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Characterizing and Optimizing Protease/Peptide Inhibitor Interactions, a New Application for Spot Synthesis[1]

Kai Hilpert,[*] Gerd Hansen,[*] Helga Wessner,[*] Jens Schneider-Mergener,[†] and Wolfgang Höhne[*,2]

[*]Institut für Biochemie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Monbijoustr. 2, 10117 Berlin, Germany; and [†]Institut für Medizinische Immunologie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Schumannstr. 20-21, 10117 Berlin, Germany

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A new method is presented that uses parallel peptide array synthesis on cellulose membranes to characterize protease/peptide inhibitor interactions. A peptide comprising P5-P4′ of the third domain of turkey ovomucoid inhibitor was investigated for both binding to and inhibition of porcine pancreatic elastase. Binding was studied directly on the cellulose membrane, while inhibition was measured by an assay in microtiter plates with punched out peptide spots. The importance of each residue for binding or inhibition was determined by substitutional analyses, exchanging every original amino acid with all other 19 coded amino acids. Seven hundred eighty individual peptides were investigated for binding behavior to porcine pancreatic elastase, and 320 individual peptides were measured in inhibition experiments. The results provide new insights into the interaction between the ovomucoid derived peptide and subsites in the active site of elastase. Combining these data with length analysis we designed new peptides in a stepwise fashion which in the end not only inhibited elastase 400 times more strongly than the original peptide, but are highly specific for the enzyme. In addition, the optimized inhibitor peptide was protected against exopeptidase attack by substituting D-amino acids at both termini.

Key words: inhibitor characterization, ovomuvoid inhibitor, optimizing specificity, porcine pancreatic elastase, spot synthesis.

There is considerable interest in investigating protease/inhibitor interactions because proteases are involved in many human diseases, such as coagulopathy, emphysema, several inflammatory conditions, cancer infiltration, some forms of arthritis, and many bacterial, viral, and parasitic infections. Secondly, the protease/inhibitor interaction is an interesting example and useful model system for studying protein/protein interactions and molecular recognition in general.

Some natural protease inhibitors are themselves small proteins consisting of only 25–60 amino acids, and are therefore accessible to peptide synthesis. Such inhibitors include squash seed inhibitors, the Kunitz inhibitory family, or domains of the Kazal inhibitory family (*1*). Many authors have reported the results of substitutional analyses of P1 (the amino acid position N-terminal to the scissile bond in the protease substrate; for nomenclature see Ref. *2*) with coded and non-coded amino acids using site-directed mutagenesis in the inhibitor gene or peptide synthesis (for example, see Refs. *3–5*). In a protease substrate or inhibitor molecule the amino acid at position P1 is mainly responsible for the binding specificity. For a medical approach, not only P1 is important but also other subsites, because there is a great interest in specificity, which is largely influenced by these subsites. Until now the investigation of each amino acid in the subsite of PPE, for example, from P4 to P4′, was too cumbersome and expensive to pursue in detail. Because of this we used spot synthesis on cellulose membranes to solve this problem. The advantage of this method is that it is possible to synthesize up to 500 different peptides in parallel on a cellulose sheet without special equipment, or up to 8,000 different peptides with the help of a pipetting robot (*e.g.* Refs. *6–8*). A further advantage is the possibility of using non-coded amino acids or stereoisomers of coded amino acids. For our investigation, we used porcine pancreatic elastase (PPE) and the third domain of the turkey ovomucoid inhibitor (OMTKY3). PPE is a member of the trypsin family of serine proteases that contain the catalytic triad His-57, Asp-102, and Ser-195 (chymotrypsin numbering system). PPE preferentially cleaves peptide bonds involving the carbonyl groups of amino acid residues with small hydrophobic side chains, such as Ala, or 30 times less effectively, Val, Leu, Ile, and 200 times less efficiently, Gly (*9*). Investigations of polyalanyl substrates have revealed that the active site of PPE has eight subsites S5-S3′ (*10*). The natural function of PPE is to digest many connective tissue proteins such as elastin.

The third domain of the turkey ovomucoid inhibitor, OMTKY3, is a potent protease inhibitor that comprises 56 amino acids, including six cysteines in three disulfide bridges. OMTKY3 inhibits a wide range of serine proteases such as bovine α-chymotrypsin (K_i = 5.5 × 10^{-12} M), PPE

[1] This work was partially supported by a grant from Fazit Stiftung.
[2] To whom correspondence should be addressed. Tel: +493028026273, Fax: +493028026252, wolfgang.hoehne@charite.de

(K_i = 2.4 × 10^{-11} M), subtilisin Carlsberg (K_i = 2.2 × 10^{-11} M), *Streptomyces griseus* protease A (K_i = 3.4 × 10^{-12} M), and human leukocyte elastase (K_i = 1.6 × 10^{-10} M) (*4*).

