Surface-simulation synthesis of the substrate-binding site of an enzyme

Demonstration with trypsin

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From the X-ray co-ordinates of bovine trypsin and its complexes with substrate analogues (benzamidine) and with soya-bean trypsin inhibitor, a peptide (TP) was designed and synthesized by surface-simulation synthesis, a concept previously introduced by this laboratory, to mimic the binding site of trypsin. Also, a control peptide (CTP) was synthesized that contained all the amino acids present in the TP peptide, except that their order was randomized. The radioiodinated TP peptide bound specifically to adsorbents of benzamidine, whereas the control CTP peptide exhibited no binding activity. Conjugates to succinyl (3-carboxypropionyl)-lysozyme of the TP peptide, control CTP peptide and other unrelated peptides were examined by a radiometric binding assay for the ability to bind soya-bean trypsin inhibitor and human α_1 -antitrypsin. Conjugates of the TP peptide exhibited considerable binding activity to adsorbents of soya-bean trypsin inhibitor or α_1 -antitrypsin. None of the other peptide conjugates possessed any binding activity. Action of the active-site-directed reagents phenylmethanesulphonyl fluoride and di-isopropyl phosphorofluoridate on free TP and CTP peptides resulted in the modification of a serine residue in the TP peptide whereas the CTP peptide remained unaltered. The TP peptide, either in the free form or as a conjugate on succinyl-lysozyme, had no enzymic activity on protein substrates or on tosylarginine methyl ester. These findings indicated that the binding activity of an enzyme was well mimicked by the surface-simulation peptide but that reproduction of the catalytic activity was not obtained.

Previously, this laboratory introduced the concept of 'surface-stimulation' synthesis (Atassi *et al.*, 1976; Lee & Atassi, 1976), which was subsequently further well developed (Lee & Atassi, 1977*a,b*; Atassi & Lee, 1978*a,b*; Atassi & Zablocki, 1977; Atassi, 1978, 1979). In this concept, the spatially adjacent residues constituting a protein binding site are linked directly via peptide bonds with appropriate spacing and directionality into a single peptide that does not exist in the protein but mimics a surface region of it. This has proved to be

a powerful concept in protein molecular recognition and has provided a new dimension of unlimited versatility for synthetic mimicking of protein binding sites (for review see Atassi, 1980). In fact, binding sites representing antibody combining sites have been mimicked into single surface-simulation synthetic peptides that bind the antigen and effectively compete with the antibody for the antigen (Atassi & Zablocki, 1977; Twining & Atassi, 1978; Kazim & Atassi, 1980*a*).

Abbreviations used: TP peptide, the surface-simulation synthetic peptide designed to mimic the substrate-binding site of trypsin; CTP peptide, a control peptide having the same amino acid composition as the TP peptide except that they are randomized; iPrP-F, di-isopropyl phosphorofluoridate; PhMeSO₂F, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline (0.15M-NaCl in 0.01M-sodium phosphate buffer, pH7.2); Suc, 3-carboxypropionyl.

The surface-simulation synthesis of the substrate-binding site of an enzyme has not been reported. The present work examines the application of this concept to an enzyme binding site. Bovine trypsin is selected here to test the feasibility of the approach. The substrate-binding site of trypsin is mimicked by surface-simulation synthesis into a single peptide comprising the essential contact residues with appropriate spacing. The ability of this peptide to bind substrate analogues

and organic and protein inhibitors of trypsin is reported.

Materials and methods

Materials

Bovine trypsin was obtained from Worthington Diagnostic Systems (Freehold, NJ, U.S.A.). The Merrifield resin (0.8 mequiv. of Cl/g) and the *N*-*t*-butyloxycarbonyl amino acid derivatives were from Vega Fox Biochemicals (Tucson, AZ, U.S.A.). The side-chain-protecting groups used were; arginine, nitroguanidinium; serine, *O*-benzyl ether; tyrosine, 2,6-dichlorobenzyl ether; histidine, *m*-dinitrophenyl; lysine, ϵ -chlorobenzyl-oxycarbonyl; aspartic acid, β -benzyl ester; *t*-butoxycarbonyl was employed for the protection of all α -amino groups. The purity of all the amino acid derivatives was confirmed by t.l.c. All organic solvents employed for peptide synthesis were of spectroscopic grade and were again dehydrated and further purified by fractional distillation.

