The identification of epitopic sites in human α_1 -proteinase inhibitor

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Human α_1 -proteinase inhibitor (α_1 -PI) yielded nine fragments on cleavage with CNBr. The amino acid sequences of these fragments were determined. Three of these CNBr-cleavage fragments, namely fragment I (residues 64–220), fragment II (residues 243–351) and fragment III (residues 1–63), were found to bind rabbit polyclonal antibodies against chemically oxidized α_1 -PI and mouse polyclonal antibodies against native α_1 -PI by the Bio-Dot method (enzyme-linked immunosorbent assay on nitrocellulose). These fragments, I, II and III, inhibited by 60%, 25% and 5% respectively the binding between α_1 -PI and the rabbit antibodies. Fragments I, II and III were subjected to proteolytic digestion, and 15, ten and five peptides were obtained from these fragments respectively. Only four of these peptides showed binding to the mouse antibodies against native α_1 -PI. These were residues 40–63, 79–86, 176–206 and 299–323. A panel of monoclonal antibodies was prepared by conventional hybridoma technology, with chemically oxidized α_1 -PI as the antigen. The ability of the monoclonal antibodies to bind native α_1 -PI and CNBr-cleavage fragments I–III was determined. The monoclonal antibodies fell into three categories. Most (over 90%) belonged to group I, which was capable of binding α_1 -PI and only fragment I. Antibodies in groups II and III bound α_1 -PI and either fragment II or fragment III respectively. The ability of the peptides derived from proteolytic digestion of fragments I, II and III to bind three monoclonal antibodies representing each of the three groups was determined. Among all the peptides tested, only one (residues 176–206) derived from fragment I showed binding to the antibodies from group I, one (residues 299–323) derived from fragment II showed binding to the antibodies from group II, and one (residues 40–63) from fragment III showed binding to the antibodies from group III. Each of these three peptides also inhibited the binding between α_1 -PI and the corresponding monoclonal antibodies. From these data we concluded that at least four epitopic regions (residues 40–63, 79–86, 176–206 and 299–323) were present in α_1 -PI. Specific monoclonal antibodies to three of these sites were obtained.

INTRODUCTION

The chemical structure of human α_1 -proteinase inhibitor (α_1 -PI) has been extensively investigated. The primary structure of α_1 -PI both in terms of amino acid sequence (Chan, 1982; Carrell *et al.*, 1982) and the oligosaccharide structure (Hodges *et al.*, 1979; Mega *et al.*, 1980; Vaughan *et al.*, 1982; Hodges & Chan, 1982) has been reported. In addition, the cDNAs for α_1 -PI from baboons and from humans have been cloned and the nucleotide sequences elucidated (Kurachi *et al.*, 1981).

With this structural information on hand, our laboratory has established a research programme to identify the major epitopic sites of the protein molecule. The present paper describes experiments from which several epitopic sites of the protein molecule were identified.

MATERIALS AND METHODS

Chemicals

All chemicals used in these experiments, unless otherwise indicated, were of reagent-grade quality. All organic solvents were redistilled before use. Ultrogel Aca 54 was obtained from LKB Produkter (Bromma,

Sweden). Activated thio-Sepharose 4B, concanavalin A-Sepharose and all other Sephadex products were purchased from Pharmacia (Uppsala, Sweden). Bio-Gel P-4 (200–400 mesh), Bio-Gel P-6 (200–400 mesh) and the cationic-exchange Aminex A-5 were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). CNBr, as well as polyacrylamide (electrophoresis grade) and all associated reagents for gel electrophoresis, were obtained from Eastman Kodak (Rochester, NY, U.S.A.). Ultrapure guanidinium chloride was from Schwarz–Mann (Orangeburg, NY, U.S.A.). Constant-boiling HCl, iodoacetamide, fluorescamine and the Sequenal-grade reagents used for manual Edman degradations were from Pierce Chemical Co. (Rockford, IL, U.S.A.). The reagents used in the gas-phase sequencer were supplied by the Applied Biosystems Corp. (Foster City, CA, U.S.A.). Trypsin (tosylphenylalanylchloromethane-treated; catalogue no. TRTPCK-33M945) was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.) and the *Staphylococcus aureus* strain VI proteinase from Miles Laboratories (Elkhart, IN, U.S.A.).

Purification of α_1 -PI and oxidized α_1 -PI

The source for human α_1 -PI was outdated human plasma. Procedures for the purification as described previously (Shochat *et al.*, 1978) involved affinity

Abbreviation used: α_1 -PI, α_1 -proteinase inhibitor (formerly α_1 -antitrypsin).

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chromatography on activated thio-Sepharose followed by concanavalin A-Sepharose. The final product was obtained after gel filtration on Sephadex G-100. The purified protein appeared homogeneous by electrophoresis on polyacrylamide gel in the absence and in the presence of SDS. Native α_1 -PI was oxidized by *N*-chlorosuccinimide as described in the preceding paper (Zhu & Chan, 1987). The final oxidized product lost all anti-elastase activity but maintained 95% of the original anti-trypsin activity.

Preparation of CNBr-cleavage fragments

Native α_1 -PI was carboxamidomethylated and fragmented with CNBr as previously described (Shochat *et al.*, 1978). The mixture of CNBr-cleavage peptides (500 nmol) was fractionated by gel filtration on Ultrogel AcA 54 in a column (2.5 cm $\times$ 100 cm) equilibrated with 50 mM-NH₄HCO₃. The effluent was monitored by its absorbance at 225 nm (Shochat *et al.*, 1978).

The smaller CNBr-cleavage fragments, IV–IX, were collected as a single peak from the Ultrogel column and were further resolved by gel filtration on Bio-Gel P-6 in a column (1.5 cm $\times$ 100 cm) equilibrated with 50 mM-*N*-ethylmorpholine/acetate buffer, pH 8.6. The effluent was monitored by relative fluorescence intensity exhibited after reaction with fluorescamine.