Here we present a combination of two simple experiments that can be used to investigate the elastase/inhibitor interaction with a peptide comprising P5-P4′ of the third domain of the turkey ovomucoid inhibitor. The importance of each single residue in the binding interaction or inhibition was determined. In addition, we show that with this method it is possible to increase the affinity and specificity of the inhibitory peptide. The influence of disulfide bridges on this interaction was investigated.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Cellulose bound peptides for binding experiments were prepared using a pipetting robot (Abimed, Langenfeld, Germany) and Whatman 50 cellulose membranes (Whatman, Maidstone, United Kingdom) as described before (*6*, *11*), with modifications in the protection group cleavage procedure in 90% trifluoroacetic acid (Fluka, Deisenhofen, Germany), 2% triisopropylsilan (Lancaster Synthesis GmbH, Mühlheim am Main, Germany), 5% water, and 3% phenol (Fluka, Deisenhofen, Germany) for 3 h. Cyclization of the peptides bound on the membrane was performed with 20% DMSO in water at pH 8.0 for 24 h. Further investigations of the cyclization of the cellulose bound peptides show that a more effective method is the use of Tris-buffer, pH 7.5, and charcoal in a 1:1 mass ratio of charcoal to membrane. The peptides used for K_i determination and hydrolysis investigations were prepared by Interactiva (Ulm, Germany), or in the lab of J. Schneider-Mergener according to standard Fmoc machine protocols using a multiple peptide synthesizer (Abimed, Langenfeld, Germany). The cyclization protocols were described before (*12*).

The degree of cyclization was characterized for both soluble and cellulose bound peptides by determining the amount of free cysteine with 5,5′-dithiobis(2-nitrobenzoic acid). The cyclization procedure was applied until no free cysteine was detectable.

Binding Experiments with Cellulose Bound Peptides—To detect PPE binding, the enzyme was labeled with horseradish peroxidase (POD) by the method of Wilson and Nakame (*13*). The activity of the labeled enzyme (PPE*) was determined to be 75% of the native enzyme activity. Cellulose membranes were washed with 96% ethanol for 5 min and equilibrated with 0.1 M Tris (Applichem GmbH, Darmstadt, Germany) buffer, pH 8.5, then blocked by incubation with blocking buffer (Genosys, Cambridge, UK) in 0.1 M Tris buffer, pH 8.5, for 2 h at room temperature, washed three times with 0.1 M Tris buffer, pH 8.5, for 3 min each, and incubated for 30 min at room temperature with 22 pM PPE* in 0.1 M Tris buffer, pH 8.5. The membranes were finally washed with 0.1 M Tris buffer, pH 8.5, once for 5 min and five times for 10 min, and then incubated with a chemiluminescence kit (Pierce, Illinios, USA) for three minutes. The light arising from spots with bound PPE* was measured with a Lumi-Imager™ (Boehringer, Mannheim, Germany).

Elastase Inhibition Experiments with Cellulose-Synthesized Peptides—For cleavage of the peptides from the cellulose support, the sheets were placed in an exsiccator filled with NH_3 gas and incubated overnight. For both cleaved and uncleaved peptides, spots with a diameter of 5 mm were punched out with a common punch and transferred into the wells of a 96-well microtiter plate. Each well contained 50 μl 0.1 M Tris buffer, pH 8.5, 1 μl 0.025 M suc-Ala-Ala-Ala-pNA (Serva, Heidelberg, Germany) in DMSO (Merck KGaA, Darmstadt, Germany) and 2.5 μl containing 18 units PPE (Serva, Heidelberg, Germany) in 0.1 M Tris buffer, pH 8.5. The reaction was allowed to proceed for 40 min at room temperature. For better comparability, it is recommended to mix the components before transfer into the wells with a multi-channel pipette, although this means the reaction starts before the inhibitor is available. Thus the inhibition data are relative and never reach complete inhibition. After the corresponding reaction time, the assay volume was increased to 75 μl, and 50 μl of this solution was transferred to another microtiter plate. The absorption was measured by a microtiter reader (Anthos htII, Salzburg, Austria) at 402 nm. As a control, 100% activity data were measured using punched peptide-free cellulose from the sheet applied to peptide synthesis. One unit of elastase catalyzes the hydrolysis of 1 μmol of ac-Ala-Ala-Ala-ME per minute at 25°C, pH 8.5.