Synthesis and purification of the peptides

The peptides (see Table 4 for the structures) were synthesized by the solid-phase method in accordance with previously described procedures (Koketsu & Atassi, 1973, 1974a), except that in the present work the *t*-butyloxycarbonyl group was removed by treatment with trifluoroacetic acid in dichloromethane. The peptides were cleaved from the resin by HF at -10°C for 30 min. The dinitrophenyl protecting group on the imidazole side chain was removed by thiolation. The peptides were purified by gel filtration on Sephadex G-25 in 30% (w/v) dimethylformamide, followed by chromatography on Sephadex LH-20 in dimethylformamide and finally by ion-exchange chromatography on CM-Sephadex C-50 (Koketsu & Atassi, 1973, 1974a,b) in buffers containing 30% dimethylformamide. Peptide purity was checked by peptide mapping (Atassi & Saplin, 1968), h.p.l.c. on an analytical C_{18} column and amino acid analysis.

Preparation of Sepharose adsorbents

Coupling of trypsin inhibitor to CNBr-activated (March *et al.*, 1974) Sepharose CL-4B was carried out under the optimum conditions for the preparation of active adsorbents (Twining & Atassi, 1979). Adsorbents of unrelated proteins (bovine serum albumin, sperm-whale myoglobin and human haemoglobin A) and synthetic peptides [myoglobin-(132-153)-peptide (Young & Atassi, 1983), human haemoglobin-(1-15)-peptide (Kazim & Atassi, 1980b) and CTP peptide synthesized in the present work] were used as controls to determine non-specific background binding. The adsorbents

contained 1-1.5 mg of protein/ml of packed volume.

Preparation of adsorbents of *p*- and *m*-aminobenzamidine

A 1 ml portion of *p*-aminobenzamidine or *m*-aminobenzamidine (10 mg) in 0.2 M- NaHCO_3 , pH 9.5, was coupled overnight at 4°C to CNBr-activated Sepharose CL-4B. After the coupling, the Sepharose was washed to remove free uncoupled ligand (checked by reading the absorbance of the washing at 280 nm). The adsorbent was then masked by incubation at 4°C (with gentle rotation) with 1 M-2-aminoethanol (1 ml/ml of packed volume). This was followed by the following series of washes (three times each): 0.1 M-sodium acetate in 0.5 M-NaCl, pH 10; 2 M-urea in 0.5 M-NaCl; 0.1 M- NaHCO_3 in 0.5 M-NaCl, pH 9.0; and finally PBS. Sepharose adsorbents were stored at 4°C in an equal volume of PBS containing merthiolate.

Coupling of peptide to protein carrier

Because of the hydrophobic nature of the trypsin cavity the surface-simulation peptides were found to possess very low solubility in aqueous solvents. Therefore, in order to monitor their binding to trypsin inhibitors and substrates conveniently, we resorted to coupling them to very soluble carriers. For this purpose, succinylated (3-carboxypropionylated) lysozyme was mostly used. Synthetic peptides were coupled to Suc-lysozyme after activation of the carrier carboxy groups by conversion into the *p*-nitrophenyl ester groups (Atassi *et al.*, 1981). The reaction is performed in pure dimethylformamide, and has therefore proved to be convenient for the TP and CTP peptides because they are soluble in dimethylformamide. The extent of coupling was determined by amino acid analysis of acid hydrolysates of the peptide-protein conjugates.