Tryptic and *S. aureus*-proteinase digestion of CNBr-cleavage fragments I and III

A 1 μ mol portion of purified fragment III was dissolved in 5 ml of 50 mM-NH₄HCO₃ and digested with 1.0 mg of trypsin. The digestion proceeded at 37 °C for 8 h, and another 1.0 mg of trypsin was added. After a total of 24 h of digestion, the reaction mixture was freeze-dried.

A 1 μ mol portion of fragment I was dissolved in 2 ml of 50 mM-NH₄HCO₃ and digested with 0.5 mg of *S. aureus* proteinase. The digestion was carried out at 37 °C for 8 h. At that time another 0.5 mg of the proteinase was added. The digestion was allowed to proceed for another 16 h, and the reaction mixture was freeze-dried.

Purification of peptides from tryptic and *S. aureus*-proteinase digestion of CNBr-cleavage fragments I and III

Peptide mixtures after enzymic digestion were resolved by gel filtration on a column (1.5 cm $\times$ 90 cm) of Sephadex G-50 (superfine grade). Subsequent fractionation was carried out by chromatography at 40 °C on a column (0.9 cm $\times$ 100 cm) of Aminex A-5 followed by gel filtration on a column (0.9 cm $\times$ 100 cm) of Bio-Gel P-4, Bio-Gel P-6 or Sephadex G-25.

Manual Edman degradation procedure

Manual Edman degradations were performed according to published methods (Peterson *et al.*, 1972; Tarr, 1975). The identification of amino acid phenylthiohydantoin derivatives was made by h.p.l.c. in accordance with Archer (1980).

Edman degradation by an automatic sequencer

Several peptides were sequenced by an Applied Biosystems gas-phase sequencer. The procedures for the degradation and the identification of amino acid phenylthiohydantoin derivatives by h.p.l.c. were described in Beach *et al.* (1985).

Fig. 1. Elution profile of the CNBr-cleavage fragments of α_1 -PI on Ultrogel AcA 54

The fraction size was 4 ml and the flow rate was 25 ml/h. Fractions that were pooled are marked by horizontal bars. The inset shows the results of SDS/7.5%-polyacrylamide-gel electrophoresis of fractions I, II and III.

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis (20% acrylamide) in the presence of SDS was performed according to the procedure of Weber & Osborn (1979).

Amino acid analysis

Peptides were hydrolysed in constant-boiling HCl at 110 °C *in vacuo* for 18 h. Amino acids were analysed on an automated amino acid analyser with a stepwise gradient of buffers (Liao *et al.*, 1973). Norleucine was used as the internal standard in all analyses.

Polyclonal-antibody production

Chemically oxidized α_1 -PI and CNBr-cleavage fragments I and II were used as antigens, and polyclonal antibodies against these antigens were raised in New Zealand White rabbits. Two rabbits were immunized with each antigen, and antiserum from the rabbit that had the highest antibody titre in each case was used in the experiments described in the present paper. The IgG fraction of the antiserum was isolated by (NH₄)₂SO₄ precipitation and DEAE-cellulose column chromatography (Hura & Chantler, 1980).

Another set of polyclonal antibodies was raised in BALB/c mice with the use of native α_1 -PI as an antigen. Three mice were immunized, and antiserum from the mouse that had the highest antibody titre was used in the experiment. This antiserum preparation was used without further purification.

Monoclonal-antibody production and screening for binding capacity

The antigen used was chemically oxidized α_1 -PI. The procedures for producing the hybridomas and preparing the monoclonal antibodies in accordance with Zola & Brooks (1982) were as described in the preceding paper (Zhu & Chan, 1987). The ability of peptides to bind antibodies was determined by the Bio-Dot immunoassay (Hawkes *et al.*, 1982). The assay procedure was as described in the preceding paper (Zhu & Chan, 1987).

Competitive binding experiments were performed by the classical enzyme-linked-immunosorbent-assay technique carried out on micro-titre plates (Engvall, 1980) or by the Bio-Dot enzyme-linked immunosorbent assay on nitrocellulose (Hawkes *et al.*, 1982).

Fig. 2. Elution profile of the smaller CNBr-cleavage fragments of α_1 -PI on Bio-Gel P-6

The fraction size was 2 ml and the flow rate was 7 ml/h. Fractions that were pooled are marked by horizontal bars.

Western blot technique

The technique was carried out in accordance with Burnette (1981). Samples of α_1 -PI or the CNBr-cleavage fragments were subjected to SDS/polyacrylamide-gel electrophoresis. Afterwards, the protein bands in the gels were electrophoretically transferred to a piece of nitrocellulose paper. The paper was then incubated with primary and secondary antibodies and developed as in the Bio-Dot immunoassay (Hawkes *et al.*, 1982) as specified in each experiment.

RESULTS

Purification of CNBr-cleavage fragments

There are nine methionine residues in α_1 -PI. Cleavage of the protein with CNBr should result in ten fragments. Because of the presence of a Met-Met sequence in the protein, nine peptide fragments were obtained. The elution profile is shown in Fig. 1. Five major peaks were obtained, which were analysed by SDS/polyacrylamide-gel electrophoresis. The first peak consisted of an aggregate of peptide fragments. The second, third and fourth were CNBr-cleavage fragments I, II and III respectively. The fifth peak contained the smaller fragments, which could not be detected by SDS/polyacrylamide-gel electrophoresis. Peptide fractions were pooled as shown and freeze-dried. Fragments I, II and III were further purified by rechromatography on the same column. The species contained in the fifth peak were further fractionated on Bio-Gel P-6. The elution profile is shown in Fig. 2. Six peptide fractions were obtained, which corresponded to fragments IV–IX. The amino acid compositions of the nine CNBr-cleavage fragments are given in Table 1. The complete amino acid sequence of CNBr-cleavage fragment II had previously been reported (Shochat *et al.*, 1978), so no further sequencing was performed.

Purification of the peptides derived from proteolytic digestion of CNBr-cleavage fragments I and III

Peptides produced from CNBr-cleavage fragments I and III after digestion with *S. aureus* proteinase and trypsin respectively were resolved by chromatography on Sephadex G-50, and the elution profiles are shown in

Table 1. Amino acid compositions of the CNBr-cleavage fragments of α_1 -PI

In this and subsequent Tables the values given are those calculated from the amino acid analysis of the peptides, with in parentheses the numbers of residues present in the peptides.