K_i and Stability Determination—The activity of PPE was measured by monitoring the hydrolysis of the substrate suc-Ala-Ala-Ala-pNA on an UV-160A recording spectrophotometer (Shimadzu, Duisburg, Germany). Varying the substrate (0.25–1.3 mM) and inhibitor (0.9 μM–0.3 mM) concentrations, the K_i values were determined with 0.18 unit (in 200 μl assay volume) PPE in 0.1 M Tris buffer, pH 8.5, at 25°C. The determination of chymotrypsin activity (Serva, Heidelberg, Germany) used solutions of 0.025 M suc-Ala-Ala-Phe-pNA (Serva, Heidelberg, Germany) in DMSO and 24 units (in 200 μl) chymotrypsin in 0.1 M Tris buffer, pH 7.5, where the substrate and inhibitor concentrations were varied as for the PPE assay indicated above. The same protocol was applied to the determination of HLE (Serva, Heidelberg, Germany) activity using solutions of 0.025 M suc-Ala-Ala-Val-pNA (Serva, Heidelberg, Germany) as a substrate with 4.5 units (in 200 μl) HLE in 0.1 M Tris, pH 8.5.

The stability of the peptide PMTLEYR was measured (determined *via* PPE activity) by preincubation of the inhibitor at different concentrations (180–500 μM) and PPE (150 units in 150 μl assay volume) for between 2 min and 4 h in 0.1 M Tris buffer, pH 8.5, at 25°C.

One unit of chymotrypsin catalyzes the hydrolysis of 1 μmol of *N*-benzoyl-L-tyrosine ethyl ester per minute at 25°C, pH 7.8. One unit HLE releases 1 μmol *p*-nitroanilide per minute from MeO-suc-Ala-Ala-Pro-Val-pNA at 25°C, pH 8.0.

RESULTS

Design of an Inhibitory Peptide from the Ovomucoid Third Domain—Using the information from the crystal structure of the complex between human leukocyte elastase (HLE, highly homologous in structure to PPE) and OMTKY3 (*14*), we designed a peptide (PACTLEYR) that represents most of the amino acids in the inhibitor that builds contacts with the protease. In addition, to maintain a conformation similar to that in the OMTKY3-HLE complex, we introduced a new disulfide bridge by adding a C-

terminal cysteine to the peptide. The resulting peptide PACTLEYRC comprises only about one-fifth of the third domain of the OMTKY3 inhibitor.

First, the inhibition constant of the peptide PACTLEYRC with PPE was determined. The peptide was synthesized on resin and the inhibition constant of the soluble disulfide cyclized peptide was measured as a K_i of 1.7 ($\pm$0.4) $\times$ 10^{-4} M at pH 8.5 and 25°C. This result showed that the designed peptide can inhibit elastase, albeit with low affinity.

Initially, we had to determine whether the synthesis of the peptide PACTLEYRC on cellulose membranes influences its interaction with elastase. In addition, to check the specificity of this interaction, two peptides were synthesized with shuffled sequences but maintaining the amino acid composition. The binding of PPE to cellulose-supported peptides was determined using POD-labeled PPE (PPE*) by measuring the light produced by a chemiluminescent substrate. The light emission signal (which is proportional to the strength of PPE binding) of the PACTLEYRC peptide spot was much higher in comparison with the two shuffled peptides, which were nearly undetectable, confirming the specificity of the interaction (data not shown).

Characterization of the Inhibitory Peptide by Substitutional Analysis: Binding Experiments—A substitutional matrix for PACTLEYRC was synthesized on a cellulose membrane and all amino acids along the sequence were substituted with all 19 coded amino acids (*11*). The results of PPE* binding to the peptide matrix are presented in Fig. 1A. The key amino acids for this interaction were found at P1, P1′, and P2, since at these positions no signals higher than those from the original amino acid were detected, and most substitutions showed a drastic reduction in the signal. On the other hand, it is obvious that most of the substitutions at positions P3 and P4′ resulted in stronger signals than those from the wild type peptide. Furthermore, the results show that P5, and to a lesser extent P4, P2′, and P3′, can be replaced by several other amino acids without a loss of signal.