Radiolabelling of proteins and peptides

Trypsin inhibitor, antibodies, protein A, peptides or peptide-protein conjugates (e.g. TP-Suc-lysozyme) were radiolabelled with ^{125}I (Amersham Corp., Arlington Heights, IL, U.S.A.) by using the chloramine-T method (Hunter & Greenwood, 1962). Unbound ^{125}I was separated from the radiolabelled sample by gel filtration on Sephadex G-15 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Protein-associated ^{125}I was assayed by precipitation with 10% (w/v) trichloroacetic acid.

Adsorbent binding studies

Quantitative adsorbent binding studies were performed with fixed amounts of adsorbents (50 μl) and increasing amounts of soluble ^{125}I -labelled peptide in glass tubes by the procedures described

previously (Twining & Atassi, 1979). The solvent for the titration studies was 20% (v/v) dimethylformamide/16% (w/v) glycine in PBS. Adsorbents of unrelated proteins and peptides and of the carrier protein (Suc-lysozyme, when peptide-protein conjugates were titrated) were used as controls for background binding.

Peptide binding to soya-bean trypsin inhibitor and α_1 -antitrypsin by plate assay

We have developed a convenient assay for rapid screening of the synthetic peptides that are designed to mimic the binding site of trypsin. The assay is a variation of a solid-phase radioimmunoassay previously described (Sakata & Atassi, 1981; Schmitz *et al.*, 1982). Briefly, the assay is carried out as follows. Polyvinyl radioimmunoassay plates (Costar, Cambridge, MD, U.S.A.) are incubated with trypsin inhibitor (5 μ g in 50 μ l of PBS) for 1 h at 37°C. After being washed with PBS (six times) the plates are incubated with 2% bovine serum albumin (100 μ l/well) for 1 h at 37°C. The plates are then washed (six times) and incubated with a peptide-Suc-lysozyme conjugate (5 μ g) in 0.1% bovine serum albumin in PBS (50 μ l/well) for 3 h at 37°C. After being washed with PBS (six times) the plates are incubated with mouse anti-lysozyme (1:1000 dilution in 0.1% bovine serum albumin in PBS) (50 μ l/well) for 2 h at 37°C. The plates are then washed with PBS (six times) and incubated with rabbit anti-mouse IgG (1:1000 dilution in 0.1% bovine serum albumin in PBS) (50 μ l/well) for 2 h at 37°C. Finally, the plates are washed with PBS (six times), incubated with 125 I-labelled protein A in 0.1% bovine serum albumin in PBS (2×10^5 c.p.m. in 50 μ l/well) for 2 h at room temperature, washed with PBS (six times), dried, cut into individual wells and their radioactivities counted on a γ -radiation counter. Plates coated with bovine serum albumin and with Suc-lysozyme (instead of trypsin inhibitor) are used as controls. Also Suc-lysozyme conjugates of unrelated peptides were used as additional controls.

Reaction of the TP peptides with iPrP-F and with PhMeSO₂F

For reaction with iPrP-F, the TP peptides were used in their free form because it is easier to determine, in amino acid analysis, the change in the amino acid composition when the free peptides are employed. Because the TP peptides are not soluble in aqueous solvents, dimethylformamide (at least 15%) was required in the reaction solvent.

For reaction with iPrP-F, the TP peptide (0.5 mg) was mixed with 10 molar excess of iPrP-F (0.5 mg) in 15% dimethylformamide/0.1 M-sodium phosphate buffer, pH 8.0. The total reaction volume was 250 μ l. The solution was stirred

magnetically for 3 h at room temperature. The peptide was then precipitated with cold (0°C) diethyl ether (3.0 ml), centrifuged, washed with ether five times on the centrifuge and subjected to hydrolysis and amino acid analysis. Reaction with PhMeSO₂F was carried out in an identical manner.