Amino acid	Fragment ...	Composition (mol of residue/mol)								
		I	II	III	IV	V	VI	VII	VIII	IX
Aspartic acid		20.8 (20)	10.2 (10)	9.1 (9)		1.1 (1)	1.2 (1)	0.8 (1)	0.8 (1)	
Threonine		12.3 (13)	8.9 (9)	5.5 (6)		0.8 (1)			0.8 (1)	
Serine		3.7 (4)	7.2 (7)	5.6 (6)		0.7 (1)	0.7 (1)	2.2 (2)		
Homoserine		0.5 (1)	0.5 (1)	0.4 (1)	0.4 (1)	0.3 (1)	0.5 (1)	0.4 (1)	0.3 (1)	0.6 (1)
Glutamic acid		26.3 (26)	11.2 (11)	7.2 (7)	1.2 (1)	2.3 (2)	1.1 (1)	1.1 (1)	1.2 (1)	
Proline		3.8 (4)	3.3 (3)	4.1 (4)	1.0 (1)	1.1 (1)	3.2 (3)		0.9 (1)	
Glycine		10.9 (10)	8.9 (9)	1.2 (1)					1.2 (1)	1.1 (1)
Alanine		4.7 (5)	9.8 (10)	8.2 (8)	0.9 (1)					
Valine		13.3 (13)	5.2 (5)	1.1 (1)			2.1 (2)	1.1 (1)	1.7 (2)	
Isoleucine		5.9 (6)	5.7 (6)	3.2 (3)	0.9 (1)	1.1 (1)	0.9 (1)	1.1 (1)		
Leucine		18.5 (19)	15.9 (16)	2.8 (3)	1.1 (1)	0.9 (1)	0.9 (1)	3.0 (3)		0.9 (1)
Tyrosine		2.8 (3)	1.8 (1)	0.8 (1)						
Phenylalanine		10.8 (11)	4.7 (5)	6.2 (6)	0.9 (1)	1.1 (1)	2.8 (3)	0.8 (1)		
Histidine		3.9 (4)	3.8 (4)	4.2 (4)				1.1 (1)		
Lysine		14.4 (14)	9.6 (10)	1.8 (2)		0.9 (1)	2.1 (1)	2.1 (2)	1.8 (2)	0.9 (1)
Arginine		2.7 (3)	2.3 (2)	1.1 (1)						1.1 (1)
Tryptophan		+						+		
S-Carboxyamido-methylcysteine								+		
Total no. of residues		157	110	63	7	11	16	16	9	5
Yield (%)		69	75	42	59	72	48	5	75	80
Purification method		AcA 54	AcA 54	AcA 54	P-6, G-25	P-6, G-25	P-6, G-25	P-6, G-25	P-6, G-25	P-6, G-25

Fig. 3. Elution profiles of peptide mixtures on Sephadex G-50

The fraction size was 2 ml and the flow rate was 12 ml/h. Fractions that were pooled are marked by horizontal bars. (a) Peptide mixture of *S. aureus*-proteinase digest of CNBr-cleavage fragment I of α_1 -PI. (b) Peptide mixture of tryptic digest of CNBr-cleavage fragment III of α_1 -PI.

Figs. 3(a) and 3(b). In Fig. 3(a) three distinctive peaks were visible. A single peptide was present in peak P-I, showing leucine as the single *N*-terminal amino acid. The amino acid composition of peptide P-I is shown in Table 2. Likewise, only a single peptide was present in peak P-II. On the basis of its unique amino acid composition (see Table 2), it was assumed that this was a glycopeptide whose amino acid sequence had previously been established and published (Hodges & Chan, 1982). Since there were ten peptides present in peak P-III, these were resolved on an Aminex A-5 column (Fig. 4a), and each resulting peptide was subsequently purified on Bio-Gels or Sephadex columns as indicated in Table 2. The amino acid composition of each of the purified peptides is given in Table 2.

CNBr-cleavage fragment III was very resistant to proteolysis. Repeated attempts to digest the fragment with *S. aureus* proteinase were unsuccessful. When the fragment was digested with trypsin, limited cleavage was obtained, with a large proportion of the fragment remaining intact. Fractionation of the peptides generated by limited tryptic digestion of CNBr-cleavage fragment III is shown in Fig. 3(b). Three peaks were resolved. The first consisted of aggregated and undigested materials and was not investigated further. The second peak contained a single peptide T-II, the amino acid composition of which is shown in Table 3. The peptide mixture present in the third peak was resolved on an Aminex A-5 column (Fig. 4b) and subsequently further

purified on Bio-Gels or Sephadex columns as indicated in Table 3. The yield of peptides in this digest was relatively low. The amino acid composition of each of the purified peptides is given in Table 3.

Amino acid sequences of the peptides derived from CNBr-cleavage fragment I after digestion with *S. aureus* proteinase

Peptide P-III-6: Leu-Ser-Leu-Gly-Thr-Lys-Ala-Asp-Thr-His-Asp-Glu (residues 64–75). Eleven steps of manual Edman degradation were performed on this and the structure was established as shown.

Peptide P-III-4b: Ile-Leu-Glu (residues 76–78). Two steps of manual Edman degradation established the structure of this peptide.

CHO

Peptide P-II: Gly-Leu-Asn-Phe-Asn-Leu-Thr-Glu (residues 79–86). The amino acid sequence of this glycopeptide has been established and published (Hodges & Chan, 1982).

Peptide P-III-2: Ile-Pro-Glu (residues 87–89). Two steps of manual Edman degradation established the structure of this peptide.

Peptide P-III-7: Ala-Gln-Ile-His-Glu (residues 90–94). The amino acid sequence of this peptide was established by four steps of manual Edman degradation procedure.

Peptide P-III-3: Gly-Phe-Gln-Glu (residues 95–98). Three steps of manual Edman degradation established the structure of this peptide.