Characterization of the Inhibitory Peptide by Substitutional Analysis: Inhibition Experiments—The results of these experiments are based on the binding of the POD-labeled elastase to the immobilized peptides (incubating POD without elastase showed no signal). To ascertain whether the binding results are comparable with the capability of the corresponding peptides to inhibit PPE enzymatic activity, we took advantage of parallel synthesis on cellulose combined with an assay where the enzyme reaction can be investigated under defined conditions. Follow-

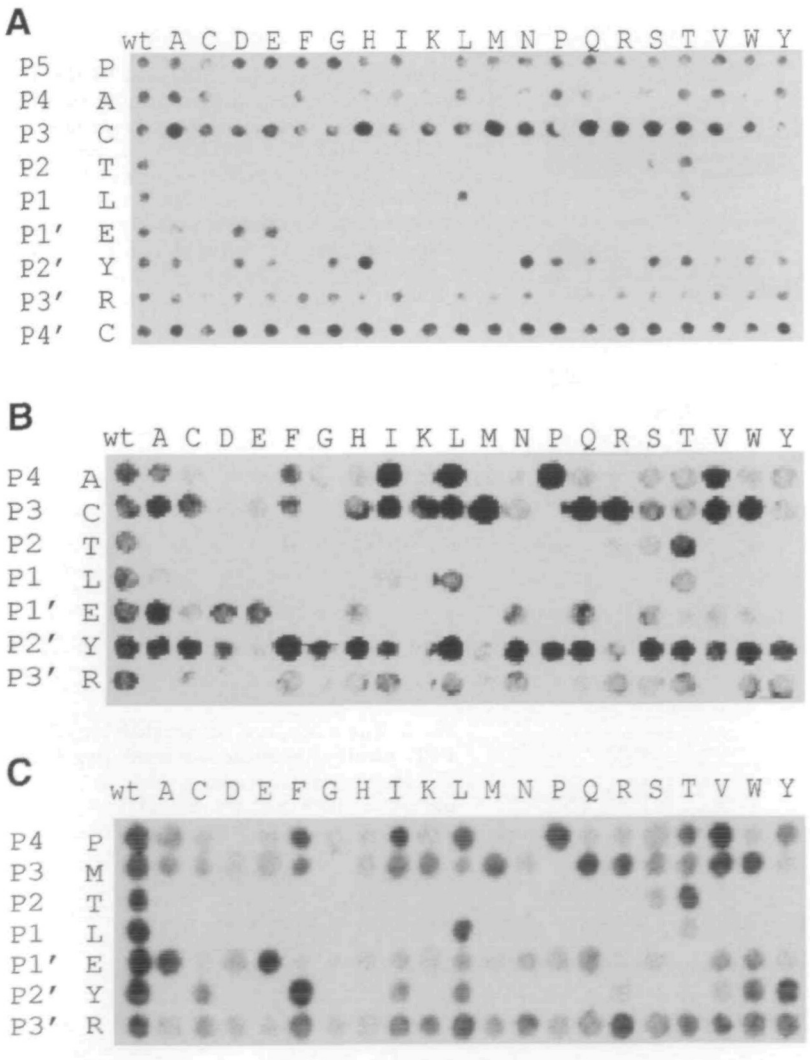

Fig. 1. **POD-labeled PPE binding to a substitutional matrix of inhibitory peptides.** The first column on the left represents peptides with the wild type sequence. The rows represent peptides substituted at that position by all other 19 coded amino acids. Bound POD-labeled PPE was detected as the chemiluminescent product of a POD-substrate. The light signal is shown in inverse form, the strongest binding resulting in the darkest spot. Substitutional matrix of (A) peptide PACTLEYRC (B) peptide ACTLEYR, and (C) peptide PMTLEYR.

ing peptide synthesis of the complete substitutional matrix on a cellulose membrane, peptide spots were punched out and transferred to a microtiter plate. The cellulose sheet was pretreated to allow the peptides to be eluted from the matrix. Buffer solution, a chromophoric substrate, and PPE were added to each well, and the absorption was measured after an appropriate incubation time (see Fig. 2A). In these experiments, the key amino acids were P1, as expected, but also P2 as implicated in the binding studies. Interestingly, position P1′ was not as specific as found in the binding experiments, in that substitutions against a larger set of amino acids were tolerated without a loss of inhibition. Also in agreement with the binding experiments, substitutions at position P3 or P4′, in most cases, showed the same or stronger inhibition compared to the wild type.

An analysis of binding, which may not necessarily be at the active site, and inhibition provides two alternate systems for investigating enzyme/inhibitor interactions. Generally, the results of both systems were comparable, for example, regarding the key amino acid positions. In some cases there were differences, especially at position P5, P4, and P3′, such as the substitution of alanine by proline at P4 resulting in a binding signal but in no significant inhibition (see Figs. 1A and 2A). The average error between these two different methods was about 30%.

