arg-155) (Table 2a) of [14 C]PGO/PGO-BASI were at least 2-fold greater in [14 C]PGO-BASI_{complex} (Table 3). We conclude on this basis that Arg-127 is protected by AMY2, which is further supported by Arg-155 following the same pattern (Table 3).

The differentially labelled [14 C]PGO/PGO-BASI contains all information necessary for identification of reactive and protected arginines in BASI-AMY2. The [14 C]PGO labelling of free BASI and of BASI-AMY2 further strengthens the evidence of the overall reactivity of the 12 arginine residues in BASI, as summarized in Table 4. Thus Arg-27, Arg-155 and possibly Arg-127, found to be protected by AMY2, are not radiolabelled in [14 C]PGO-BASI_{complex}, while peptides identified from [14 C]PGO-BASI_{free} have radiolabelled Arg-27 and Arg-155, although not Arg-127 (Figure 1c; Table 2b). Approximately 35% unmodified arginines are isolated from differentially labelled BASI (fraction 23, Table 2a) even for the reactive side chains, with the exception of Arg-61. We assume that PGO has been lost from these groups during 2-pyridylethylation, amounting to a total loss of approx. 1.5 [14 C]PGO per molecule of derivatized BASI, as expected based on radioactivity measurements.

MAbs to BASI and BASI oligopeptide mimetics

Following SDS/PAGE, BASI (1 ng) was clearly detected by MAbI and MAbII in an immunoblot (results not shown). MAbI prevented BASI from inhibiting AMY2 (Figure 2), while MAbII was not inhibitory (results not shown), suggesting that the MAbs recognize different epitopes, and that the one specific for MAbI is at the protein-protein interface in BASI-AMY2. The three synthetic 11–16-amino-acid BASI fragments (see the Materials and methods section), containing Arg-27, Arg-106/Arg-107 or Arg-127, neither alone nor in combination inhibited AMY2 or competed with BASI in AMY2 inhibition, when added in up to 10^4 -fold molar excess. Reduced denatured BASI also did not inhibit AMY2. Finally, the three peptides (alone or in combination) were unable at a 10^4 -fold molar excess to suppress the MAbI inhibition of the formation of the BASI-AMY2 complex (results not shown).

DISCUSSION

Compared with a number of recent studies in which functionally important arginines were identified by [14 C]PGO labelling [29–31], the present work addresses much more complex derivatives, because BASI has a relatively high content of arginine [3] and several residues are protected by AMY2, the target enzyme [5]. Therefore, due to (1) the complexity of the PGO reaction, (2) the limited stability of its products and (3) the inherent difficulties in differential labelling and identification through peptide mapping of a protein-protein interaction involving several arginines, a thorough record is not available on all arginines in BASI with respect to PGO reactivity. The chosen strategy including chymotryptic degradation ensured isolation of 10 of the 12 arginines present in BASI in separate fragments suitable for sequencing. This enabled characterization of individual arginines as being either non-reactive or exposed in the BASI-AMY2 complex (Table 4). Arg-27, Arg-155 and most likely Arg-127 are radiolabelled in differentially labelled BASI and thus protected by AMY2. Arg-127 was not found to react in free BASI. However, we propose that it is protected by AMY2, supported by the observation that there is an increased amount of unmodified Arg-127 found in directly labelled BASI-AMY2 as compared with differentially labelled BASI and directly labelled free BASI. Perhaps prior PGO modification of BASI-AMY2 facilitated reaction of Arg-127 after dissociation. Arg-75, Arg-81, Arg-85 and Arg-122 were not found to react with PGO. Incomplete [14 C]PGO labelling of arginines protected by AMY2 may stem from modification by PGO subsequent to dissociation of BASI-AMY2 and prior to addition of [14 C]PGO to the derivatized BASI purified from the complex. Because modification of just one of the arginines in the binding site makes reassociation of AMY2 and BASI unlikely, such overmodified BASI is probably subject to additional aberrant modification. The results of the present study agree with earlier findings where 8 of 12 arginines present in BASI were modified by PGO, four of which were protected by AMY2 [5]. We propose that the single arginine identified kinetically as being critical for the inhibitory activity [5] is one of Arg-27 and Arg-155, which are both protected by AMY2. As explained below, we favour Arg-155 for this role.