Analytical methods

Completed synthetic peptides, partial synthetic products at selected stages during synthesis, conjugates to carriers, and products of reaction with iPrP-F or PhMeSO₂F of free peptides were all checked by amino acid analysis. For amino acid analysis, protein or peptide samples (0.05 mg) were each dissolved in 0.5 ml of constant-boiling HCl (triple-distilled) in tubes that were then flushed with N₂, evacuated, sealed, and heated at 110°C for 22, 48 or 72 h. The hydrolysates were then freed of excess HCl on a rotary evaporator. Amino acid analyses were performed on a Beckman System 6300 high-performance analyser. The values for serine were obtained by extrapolation to zero hydrolysis time. Careful determination of the serine value is critical, because it is an essential active-site residue and is the site of covalent attachment of phosphorofluoridate compounds (e.g. see reaction with iPrP-F). Tryptophan was determined by amino acid analysis of hydrolysates with toluene-*p*-sulphonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu & Chang, 1971). The hydrolysates were assayed on a Beckman model 6300 amino acid analyser. Purity of synthetic peptides was checked by high-voltage (3000 V, 1 h) paper electrophoresis in pyridine/acetate buffer, pH 3.65, chromatography on an analytical-size h.p.l.c. C₁₈ column in an acetonitrile/0.5% H₃PO₄ (pH 2.3) gradient, and amino acid analysis.

Results

Purity of the synthetic peptides

After purification, the peptides were homogeneous by high-voltage electrophoresis and analytical h.p.l.c. and had amino acid compositions that were in full agreement with those expected from their respective covalent structures (see Table 3).

Binding of the peptides to trypsin inhibitor and to benzamidine adsorbents

Benzamidine has been employed extensively as a substrate analogue in binding studies by trypsin. The binding of free peptides to benzamidine was studied on adsorbents of benzamidine. However, since benzamidine has no reactive functional group by which to attach it to Sepharose, we used two derivatives for that purpose, namely *p*-aminobenzamidine and *m*-aminobenzamidine.

Both the *p*-aminobenzamidine and the *m*-aminobenzamidine derivatives were used in order to examine whether the point of attachment to the Sepharose anchor relative to the amidine group influences the binding of the peptides. Because of the insolubility of the peptides, they were labelled with ^{125}I and titrated with adsorbents, with as solvent 20% dimethylformamide in 1.6% glycine/PBS. Previously, it was determined by using different protein-free solvents and carrier that this was the most adequate solvent. Results of such a titration are summarized in Table 1.

It is evident from these results that binding of labelled TP peptide to the substrate and to trypsin-inhibitor adsorbents did occur, and that it was specific since labelled CTP peptide exhibited no binding to these adsorbents. The small amount (710 c.p.m.) of CTP peptide bound when 3×10^5 c.p.m. was added was evidently non-specific, because no binding was obtained when lower or higher (5×10^5 c.p.m.) amounts of labelled peptide were added. However, the small amount of label bound, relative to amount of label added, indicated that the conditions of the binding needed to be improved. The difficulty most probably stemmed from the insolubility of these peptides and the fact that the solvent conditions of the experiment were unsatisfactory for binding. It was apparent that a different experimental strategy had to be found, and we resorted to coupling the peptides to a soluble protein carrier.

Binding of peptide-Suc-lysozyme conjugates to trypsin inhibitor

For a soluble carrier, hen lysozyme was selected because of its convenient size and high solubility. The solubility was further improved by extensive succinylation of the protein (Suc-lysozyme) before it was employed as a carrier. Obviously carriers of other proteins or amino acid polymers could be used, but we have not yet employed them in this work. The peptide-Suc-lysozyme conjugates were completely soluble in PBS.

For convenience, we developed a rapid plate binding assay (see the Materials and methods section) to monitor the binding of the peptides to trypsin inhibitor. In this assay, trypsin inhibitor is bound to the wells. This is followed with the peptide-Suc-lysozyme conjugate, then with mouse anti-lysozyme (1:1000, v/v), then with ^{125}I -labelled protein A. The results are summarized in Table 2. It is shown in Table 2 that the TP peptide-protein conjugate exhibited considerable binding to trypsin inhibitors. The specificity of the binding is indicated from the finding that the control CTP peptide-protein conjugate or conjugates of other unrelated peptides to Suc-lysozyme, or Suc-lysozyme alone, exhibited no binding activity.