Both experiments showed that the cysteines involved in the disulfide bridges could be replaced by almost any other amino acid, most replacements resulting in a higher signal. To probe the role of the introduced disulfide bridge, we compared the inhibition activity of the resin-synthesized peptide PACTLEYRC under reducing and non-reducing conditions (data not shown). The experiments gave the same results as with peptides synthesized on cellulose supports, namely that the disulfide bridge is unfavorable for inhibition function.

Length Analysis—The amino acids at P5, P4, and P4′ appeared to be not so important for the interaction, but non-key amino acids that can be replaced by several other amino acid residues are not necessarily expendable without a loss of binding. The importance of these amino acid positions can be determined by length variation analysis where the peptide of interest is shortened at the C- or N-terminus, or both (Fig. 3). The results of such experiments comparing the original nonamer with eight truncated peptides synthesized on cellulose revealed that P4′ and P5 are not essential for the protease/inhibitor interaction, in agreement with the results of the binding and inhibition experiments.

Optimization Strategy—The shortened peptide (ACTLEYR) with the best binding (Fig. 3) was chosen for further binding analysis using a substitutional matrix (Fig. 1B). When the amino acid cysteine at position P3, as in the original OMTKY3 wt sequence, was replaced with methionine,

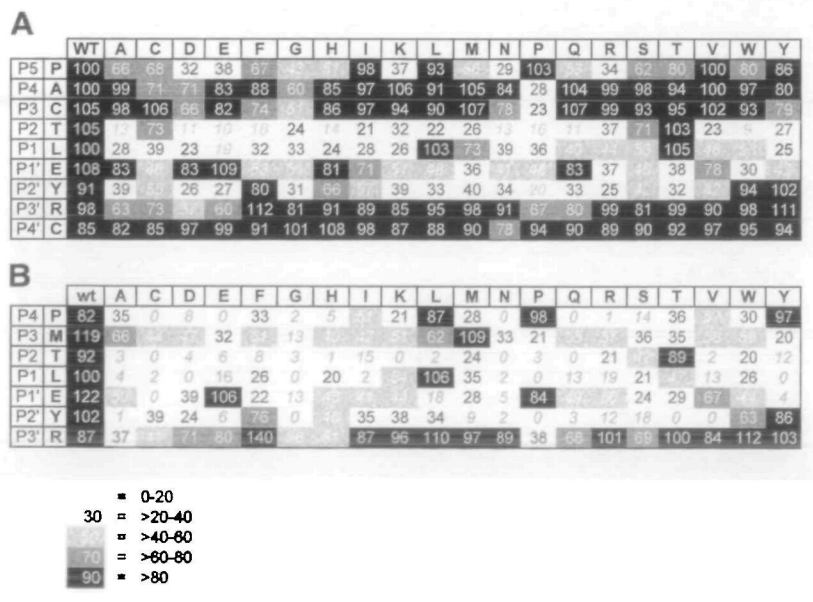

Fig. 2. **Percent relative inhibition of PPE activity in the presence of peptides from a substitutional matrix of the inhibitory peptides (A) PACTLEYRC and (B) PMTLEYR.** The PPE, chromogenic substrate and buffer were transferred to wells containing the cellulose support with the peptide. After a specified reaction time, the absorption was measured (see "MATERIALS AND METHODS"). The average inhibition by all wt peptides was set to 100%. The data were derived by averaging the results of two independent inhibition experiments.

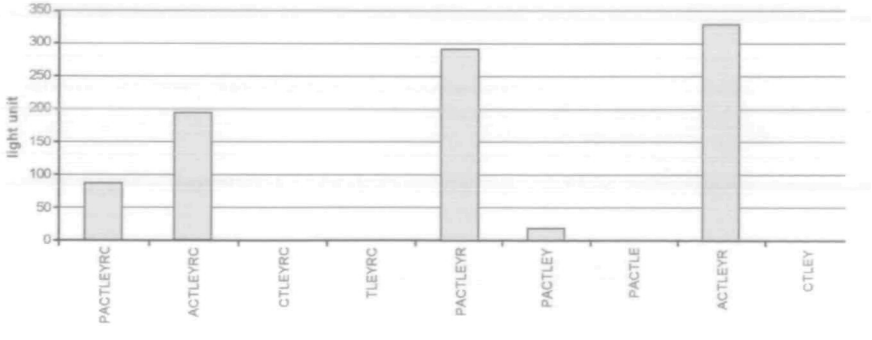

Fig. 3. **The influence of peptide length on PPE binding (cellulose-bound peptides).** The peptide was truncated at the C- or N-terminus, or at both termini. Bound POD-labeled PPE was detected as the chemiluminescent product of a POD-substrate and represented as light units. High light unit levels correspond to strong binding.