Fig. 4. Elution profiles of peptide mixtures on Aminex A-5

The fraction size was 2 ml and the flow rate was 25 ml/hour. Fractions that were pooled are marked by horizontal bars. (a) Peptide mixture of fraction P-III of CNBr-cleavage fragment I of α_1 -PI. (b) Peptide mixture of fraction T-III of CNBr-cleavage fragment III of α_1 -PI.

Table 2. Amino acid compositions of the peptides from a *S. aureus* proteinase digest of CNBr-cleavage fragment I of α_1 -PI

Amino acid	Peptide ...	Composition (mol of residue/mol)													
		P-I	P-II	P-III-1a	P-III-1b	P-III-2	P-III-3	P-III-4a	P-III-4b	P-III-5	P-III-6	P-III-7	P-III-8a	P-III-8b	P-III-9
Aspartic acid		4.3 (4)	0.8 (2)	1.4 (1)	1.8 (2)			2.0 (2)		1.7 (2)	2.0 (2)		1.1 (1)	1.1 (1)	2.3 (2)
Threonine		1.8 (2)	0.9 (1)	1.5 (2)	1.9 (2)			2.1 (2)		1.1 (1)	1.8 (2)		1.2 (1)		
Serine				1.7 (2)						1.0 (1)					0.9 (1)
Homoserine		0.4 (1)						0.6 (1)							
Glutamic acid		4.8 (5)	1.1 (1)	3.1 (3)	2.0 (2)			1.4 (1)	1.0 (1)	1.5 (1)	1.2 (1)	2.3 (2)	2.0 (2)	1.1 (1)	2.3 (2)
Proline		1.2 (1)			0.8 (1)			0.8 (1)		0.9 (1)					
Glycine		1.3 (1)	1.1 (1)	2.1 (2)	1.5 (1)		1.0 (1)				1.1 (1)	1.0 (1)	2.0 (2)	1.2 (1)	1.1 (1)
Alanine		0.9 (1)			0.7 (1)						1.0 (1)				
Valine		2.8 (3)			0.8 (1)			3.0 (4)							0.8 (1)
Isoleucine		1.3 (1)	2.2 (2)	3.9 (4)					0.9 (1)	2.9 (3)	1.8 (2)	0.8 (1)	1.7 (2)	0.9 (1)	0.9 (1)
Leucine		1.8 (2)							1.0 (1)				1.2 (1)	2.8 (3)	1.0 (1)
Tyrosine		0.8 (1)	0.9 (1)	1.3 (1)	2.3 (2)									0.9 (1)	0.8 (1)
Phenylalanine		4.2 (4)				0.96 (1)		0.7 (1)			0.9 (1)	0.8 (1)			1.0 (1)
Histidine		3.3 (3)						1.4 (1)			0.8 (1)		2.8 (3)	1.7 (2)	1.6 (2)
Lysine		2.2 (2)						1.1 (1)		0.8 (1)					
Arginine															
Tryptophan		+													
Total no. of residues		31	8	15	11	3	4	14	3	9	12	5	13	10	9
Yield (%)		78	82	37	28	40	20	44	46	60	40	64	30	40	46
Purification method		G-50	G-50	A-5, P-4	A-5, P-4	A-5	A-5	A-5, P-4	A-5, P-4	A-5	A-5	A-5	A-5, P-4	A-5, P-4	A-5, P-4

Table 3. Amino acid compositions of the peptides from a tryptic digest of CNBr-cleavage fragment III of α_1 -PI

Amino acid	Peptide ...	Composition (mol of residue/mol)								
		T-II	T-II-1a	T-III-1b	T-III-2a	T-III-2b	T-III-3a	T-III-4	T-III-5	T-III-6
Aspartic acid		2.3 (2)	1.9 (2)	1.1 (1)			2.1 (2)	3.9 (4)	3.7 (4)	
Threonine		1.6 (2)		1.1 (1)				2.7 (3)	2.6 (3)	
Serine		3.5 (4)						0.8 (1)	0.9 (1)	1.0 (1)
Homoserine		0.4 (1)								
Glutamic acid		2.2 (2)	2.7 (3)		1.2 (1)	0.6 (1)	2.9 (3)	1.4 (1)	1.3 (1)	
Proline		1.0 (1)	1.4 (1)	0.8 (1)			1.1 (1)	1.1 (1)	1.1 (1)	
Glycine			1.0 (1)				0.9 (1)			
Alanine		4.2 (4)	1.7 (2)		1.9 (2)	1.2 (1)	1.9 (2)			
Valine		1.1 (1)								
Isoleucine		2.3 (2)		0.8 (1)						
Leucine		1.1 (1)			1.0 (1)	1.2 (1)				1.0 (1)
Tyrosine										0.9 (1)
Phenylalanine		2.7 (3)			1.9 (2)	1.0 (1)		1.2 (1)	1.2 (1)	
Histidine		0.9 (1)						2.8 (3)	2.9 (3)	
Lysine			1.2 (1)				0.9 (1)	1.1 (1)	1.1 (1)	
Arginine										1.1 (1)
Tryptophan										
Total no. of residues		24	10	4	6	4	10	15	15	4
Yield (%)		85	5	20	15	17	36	55	35	40
Purification method		G-50	A-5, P-4	A-5, P-4	A-5, P-2	A-5, P-2	A-5, P-4	A-5, P-4	A-5, P-4	A-5, P-2

Peptide P-III-5: Leu-Leu-Arg-Thr-Leu-Asn-Gln-Pro-Asp (residues 99–107). Nine steps of manual Edman degradation established the structure of this peptide.

Peptide P-III-1a: Ser-Gln-Leu-Gln-Leu-Thr-Thr-Gly-Asn-Gly-Leu-Phe-Leu-Ser-Glu (residues 108–122). Sequential (14 steps) degradation by the manual Edman procedure yielded the amino acid sequence as shown.