In the model of WASI [8], a wheat homologue with 90% positional identity to BASI (Figure 3), Arg-27, Arg-127 and Arg-155 are located in the same region of the surface, which strongly supports our present identification of AMY2-protected arginines

Figure 3 Sequence comparison of BASI, WASI and RASI

The 12 β -strands (numbered 1–12) connected by loops in the β -trefoil fold are indicated according to [10]. Amino acid numbering is for mature BASI [3]. Arginines protected in BASI-AMY2 are indicated by arrows. Residues in lower case in RASI and WASI differ from those in BASI.

in BASI. The residues in WASI [8] that correspond to Arg-15, Arg-61, Arg-81 and Arg-85 in BASI (Figure 3) also group within a specific, but different, area of the surface. This agrees with our conclusion that Arg-81 and Arg-85, as clearly shown experimentally for only Arg-15 and Arg-61, are not at the interface of the complex. Interestingly a rice homologue, RASI, with 59 % sequence identity to BASI (Figure 3) inhibits insect α -amylase, but not cereal α -amylase [32], and the residues in RASI equivalent to Arg-15, Arg-27, Arg-41, Arg-85 and Arg-106 in BASI are non-conservatively substituted (Figure 3). Of these, only Arg-27 in BASI was protected by AMY2. We speculate, therefore, that Arg-27 is critical for the target-enzyme specificity of BASI. BASI Arg-127 and Arg-155 are conserved in RASI, and since Arg-155 is more reactive, Arg-155 is probably the residue indicated previously by the PGO inactivation kinetics to be essential for the inhibitory activity [5].

In an attempt to create mimetics of BASI, three oligopeptides were synthesized to contain Arg-27, Arg-106/Arg-107 and Arg-127 respectively. These either represent, or are located near, β -hairpin loops seen in the crystal structure of WASI (Figure 3; [8]). Since the oligopeptides were unable to substitute for BASI either in inhibition of AMY2 or in binding of the inhibitory MAbI, we presume that the β -trefoil scaffold is required to maintain the integrity of the inhibitory site in BASI.

A common structural region dictating proteinase-inhibitory or other functions does not seem to exist in the β -trefoil proteins [9], as supported by the following observations. The *Erythrina caffra* trypsin inhibitor has in the active site an arginine located in loop 4 of the β -trefoil structure [10]. The equivalent region in BASI encompasses Arg-75 (Figure 3). Arg-75, however, failed to react with PGO, and may not be available for BASI-subtilisin interaction. Moreover, the proteinase target structure of the homologue WASI, according to the crystal structure of its complex with proteinase K, is the bond between the conserved Thr-88 and Cys-89 [19] in loop 5 of the β -trefoil fold (Figure 3). Since inhibition studies indicated that formation of a ternary complex of AMY2, BASI and subtilisin can take place [2], distinct areas on the surface should include Arg-27, Arg-127 and Arg-155 respectively, and Thr-88 and Cys-89, as confirmed by the crystal structure of WASI [8]. However, a functional area can also be shared among members of the β -trefoil family. In IL-1 β a β -strand-connecting stretch analogous to Thr-111-Phe-126 in BASI, adjacent to the AMY2-protected Arg-127, is thus essential for biological activity, as identified by chemical modification [33]. In these β -trefoil proteins, however, topographically distinct parts also interact with the target proteins. Arg-15 in BASI is thus exposed and not protected by AMY2, whereas the equivalent arginine in IL-1 α and IL-1 β is critical in the cell surface receptor recognition that mediates signal transduction [29,34,35]. Nevertheless, the β -trefoil fold can be useful as a scaffold for structural elements that are important in the variety of protein-protein interactions. Loop 4 in IL-1 β , which forms a tight turn which is involved in receptor binding, has thus been successfully replaced by the longer loop 4 of soybean trypsin inhibitor to confer trypsin recognition and sensitivity on the resulting hybrid protein [36].

By using hybrids of the AMY1 and AMY2 isoenzymes [37], an important determinant for BASI binding has been localized to the small domain B of AMY2 that comprises Val-89-Leu-152 and protrudes from the (β/α)₈-fold of the catalytic domain between the third β -strand and helix [38]. Only AMY2 and hybrids with domain B of AMY2 origin were sensitive to BASI. The surface of BASI containing Arg-27, Arg-127 and Arg-155 thus presumably interacts with AMY2 at or near domain B, which is close to the catalytic site of AMY2. Domain B of AMY2

contains a highly conserved His-92 that is important in transition state stabilization [38], but does not contain residues acting in the chemical catalysis of glycoside bond hydrolysis [39,40]. BASI-AMY2 was crystallized recently [41] and the future determination of the three-dimensional structure, in conjunction with site-directed mutagenesis of BASI, AMY2 and AMY1/AMY2 hybrids [37,42], will allow mapping of the contribution of specific residues to the stability of BASI-AMY2. This will improve our insight into the molecular details of target enzyme specificity and the mechanism of inhibition of α -amylase by this type of proteinaceous inhibitor.

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