a significantly higher signal than with the wild type peptide was obtained. This was confirmed by comparing the K_i of the resin-synthesized peptide ACTLEYR, determined as 7.3 (±0.4) × 10^{-5} M, to that of the resin-synthesized peptide, AMTLEYR, 1.3 (±0.4) × 10^{-5} M, at 25°C and pH 8.5. Therefore, for further optimization, a substitutional analysis of AMTLEYR was performed where the replacement of the alanine at position P4 with Ile, Leu, Val, or Pro resulted in a significantly higher signal. Interestingly, position P2′ now changes to become quite exchange inert (data not shown). For a further round of optimization, the alanine at position P4 was replaced with proline for a substitutional binding analysis of PMTLEYR (Fig. 1C).

At this step, an inhibition experiment was performed with the substitutional matrix of PMTLEYR as described above, except that the peptides were left bound to the cellulose membrane, to verify the results of the binding experiments (Fig. 2B). The results of both experiments matched very well. The K_i for PMTLEYR was determined to be 6.6 (±0.4) × 10^{-7} M at 25°C and pH 8.5, representing a 250-fold better inhibition than with the peptide before optimization (PACTLEYRC). A further optimization attempt introducing first an phenylalanine at position P2′ and then at P3′ (data not shown), based on the stronger binding signal in the corresponding substitutional analyses, did not result in stronger inhibition. The rate of hydrolysis of PMTLEYR by PPE was determined to be 15 nmol/h·u at 25°C and pH 8.5. In fact, the inhibitor was hydrolyzed 1.1 × 10^4 fold more slowly as the substrate suc-Ala-Ala-Ala-pNA.

One important feature of affinity optimization is the specificity of the peptide at the end of the process. To investigate this, we measured the inhibitory effect of peptides PACTLEYRC and PMTLEYR on two other proteases that are strongly inhibited by OMTKY3: human leukocyte elastase (HLE) and α-chymotrypsin from bovine pancreas (Table I). The results show that the optimization was highly specific for the target protease, as expected, but, in addition, demonstrate that a peptide derived from a relatively unspecific inhibitor (OMTKY3) can be optimized to become a specific peptide inhibitor, since PMTLEYR now inhibits PPE at least 100-fold more efficiently than either HLE or chymotrypsin.

In medical applications, short peptides would be degraded by exopeptidases. To reduce such hydrolysis, a substitutional matrix of PMTLEYR introducing D-amino acids into the peptide was synthesized (data not shown). Exchanging the C- and N-termini with appropriate D-amino acids resulted in the peptide vMTLEYl. Its K_i was determined to be 5.2 (±0.7) × 10^{-6} M, indicating that D-amino acids can be introduced at both the C- and N-termini without much loss in affinity.

TABLE I. **K_i determination (in molar values) for the starting peptide PACTLEYRC and the optimized peptide PMTLEYR.** Both peptides were analyzed with three proteases: porcine pancreatic elastase (PPE), human leukocyte elastase (HLE), and α-chymotrypsin (BPC) from bovine pancreas. The data for the third domain of the ovomucoid inhibitor (OMTKY3) were taken from literature (*4*).

	PPE	HLE	BPC
OMTKY3	2.4×10^{-11}	1.6×10^{-10}	5.5×10^{-12}
PACTLEYRC	1.7×10^{-4}	2.5×10^{-4}	1.0×10^{-4}
PMTLEYR	6.7×10^{-7}	1.1×10^{-4}	1.3×10^{-4}

At the beginning of our investigations of the ovomucoid derived peptide PACTLEYR, an additional Cys was C-terminally introduced to obtain a conformation similar to that of the corresponding OMTKY3. But, obviously, the resulting disulfide bridge stabilized an unfavorable conformation for the PPE/peptide interaction. To solve this problem of whether there is another combination of two cysteines that will form a disulfide bridge that stabilizes a conformation similar to that of OMTKY3, the optimized peptide was extended for two positions at the C-terminus and three positions at the N-terminus with amino acids corresponding to OMTKY3. A "cysteine walk" (most combinations of two Cys positions in the sequence) was then synthesized, and the inhibition of PPE was investigated (see Table II). The results clearly demonstrate that most variants of the disulfide cyclized peptides show a decrease in inhibition compared with the non-disulfide cyclized peptides. Cyclic peptide variants that inhibit PPE comparably to the non-cyclized peptide PKWPMTLEYRPL (K_i = 3.0 ± 0.3 × 10^{-7} M) are connected by well-separated cysteines. Two of these peptides that show strong inhibition were selected for K_i determination, resulting in K_i = 6.8 (±0.79) × 10^{-7} M for peptide PCWPMTLEYRCL and K_i = 4.2 (±0.62) × 10^{-7} M for peptide PKCPMTLEYRPC, which is a 400-fold increase in affinity compared with the starting peptide PACTLEYRC. In all cases, the peptide inhibitions showed competitive behavior.