Peptide P-III-8b: Gly-Leu-Lys-Leu-Val-Asp-Lys-Phe-Leu-Glu (residues 123–132). Nine steps of manual Edman degradation established the structure of this peptide.

Peptide P-III-10: Asp-Val-Lys-Lys-Leu-Tyr-His-Ser-Glu (residues 133–141). Eight steps of manual Edman degradation established the structure of this peptide.

Peptide P-III-1b: Ala-Phe-Thr-Val-Asn-Phe-Gly-Asp-Thr-Glu-Glu (residues 142–152). Ten steps of manual Edman degradation were performed on this peptide. The amino acid composition of the peptide (see Table 2) showed the presence of 2 mol of glutamic acid residues/mol of peptide. It was concluded that the C-terminus of the peptide was occupied by a Glu-Glu sequence.

Peptide P-III-9: Ala-Lys-Lys-Gln-Ile-Asn-Asp-Tyr-Val-Glu (residues 153–162). Nine steps of manual Edman degradation were successfully carried out on this peptide, and the structure was established as shown.

Peptide P-III-8a: Lys-Gly-Thr-Gln-Gly-Lys-Ile-Val-Asp-Leu-Val-Lys-Glu (residues 163–175). Twelve steps of manual Edman degradation established the structure of this peptide.

Peptide P-1: Leu-Asp-Arg-Asp-Thr-Val-Phe-Ala-Leu-Val-Asn-Tyr-Ile-Phe-Phe-Lys-Gly-Lys-Trp-Glu-Arg-Pro-Phe-Glu-Val-Lys-Asp-Thr-Glu-Glu-Glu (residues 176–206). The amino acid composition (Table 2) of this peptide showed the presence of five glutamic acid residues. Degradation (31 steps) by the Edman procedure with an automated gas-phase sequencer yielded the amino acid sequence as shown.

Peptide P-III-4a: Asp-Phe-His-Val-Asp-Gln-Val-Thr-Thr-Val-Lys-Val-Pro-Hse (residues 207–220). The presence of homoserine (see Table 2) in this peptide indicated that this peptide occupied the C-terminus position of CNBr-cleavage fragment I. Thirteen steps of manual Edman degradation established the structure of the peptide. The elucidated sequence showed the presence of four valine residues in the peptide. Yet, only three valine residues were suggested from amino acid analysis of the peptide. It is possible that incomplete digestion of the peptide may have occurred during acid hydrolysis of only 16 h duration.

The complete amino acid sequence of CNBr-cleavage fragment I (residues 64–220) can be derived from the data herein presented. The alignment of the peptides was made according to the published corresponding cDNA sequence (Kurachi *et al.*, 1981). There are 157 amino acid residues in the fragment, with one glycosylation site.

Amino acid sequences of the peptides derived from CNBr-cleavage fragment III after digestion with trypsin

Peptide T-III-3a: Glu-Asp-Pro-Gln-Gly-Asp-Ala-Ala-Gln-Lys (residues 1–10). Nine steps of manual Edman degradation procedure were carried out and the structure was established as shown.

Peptide T-III-5: Thr-Asp-Thr-Ser-His-His-Asp-Gln-Asp-His-Pro-Thr-Phe-Asn-Lys (residues 11–25). Fourteen steps of manual Edman degradation procedure were carried out to establish the structure of the peptide.

Peptide T-III-1b: Ile-Thr-Pro-Asn (residues 26–29). Four steps of Edman degradation established the structure of the peptide.

Peptide T-III-2a: Leu-Ala-Glu-Phe-Ala-Phe (residues 30–35). Six steps of manual Edman degradation were performed. The structure of the peptide was established as shown.

Peptide T-III-6: Ser-Leu-Tyr-Arg (residues 36–39).

Fig. 5. Primary structure of α_1 -PI

The alignment of the CNBr-cleavage fragments is based on the published corresponding cDNA sequence (Kurachi *et al.*, 1981). The three CNBr-cleavage fragments I (residues 64–220), II (residues 243–351) and III (residues 1–63) are underlined. The four peptides that have been identified to contain epitopic sites are boxed in. CHO represents carbohydrate.

Results from three steps of manual Edman degradation established the structure of this peptide.

CHO

Peptide T-II: Gln-Leu-Ala-His-Gln-Ser-Asn-Ser-Thr-Asn-Ile-Phe-Phe-Ser-Pro-Val-Ser-Ile-Ala-Thr-Ala-Phe-Ala-Hse (residues 40–63). The presence of homoserine in this peptide (for amino acid composition see Table 3) indicated that this peptide occupied the C-terminus of CNBr-cleavage fragment III. Seven steps of manual Edman degradation procedure were carried out on this peptide. The N-terminal of the peptide was not easily amenable to the Edman degradation reaction, probably because of cyclization of glutamine on the N-terminus. Step 1, Gln (10%); step 2, Leu (8%); step 3, Ala (8%); step 4, His; step 5, Gln (5%); step 6, not identified; step 7, Asn (5%). The presence of an oligosaccharide chain in this peptide had been shown previously (Hodges & Chan, 1982). The structural analysis of this peptide was not complete in our studies. The proposed structure was based on the corresponding cDNA sequence, which was in agreement with the amino acid composition of the peptide (Table 3).

The amino acid sequence of CNBr-cleavage fragment III (residues 1–63) was deduced from these data. Peptides T-II-1a, T-III-2b and T-III-4 were not sequenced. On the basis of their amino acid compositions (see Table 3), peptide T-II-1a was probably a deaminated product of peptide T-III-3a; peptide T-III-2b was probably

derived from peptide T-III-2a, and peptide T-III-4 was probably a deaminated product of peptide T-III-5. The alignment of the peptides is again based on the published corresponding cDNA sequence. This fragment contains 63 amino acid residues and contains one glycosylation site.

Amino acid sequences of CNBr-cleavage fragments IV–IX

CNBr-cleavage fragment IV: Phe-Leu-Glu-Ala-Ile-Pro-Hse (residues 352–358). Six steps of manual Edman degradation established the structure of this peptide.