Binding of PhMeSO₂F and iPrP-F to TP and CTP peptides

To determine whether the TP peptide will mimic the binding activity of trypsin to its inhibitors, it was allowed to react with PhMeSO₂F and iPrP-F. The reaction of CTP peptide with each compound was done concurrently with controls. Since the peptides are insoluble in aqueous solvents, binding studies were done (3 h, room temperature) with as solvent 15% dimethylformamide in 0.1 M-phosphate buffer, pH 8.0 (see the Materials and methods section). After reaction, the peptides were isolated from the mixture and subjected to acid hydrolysis and amino acid analysis.

The results showed that binding of PhMeSO₂F and iPrP-F to TP peptide afforded a stable conjugate in which a covalent bond was formed between the inhibitor and a serine residue on the TP peptide (Table 3). The specificity of the binding (and the ensuing reaction) was indicated from the finding that the control CTP peptide suffered no covalent alterations under the same conditions (Table 3). We have not determined which of the three serine residues in the TP peptide is the point of attachment to the inhibitor, but it is not unreasonable to suggest that this may be the

Table 1. *Binding of ^{125}I -labelled peptides to adsorbents of benzamidine and of soya-bean trypsin inhibitor*
Results represent the average of triplicate analyses, which varied $\pm 1.5\%$ or less, and they have been corrected for non-specific binding (0.35–0.38% of total label) of the labelled peptides to uncoupled Sepharose.

		^{125}I -labelled peptide bound (c.p.m.)							
Radioactivity added ...		120 $\times 10^3$ c.p.m.		200 $\times 10^3$ c.p.m.		300 $\times 10^3$ c.p.m.		500 $\times 10^3$ c.p.m.	
Adsorbent	Peptide ...	TP	CTP	TP	CTP	TP	CTP	TP	CTP
Soya-bean trypsin inhibitor		1060	0	1650	0	2950	710	7830	0
<i>p</i> -Aminobenzamidine		1500	0	1580	0	3650	0	7600	0
<i>m</i> -Aminobenzamidine		1310	0	1770	0	2310	0	5440	0

Table 2. *Binding of peptide-Suc-lysozyme conjugates to soya-bean trypsin inhibitor and human α_1 -antitrypsin*

Values represent the average of triplicate analyses which varied $\pm 1.3\%$ or less. These values have not been corrected for the non-specific binding of the negative control. The conjugates of nonsense peptide to Suc-lysozyme or Suc-lysozyme itself do not bind to trypsin inhibitor, and therefore they serve as negative controls for the binding of peptide-Suc-lysozyme conjugates. For positive control, Suc-lysozyme was bound directly to the well (instead of trypsin inhibitor) and was followed (after appropriate washing) by 2% bovine serum albumin (instead of protein-peptide conjugate). The amount of anti-lysozyme antibodies bound to Suc-lysozyme was 19760 c.p.m. This served as an appropriate positive control for the antisera and the reagents, also gave an estimate of the maximum amount of label expected to be bound in the assay. The structure and synthesis of the nonsense peptide have been reported (Twining & Atassi, 1979).

Protein or conjugate added	Protein coated to well ...	Amount of anti-lysozyme bound (c.p.m.)	
		Soya-bean inhibitor	Human α_1 -antitrypsin
TP peptide-Suc-lysozyme		19110	18540
CTP peptide-Suc-lysozyme		410	400
(Nonsense peptide)-Suc-lysozyme		460	420
Suc-lysozyme		430	410

Table 3. *Amino acid composition of TP and CTP peptides and the products of their binding with PhMeSO₂F or iPrP-F*

Results represent the averages of triplicate analyses of acid hydrolysates at 22h, 48h and 72h. Tryptophan values were derived from duplicate hydrolyses with toluene-*p*-sulphonic acid containing 0.2% aminoethylindole. Values for serine were obtained by extrapolation to zero hydrolysis time. Abbreviation: N.D., not determined. Note that the serine content in the PhMeSO₂F and the iPrP-F products decreased by one residue, implicating a serine as the site of attachment to these compounds. The hydrolysis product of the inhibitor-serine adducts is co-eluted with alanine, and hence the alanine value is increased by one residue.