Error Analysis—As with all newly introduced techniques, it is important to determine the error of the method to judge the quality of the measured data. In a typical substitutional matrix 2*n* peptides with the same sequence (wild type) are present, where n is the number of amino acids in the peptide. Therefore, to determine the internal error of

TABLE II. **Percent relative inhibition of PPE activity in the presence of peptides from a "cysteine walk" ordered in the sequence of inhibition capacity.** The PPE, chromogenic substrate and buffer were transferred to wells containing the cellulose support with the peptide, and the absorption was measured after a specified reaction time (see "MATERIALS AND METHODS"). 100% activity data were obtained using punched peptide-free cellulose from a sheet applied to the peptide synthesis. The peptides chosen for K_i determination are indicated in bold letters.

Sequence	% relative inhibition	Sequence	% relative inhibition
PMTLEYR	84	PKWPMTLCYRPC	33
PMTLEYR	85	PKWPCTLCYRPL	29
PACTLEYRC	26	PKWPCTLEYRCL	29
PACTLEYRC	24	PCWPMTLCYRPL	27
PKWPMTLEYRPL	89	PKWPMTLCYRCL	23
PKCPMTLEYRPC	89	CKWPMTCEYRPL	22
PCWPMTLEYRCL	89	PCWPMTCEYRPL	20
PKCPMTLEYRCL	89	PKWPCTLEYRPC	20
CKWPMTLEYRCL	86	PKCPMTCEYRPL	19
PCWPMTLEYRPC	86	PKWPMCLEYYRCL	17
CKWPMTLEYRPC	85	PKWPMTCEYRCL	17
PCWPMTLEYCPL	82	PCWPMCLEYRPL	17
PKCPMTLEYCPL	75	PKWPCTCEYRPL	14
PKCPMTLCYRPL	72	PKWPCTLEYCPL	13
CKWPMTLEYCPL	69	PKWPMTCEYCPL	13
PKWCMTLEYRCL	65	PKCPMCLEYRPL	13
PKCPMTLECRPL	53	PKWPCTLECRPL	11
CKWPMTLCYRPL	47	CKWPMCLEYRPL	11
PCWPMTLECRPL	46	PKWPMTCEYRPC	10
CKWPCTLEYRPL	44	PKWCMTLECRPL	9
PKWCMTLEYCPL	42	PKWPMCLECRPL	7
PKWCMTLEYRPC	40	PKWCMTLCYRPL	6
PCWPCTLEYRPL	39	PKWPMCLEYRPC	0
CKWPMTLECRPL	38		

any experiment using substitutional analysis we compared the binding or inhibition signals for the same wild-type peptides but different spots. The internal average error in two equivalent sets of measurements for a binding experiment were 12 and 22%, and for two inhibition experiments, 8.6 and 9.8%. The average error for the two binding experiments taken together and all peptides involved amounts to 41%, but decreases to 12% if only the values exceeding a signal value 2 times the rms deviation are considered. The average error over the two inhibition experiments is 25% including all data. The average error between the inhibition and binding experiments was about 30%. In addition, because of a saturation effect, binding signals that look similar can differ in K_d by one or two orders of magnitude. The inhibition experiments are more sensitive and reflect more properly the behavior of the soluble peptides. It should be mentioned that the inhibition data measured have certainly to be considered as semiquantitative because of possible limitations in the solubility of the peptides cleaved from the cellulose.

DISCUSSION

Reducing the OMTKY3 inhibitor to a peptide derived from the corresponding binding region led to a drastic decrease in PPE inhibition. This was probably the result of the loss of structural constraints, and thus the large positive entropy associated with the binding energy (*15*).

Both positions P1 (Leu) and, surprisingly, P2 (Thr) in the peptide PACTLEYRC were especially important for the binding and inhibition of PPE. For position P1, a binding free energy of about –7 kJ/mol was calculated for OMTKY3 and PPE (*15*), and, indeed, this position was very sensitive to substitution. In contrast to the investigations with PPE and synthetic substrates where at P1 Ala, Ser, and Val are the preferred amino acids (*9*); in our the binding experiments, no signal at these positions are detectable. The inhibition experiments show strong inhibition only with Leu and Thr and to somewhat lesser extent with Met at P1. The substitutions at P1 with Ser or Val show 50% decrease in inhibition, in the case of Ala 70%.