CNBr-cleavage fragment V: Ile-Glu-Gln-Asn-Thr-Lys-Ser-Pro-Leu-Phe-Hse (residues 375–385). Ten steps of manual Edman degradation established the structure of this peptide.

CNBr-cleavage fragment VI: Ser-Ile-Pro-Pro-Glu-Val-Lys-Phe-Asn-Lys-Pro-Phe-Val-Phe-Leu-Hse (residues 359–374). Fifteen steps of manual Edman degradation were carried out. The structure of the peptide was established as shown.

CNBr-cleavage fragment VII: Phe-Asn-Ile-Gln-His-Cys-Lys-Lys-Leu-Ser-Ser-Trp-Val-Leu-Leu-Hse (residues 227–242). Fifteen steps of manual Edman degradation were carried out. No positive identification of the amino acid residue in step 6 was made. The assignment of a cysteine residue in this position was made on results from another experiment. The protein (α_1 -PI) was carboxymethylated with iodo[1- ^{14} C]acetic

acid before cleavage with CNBr. CNBr-cleavage fragment VII was the only CNBr-cleavage fragment that contained radioactivity.

CNBr-cleavage fragment VIII: Gly-Lys-Val-Val-Asn-Pro-Thr-Gln-Lys (residues 386–394). The absence of homoserine from the peptide indicated that this CNBr-cleavage fragment occupied the C-terminus of intact α_1 -PI. Eight steps of manual Edman degradation established the structure of the peptide.

CNBr-cleavage fragment IX: Lys-Arg-Leu-Gly-Hse (residues 222–226). Four steps of manual Edman degradation established the structure of this peptide.

Primary structure of α_1 -PI

The complete amino acid sequence was constructed on the basis of the data herein presented and shown in Fig. 5. The alignment of the CNBr-cleavage fragments was based on the published corresponding cDNA sequence. From the N-terminus to the C-terminus, the arrangement of the CNBr-cleavage fragments was in the following order: III–I–IX–VII–II–IV–VI–V–VIII. Insertion of a methionine residue between CNBr-cleavage fragments I and IX was made according to the cDNA sequence, in agreement with the presence of free homoserine in samples of α_1 -PI after CNBr cleavage. The only difference between this proposed sequence and that predicted by the cDNA sequence as pointed out previously (Chan, 1982; Carrell *et al.*, 1982) was in residue 213, where we detected the presence of valine whereas the cDNA sequence indicated alanine.

Binding properties of the CNBr-cleavage fragments and peptides derived from the fragments to antibodies

The binding capacity of the nine CNBr-cleavage fragments to rabbit antibodies against oxidized α_1 -PI was determined by the Bio-Dot assay. The results are shown in Fig. 6(a). Reaction was observed for CNBr-cleavage fragments I, II and III but not for CNBr-cleavage fragments IV–IX. These results indicated that epitopic sites were present in CNBr-cleavage fragments I–III, and the epitopic structures in these fragments were reasonably maintained when the protein was subjected to CNBr cleavage. In a preliminary communication (Zhu *et al.*, 1984) it was reported that binding to the antibodies occurred with CNBr-cleavage fragment IV. It was found subsequently that a slight contamination by CNBr-cleavage fragment III in the preparation of CNBr-cleavage fragment IV was responsible for the binding observed. CNBr-cleavage fragment IV after repurification did not show binding to the rabbit antibodies. Another experiment was carried out in which native α_1 -PI was used as the antigen to raise antibodies in mice. As shown in Fig. 6(b), binding properties were similar to those shown in Fig. 6(a), except that the binding of CNBr-cleavage fragment III to the mouse antibodies was weaker than to the rabbit antibodies.

Competitive binding experiments were carried out to determine whether CNBr-cleavage fragments I, II and III from native α_1 -PI could inhibit the binding between native α_1 -PI and the rabbit antibodies against oxidized α_1 -PI. The results are shown in Fig. 7. CNBr-cleavage fragment I inhibited binding by as much as 60%, fragment II inhibited it by 25%, but inhibition by fragment III could only barely be demonstrated. These results showed that the majority of the rabbit antibodies against oxidized α_1 -PI were directed towards the epitopic

Fig. 6. Binding of CNBr-cleavage fragments to polyclonal antibodies against oxidized α_1 -PI

Samples (50 μ l) of CNBr-cleavage fragments I–IX and α_1 -PI (I and II, 1 nmol/ml; III, 5 nmol/ml; IV–IX, 20 nmol/ml each; α_1 -PI, 0.1 nmol/ml) were applied to two pieces of nitrocellulose paper in positions A-1, B-1, C-1, D-1, E-1, A-2, B-2, C-2, D-2 and E-2 respectively. (a) The primary and secondary antibodies used in this experiment were rabbit anti-(oxidized α_1 -PI) antibody and peroxidase-linked goat anti-(rabbit IgG) antibody respectively. The substrate used was 4-chloro-1-naphthol. (b) The primary and secondary antibodies used in this experiment were mouse anti- α_1 -PI antibody and peroxidase-linked goat anti-(mouse IgG) antibody respectively.

Fig. 7. Two-step competitive enzyme-linked immunosorbent assay: inhibition of binding between rabbit antibodies and α_1 -PI by CNBr-cleavage fragments and α_1 -PI

Each well of a 96-well micro-titre plate was coated with 100 μ l of α_1 -PI (0.2 nmol/ml), followed by blocking with 1% (w/v) bovine serum albumin. Inhibitors ($\circ$, α_1 -PI; $\bullet$, fragment I; $\blacktriangle$, fragment II; $\blacksquare$, fragment III) of increasing concentration as indicated were mixed with a constant amount of rabbit antibodies and the mixture was added to the wells. After incubation for 4 h at room temperature, biotinylated goat anti-(rabbit IgG) antibody was introduced to each well. Avidin and biotinylated β -galactosidase were sequentially added, and *o*-nitrophenyl galactoside was used as the substrate. The colour intensity given by the product was monitored at 405 nm with a Titertek multiscan photometer.