Amino acid composition (mol of residue/mol)								
Amino acid	TP peptide				CTP peptide			
	Expected	Found	Product with PhMeSO ₂ F	Product with iPrP-F	Expected	Found	Product with PhMeSO ₂ F	Product with iPrP-F
Asp	3	3.21	2.99	2.90	3	3.13	3.04	3.02
Ser	3	2.96	1.89	1.93	3	3.16	3.00	3.01
Glu	1	0.88	0.78	0.82	1	0.76	0.98	1.00
Gly	10	10.2	10.3	9.62	10	9.47	10.30	9.90
Ala	2	1.95	3.11	3.02	2	1.85	2.05	1.98
Val	1	1.02	1.10	1.16	1	0.90	1.14	1.15
Leu	1	0.99	0.98	1.11	1	1.36	1.07	1.38
Tyr	1	1.18	1.25	1.02	1	1.33	0.84	0.97
Phe	1	1.35	1.16	0.94	1	0.86	1.11	0.99
Trp	1	0.90	N.D.	N.D.	1	1.00	N.D.	N.D.
His	2	2.00	2.00	1.98	2	1.97	1.95	2.01

residue corresponding to Ser-195 (i.e. residue 7 in the peptide). However, this requires verification. But, regardless of which serine residue is involved in the attachment to the inhibitor, the fact remains that the TP peptide bound irreversibly to the inhibitor forming in the process a stable covalent bond.

Enzymic activity of the TP peptide

The finding that the TP peptide exhibited specific binding activity with α_1 -antitrypsin, soya-

bean trypsin inhibitor, substrate analogues and the serine-esterase irreversible inhibitors PhMeSO₂F and iPrP-F posed the intriguing question whether the TP peptide possessed enzymic activity. The TP peptide was incubated at pH 8.5 and 37°C with protein substrates (globin of human haemoglobin, apomyoglobin) for 2 days. Examination of the mixture by peptide mapping revealed that no free fragments were generated from the protein. However, because of the low solubility of the peptide in aqueous solvents, the reaction by necessity was a

heterogeneous reaction (the protein substrate was in solution but the peptide was in fine suspension), and it was therefore re-examined with soluble TP peptide-Suc-lysozyme conjugate. The TP peptide conjugate exhibited no enzymic activity with protein substrates.

Also, the soluble TP peptide-Suc-lysozyme conjugate had no hydrolytic activity (pH 8.1, room temperature) on *N*²-*p*-tosyl-L-arginine methyl ester, a substrate commonly used to monitor trypsin enzymic activity. Thus it was evident that the TP peptide, generated by surface-simulation, expressed the binding activity of trypsin with substrates and inhibitors but not its catalytic activity.

Discussion

The three-dimensional structure of bovine trypsin, iPr₂P-trypsin and complexes with trypsin inhibitor and benzamidine substrate analogues are known in detail (Stroud *et al.*, 1974; Chambers & Stroud, 1977; Huber & Bode, 1978). It is therefore possible to design surface-simulation peptides that will mimic the binding site of a serine esterase. The studies described in the present paper were aimed at mimicking the substrate-binding site of bovine trypsin into a surface-simulation peptide that expresses the binding activity of the enzyme molecule with substrates and inhibitors. Trypsin was chosen as the model for these studies because its X-ray structure is known in fine detail. The structure of iPrP-F-inhibited trypsin, initially determined by Stroud *et al.* (1971, 1974), was subsequently refined to 0.15 nm (1.5 Å) resolution (Chambers & Stroud, 1977). Also, the binding of trypsin to soya-bean trypsin inhibitor afforded the making of a convenient assay by which the surface-simulation peptides could be screened for binding activity.