Fig. 8. Binding of purified peptides to antibodies against native α_1 -PI

CNBr-cleavage fragments I, II and III were digested with specific proteinases and the resulting peptides were isolated and purified. Fragment I yielded 15 peptides, fragment II ten peptides and fragment III five peptides. A 50 μ l sample of each purified peptide solution was applied to a piece of nitrocellulose paper. The Bio-Dot assay was carried out as described in the Materials and methods section. The primary and secondary antibodies used in this experiment were polyclonal mouse anti-(native α_1 -PI) antibody and peroxidase-linked goat anti-(mouse IgG) antibody respectively. The purified peptides, 50 nmol/ml except otherwise indicated, were placed in the positions indicated: A-1, blank; A-2, residues 324–342; A-3, blank; A-4, residues 108–122; B-1, residues 40–63 (5 nmol/ml); B-2, residues 299–323 (5 nmol/ml); B-3, residues 207–221; B-4, residues 99–107; C-1, residues 36–39; C-2, residues 278–298; C-3, residues 176–206 (5 nmol/ml); C-4, residues 95–98; D-1, residues 26–35; D-2, residues 271–277; D-3, residues 163–175; D-4, residues 90–94; E-1, residues 11–25; E-2, residues 267–270; E-3, residues 153–162; E-4, residues 87–89; F-1, residues 1–10; F-2, residues 265–266; F-3, residues 142–152; F-4, residues 79–86 (20 nmol/ml); G-1, residues 347–351; G-2, residues 258–264; G-3, residues 133–141; G-4, residues 76–78; H-1, residues 343–346; H-2, residues 243–257; H-3, 108–122; H-4, 64–75.

sites in CNBr-cleavage fragments I and II and only minimally to CNBr-cleavage fragment III. Since the three CNBr-cleavage fragments represent 75% of the protein, it is reasonable that they comprise most of the epitopic regions of the molecule.

Because CNBr-cleavage fragments I, II and III were relatively large in size, these fragments were subjected to specific proteinase digestion. The peptides derived from these fragments were isolated, purified and sequenced, and each of the purified peptides was tested for its binding properties. First, the ability of these peptides to bind the mouse polyclonal antibodies against native α_1 -PI was determined by the Bio-Dot method. The results as shown in Fig. 8 indicated that only four peptides showed

Fig. 9. Two-step competitive enzyme-linked immunosorbent assay: inhibition of binding between rabbit antibodies to CNBr-cleavage fragments I and II and peptides derived from proteolytic digestion of fragments I and II

The experimental procedures were the same as for Fig. 7. (a) CNBr-cleavage fragment I (0.2 nmol/ml) was used to coat the wells in the plate. The inhibitors ($\circ$, fragment I; $\bullet$, peptide comprising residues 176–206; $\blacktriangle$, peptide comprising residues 79–86; $\blacksquare$, all other peptides) were mixed with a constant amount of rabbit antibodies against CNBr-cleavage fragment I. (b) CNBr-cleavage fragment II (0.2 nmol/ml) was used to coat the wells in the plate. The inhibitors ($\circ$, fragment II; $\bullet$, peptide comprising residues 299–323; $\blacksquare$, all other peptides) were mixed with the rabbit antibodies against CNBr-cleavage fragment II.

binding. These peptides were residues 40–63 (from fragment III), residues 79–86, residues 176–206 (from fragment I) and residues 299–323 (from fragment II).

In the next experiment, rabbit polyclonal antibodies were raised with the use of CNBr-cleavage fragments I and II as antigens. Those peptides derived from proteolytic digestion of CNBr-cleavage fragments I and II were tested for their ability to inhibit the binding between each of the two CNBr-cleavage fragments and their corresponding rabbit antibodies. The data are shown in Fig. 9. In Fig. 9(a) it was demonstrated that, of all peptides tested, only two peptides (residues 79–86 and 176–206) inhibited the binding between CNBr-cleavage fragment I and its rabbit antibodies, and in Fig. 9(b) that only one peptide (residues 299–323) inhibited the

Fig. 10. Binding between monoclonal antibodies and CNBr-cleavage fragments of α_1 -PI

Samples (50 μ l) of α_1 -PI and CNBr-cleavage fragments I, II and III were applied to a nitrocellulose paper. The experimental procedures were similar to those described in the legend to Fig. 6. α_1 -PI in A-1, B-1, C-1 and D-1 had a concentration of 7, 17, 31 and 62 pmol/ml respectively; CNBr-cleavage fragment I in E-1, F-1, G-1 and H-1, CNBr-cleavage fragment II in A-2, B-2, C-2 and D-2, and CNBr-cleavage fragment III in E-2, F-2, G-2 and H-2 all had concentrations of 62, 125, 250 and 500 pmol/ml respectively. The primary antibody used in (a) was from clone 6H₄-I, that used in (b) was from clone 5C₁₂-8-7, and that used in (c) was from clone 6D₄-6-18.

binding between CNBr-cleavage fragment II and its rabbit antibodies.

Binding properties of CNBr-cleavage fragments and the peptides derived from these fragments to monoclonal antibodies

By conventional hybridoma technology, hybrid cells that secreted antibodies capable of binding to native α_1 -PI were obtained. The ability of the monoclonal antibodies from these cell lines to bind α_1 -PI and CNBr-cleavage fragments I, II and III was determined. In general, the antibodies fell into three categories (results not shown). Most of the antibodies (over 90%) belonged to group I, which bound α_1 -PI and only fragment I. The other two groups, II and III, bound α_1 -PI and either fragment II or fragment III respectively. These three groups were represented by three clones, 6H₄-I, 5C₁₂-8-7 and 6D₄-6-18. The antibodies secreted by these three clones were purified on Protein A-Sepharose, and their binding properties are shown in Fig. 10.