The substrate-binding site of trypsin in complex with substrate analogues (benzamidine) and with trypsin inhibitor is known in great detail at 0.19–0.15 nm (Huber & Bode, 1978; Chambers & Stroud, 1977; Bode & Schwager, 1975; Huber *et al.*, 1974, 1975). The following summarizes the amino acids that have been identified as the contact residues of the trypsin binding site (see also Fig. 1). Tyr-39 hydrogen bonds with trypsin inhibitor. His-40 is buried in the trypsin-inhibitor complex and participates in polar interaction with Arg-17 of trypsin inhibitor. The residues Phe-41, Ala-55, Ala-56, His-57, Tyr-94 and Leu-99 are all buried in the trypsin-inhibitor complex. Phe-41 participates in hydrogen-bonding with trypsin inhibitor. Asp-189 participates in interaction with benzamidine and with ε-amino groups of lysine or

guanidino groups of arginine in the substrate. Ser-190 is buried in the trypsin-inhibitor complex and participates in the binding to benzamidine. Cys-191 also resides in the binding pocket to benzamidine. The residues Gln-192, Gly-193, Ser-195, Ser-214, Trp-215 and Gly-216 all form hydrogen bonds with trypsin inhibitor and are buried in the trypsin-inhibitor complex. Also, Trp-215 forms the hydrophobic part of the benzamidine-binding site. Finally, His-57, Asp-102 and Ser-195 are important residues in the catalytic centre of the enzyme. The distances ($C_{(\alpha)}$ to $C_{(\alpha)}$) separating the residues of the trypsin binding site are given in Table 4.

From the X-ray co-ordinates and by taking into account the participation of the residues listed

Table 4. *Design of the surface-simulation peptide mimicking the substrate-binding site of trypsin*

Column A identifies the contact residues in the binding cavity of the enzyme and the distances (from $C_{(\alpha)}$ to $C_{(\alpha)}$, in nm) separating appropriate residues. Column B indicates the surface-simulation synthetic peptide (TP) designed to mimic the binding site. Column C indicates control peptide (CTP) that has all the essential residues of the binding site except that they are randomized. The peptides are written with the *N*-terminus on the top and the *C*-terminus on the bottom.

A	B	C
Contact residues and distances	Surface-simulation synthetic peptides (TP)	Control synthetic peptide (CTP)
Asp-189	Asp (<i>N</i> -terminus)	Trp (<i>N</i> -terminus)
Ser-190	Ser	His
0.49 nm	Gly	Gly
Gln-192	Gln	Asp
Gly-193	Gly	Gly
Asp-194	Asp	Asp
Ser-195	Ser	Val
0.85 nm	{ Gly	Ala
	{ Gly	Gly
Val-213	Val	Gln
Ser-214	Ser	Phe
Trp-215	Trp	Ser
0.85 nm	{ Gly	Gly
	{ Gly	Gly
Leu-99	Leu	Asp
0.55 nm	Gly	His
Asp-102	Asp	Gly
0.53 nm	Gly	Ser
Ala-55	Ala	Gly
Ala-56	Ala	Ser
His-57	His	Gly
0.50 nm	Gly	Ala
Cys-42	Gly	Gly
Phe-41	Phe	Leu
His-40	His	Gly
Tyr-39	Tyr (<i>C</i> -terminus)	Tyr (<i>C</i> -terminus)

Fig. 1. Model showing the region of the substrate-binding site of trypsin (i.e. not the entire trypsin molecule) The upper panel gives a top view, looking down into the binding cavity. From this perspective, the following contact residues can be seen: Tyr-39, Phe-41, His-57, Ala-56, Asp-102, Leu-99, Trp-215, Ser-214, Val-213, Ser-195, Asp-194, Gly-193, Gln-192, Ser-190 and Asp-189. The lower panel gives a side view of the cavity, taken from the left side of the upper panel.

above in the function of the enzyme, a peptide (TP) was designed by surface-simulation synthesis to mimic the binding site of trypsin. It should be emphasized that this peptide does not exist in

trypsin, but rather mimicks the topographic arrangement of the residues in its substrate-binding cavity. The element of the design, which is based on spatial and distance considerations, are

shown in Table 4. A control peptide was also prepared that incorporated all the contact and spacing residues but in random fashion (CTP). The control peptide was crucial in order to confirm, should binding activity be found in the TP peptide, that such binding is indeed specific and not caused by some unusual charge or hydrophobic effects.

The finding that the radiolabelled TP peptide was able to bind to benzamidine and trypsin-inhibitor adsorbents, whereas the control CTP peptide exhibited no binding activity, was encouraging and suggested that the peptide must have mimicked the architecture of the site. The relatively small amount of label bound, which was of concern, may have been due to the fact that the binding studies were carried out in the presence of 20% dimethylformamide because of the insolubility of both peptides in purely aqueous solvents. The presence of such an organic solvent would be expected to interfere in the binding of the peptide, which involves predominantly hydrophobic interactions on the peptide.

To avoid the aforementioned complications stemming from peptide insolubility, it was decided to couple the peptide to a soluble carrier. We have used here fully succinylated lysozyme as a carrier, because of its high solubility, but clearly any soluble carrier should serve equally as well. The coupling procedure (Atassi *et al.*, 1981), which involves activation of the carboxy side chains on the carrier by conversion into their *p*-nitrophenyl ester groups, avoids carrier-carrier cross-linking (due to prior succinylation of all the amino acid groups on the protein) and peptide polymerization side reactions, since the activated carboxy groups reside only on the carrier and the reactive nucleophile is the α -amino group of the peptide. Having obtained soluble peptide conjugates, we investigated their abilities to bind to trypsin inhibitor. The binding of the TP peptide conjugates both to soya-bean trypsin inhibitor and to α_1 -antitrypsin and the absence of any binding activity in the CTP peptide conjugates confirmed the correctness of the peptide design.

We have also investigated the action of other inhibitors known to react at the substrate-binding site. The action of PhMeSO₂F and iPrP-F on acetylcholinesterase, chymotrypsin, trypsin and other serine proteinases has been well studied (e.g. Fahrney & Gold, 1967; Singer, 1963; Turini *et al.*, 1969; Morgan *et al.*, 1972). In each case, the binding between the enzyme and inhibitor results in the formation of a covalent bond via the active serine residue of the enzyme. It was gratifying, and at the same time quite surprising, to find that the TP peptide reacted with both PhMeSO₂F and iPrP-F, the reaction in each case taking place at a serine residue in the peptide. Again the lack of

reaction with the CTP peptide confirmed the specificity of reaction with the TP peptide. Although it is tempting to speculate that the reaction may have occurred with the serine residues at the seventh position in the peptide (i.e. the residue representing Ser-195 in the mimicked structure), we have not yet determined which of the three serine residues on the peptide participate in the reaction with PhMeSO₂F or iPrP-F.

The peptides exist in solution in a conformational equilibrium, the time average of which will be random, and there will exist at any given time conformational states that are energetically favoured at appropriate energy minima and whose mode of folding approximate that required for binding (Atassi & Saplin, 1968). Those species are able to bind with the target molecule and are thus removed from the equilibrium, which should then shift in their favour (Atassi & Saplin, 1968). It should also be noted that, since peptides in the conjugates are attached only via their α -amino end, they would be able to fulfil the requirement of conformational flexibility for proper wrapping around the small ligands or assuming folding that is favourable for binding with protein inhibitors. Appropriate peptide conformation can also be induced by the binding molecule (Atassi, 1975).

The absence of enzymic activity with the TP peptide was disappointing, but was not entirely unexpected. The exact mechanisms of enzymic catalysis are not really fully understood, in spite of the voluminous literature on the subject. Catalysis is also subject to very stringent conformational and distance requirements and, since the distances and conformational relationships of the residues in a surface-simulation synthetic peptide do not precisely duplicate the corresponding parameters in the intact molecule, it is not surprising that only binding but not catalysis is duplicated. Whether catalysis can be obtained by improvement in design and employment of other synthetic strategies is yet to be determined.

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