Increasing amounts of α_1 -PI and each of the three CNBr-cleavage fragments (I, II and III) were applied to

Fig. 11. Binding between monoclonal antibodies and peptides containing the epitopic regions

Samples of peptides were applied to nitrocellulose paper, and binding to monoclonal antibodies was determined by procedures described in the legend to Fig. 6. The colour intensity was determined at 420 nm with a reflective densitometer. (a) Fifteen peptides derived from proteolytic digestion of CNBr-cleavage fragment I: $\circ$, the peptide covering residues 176-206; $\blacksquare$, all other peptides. The primary antibody used was from clone 6H₄-I. (b) Ten peptides derived from proteolytic digestion of CNBr-cleavage fragment II: $\triangle$, the peptide covering residues 299-323; $\blacksquare$, all other peptides. The primary antibody used was from clone 5C₁₂-8-7. (c) Five peptides derived from proteolytic digestion of CNBr-cleavage fragment III: $\square$, the peptide covering residues 40-63; $\blacksquare$, all other peptides. The primary antibody used was from clone 6D₄-6-18.

three pieces of nitrocellulose paper, and each piece was allowed to react with each of the three monoclonal antibodies. The results clearly showed that monoclonal antibodies from clone 6H₄-I were specific to CNBr-cleavage fragment I, those from clone 5C₁₂-8-7 to CNBr-cleavage fragment II, and those from clone 6D₄-6-18 to CNBr-cleavage fragment III. It was noted that binding occurred between monoclonal antibodies from clone 6D₄-6-18 and CNBr-cleavage fragment I. This apparent discrepancy was examined by the Western blot technique. It was found that there was present a minor high- M_r component in the fragment I preparation that alone was responsible for binding to antibodies from clone 6D₄-6-18. Fragment I itself did not bind to the same antibody (results not shown). Amino acid sequence studies of this high- M_r component showed that this peptide covered both fragments I and III (results not shown), which explained why it bound to antibodies from clone 6D₄-6-18, which should be specific for fragment III only.

We have established from the monoclonal-antibody studies that there were three distinct epitopic sites, one residing in each of the three CNBr-cleavage fragments (I, II and III). It was therefore important to determine whether these three sites as identified by the use of monoclonal antibodies were the same sites as those identified by the polyclonal antibodies. Experiments were then carried out to determine which peptides derived from proteolytic digestion of CNBr-cleavage fragments I, II and III would bind the three monoclonal antibodies. The results are shown in Fig. 11. Of all the peptides tested, the only peptide that showed binding to monoclonal antibodies from clone 6H₄-I was the peptide covering residues 176–206; correspondingly, the peptide covering residues 299–323 bound monoclonal antibodies from clone 5C₁₂-8-7, and the peptide covering residues 40–65 bound monoclonal antibodies from clone 6D₄-6-18. Competitive-binding studies (results not shown) confirmed that only these three peptides were capable of inhibiting the binding between α_1 -PI and the corresponding monoclonal antibodies.

On the basis of the results herein presented, we conclude that one predominant epitopic site (residues 176–206) and two additional sites (residues 40–63 and residues 299–323) were present in α_1 -PI. These three sites were distinct, as unique monoclonal antibodies to each of these three sites were obtained. One minor site (residues 79–86) was identified by binding and competitive-binding studies with polyclonal antibodies. But no monoclonal antibodies specific to this epitopic site have been identified.

DISCUSSION

Epitopic sites of several globular proteins, such as myoglobin, lysozyme and ribonuclease, have been extensively investigated (Atassi, 1984; Benjamin, *et al.*, 1984). It has been documented that certain sites are continuous or sequence-specific, and certain others are discontinuous or conformation-specific (Atassi, 1975, 1978, 1984; Lando *et al.*, 1982; Lando & Reichlin, 1982; Benjamin *et al.*, 1984). In a given protein, the epitopic sites may be predominantly of one type or the other. In some proteins, both types of sites may be present. Atassi (1975) presented evidence that most epitopic sites in sperm-whale myoglobin are of the continuous or

sequence-specific type, although this conclusion has been questioned by others (Lando *et al.*, 1982; Lando & Reichlin, 1982). On the other hand, epitopic sites in lysozyme have been shown (Atassi, 1978) to be predominantly the discontinuous or conformation-dependent type. In this case, denaturation or fragmentation of the native protein abolished most of the immunoreactivity of the protein.

One of our goals was to obtain monoclonal antibodies to human α_1 -PI with known site-specificities, and the first step in attaining this goal was to identify the major epitopic sites in human α_1 -PI. Our plan was to obtain peptide fragments of α_1 -PI by CNBr cleavage and subsequently by specific proteinase digestion. The ability of these peptides to bind with polyclonal antibodies against α_1 -PI was then determined. Those peptides that bound the antibodies would be considered to contain the epitopic sites of the protein. Since the amino acid sequence of human α_1 -PI is known (Kurachi *et al.*, 1981; Chan, 1982; Carrell *et al.*, 1982), the locations of the epitopic sites in the protein could be pinpointed. Obviously, this method was appropriate for detecting only the continuous or sequence-specific sites. Even some sequence-specific sites may go undetected by this method, since CNBr and/or proteolytic cleavage may occur within the critical area of an epitope. However, as was shown in the Results section, four peptide fragments had been positively identified to contain epitopic regions of α_1 -PI by this approach.

Monoclonal antibodies to chemically oxidized α_1 -PI were obtained by conventional hybridoma technology. The choice of chemically oxidized α_1 -PI instead of native α_1 -PI as the antigen was dictated by another objective of our research. However, the panel of clones selected for this study all secreted antibodies that were immunoreactive with native α_1 -PI and thus were considered appropriate for the present study. As noted in the Results section, monoclonal antibodies were identified to be specific for the three peptides covering residues 176–206, 299–323 and 40–63 in the native protein. These results amply substantiated the conclusion that these segments of the protein molecule constituted the epitopic regions of α_1 -PI. Furthermore, it should be pointed out that most of the clones obtained in the present study secreted antibodies specific to the peptide covering residues 176–206, indicating that this segment of the molecule was immunodominant, at least in mice. Also, it was noted that few clones obtained in this study secreted antibodies that bound to native α_1 -PI but not to one of the three CNBr-cleavage fragments. These data suggested strongly that, if other epitopic sites were present in α_1 -PI, they were indeed minor.

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