P2/S2 is the second important contact for the interaction in the HLE/OMTKY3 complex (*16*). P2 is as sensitive to substitution as P1, although the binding free energy at position P2 was calculated as being only about one-seventh of the P1 value (*15*). The characterization of the S2 subsite in PPE with methylesters shows a broad spectrum of amino acids that fit very well in the S2 subsite: Lys, Gln, Tyr, Pro, Asn, Leu, Val, Thr, and Ala (*17*). In comparison with the characterization of the S1 subsite with methylesters, the effects of substitutions at S2 are low (*17*). This is in some contrast to the interaction of PPE with our ovomucoid inhibitor derived peptide where Thr at P2 can only be replaced by Ser in binding experiments, and additionally by Cys in inhibition experiments without a loss of activity. In our computer-modeled complex of ovomucoid-derived peptide and PPE (Fig. 4), threonine at P2 fits well in the S2 hole of the active site, but larger residues are probably not tolerated due to steric hindrance. This is an interesting aspect of the PPE and OMTKY3 interaction because the sensitivity of P2 has not been discussed previously from this point of view.

For the S3–P3 interaction, there is good agreement between characterizations with different substrates (*18*) and our results, showing that aromatic residues and residues with large side chains are preferred.

In the case of P1′, only –1 kJ/mol was calculated for the free energy of binding (*15*) and, consequently, some substitutions in our inhibition experiments were possible. Position P2′ changed from a relatively unspecific site in PACTLEYRC to a more specific one in AMTLEYR and PMTLEYR. The results agree well with the characterization of the S2′ subsite by Atlas *et al.* (*10*).

Computer-modeled complexes of PPE with the starting and optimized inhibitor peptides superimposed are shown in Fig. 4. From the model complexes it follows that the main contacts of the starting inhibitor peptide with the PPE substrate site (I: Thr-4 methyl group with PPE: Val-99; I: Leu-5 with PPE: Val-216, I: Tyr-7 with PPE: Leu-151) are maintained with the binding of the optimized peptide. But if Ala-2 in the inhibitor peptide is replaced by Pro and Cys-3 by Met, the peptide shows clearly better fit with close contacts to PPE: Phe-215 and Trp-172. Nevertheless, the contribution of individual amino acid residues in a peptide to its overall affinity cannot easily be derived from these models of interaction.

Concerning the results of the cysteine walk, it is of interest to mention that despite the fact that the peptide region of OMTKY3 that interacts with the protease is fixed within the inhibitor protein framework by a disulfide bridge, there

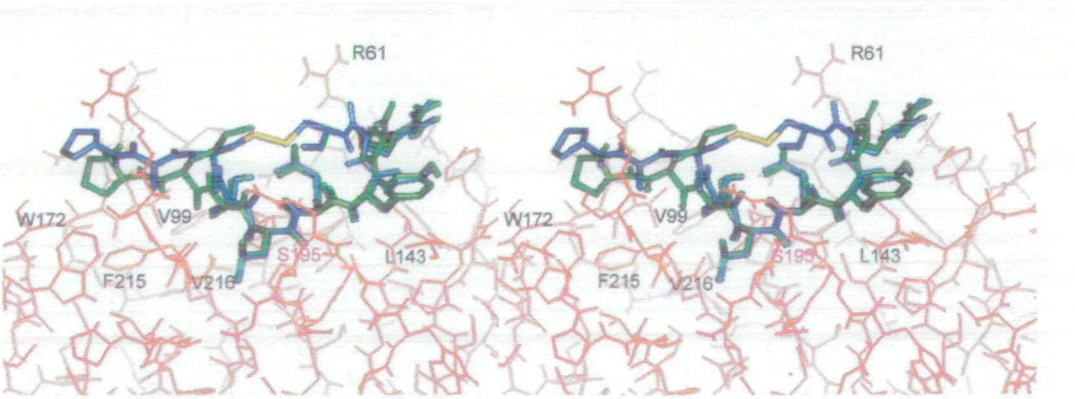

Fig. 4. **Stereo view of the PPE substrate site with the starting (blue) and optimized (green) peptides superimposed.** The most important contact residues are labeled in black, for better orientation the active site serine 195 is also indicated (in margenta). The disulfide bridge of the starting peptide is shown in yellow.

is no case in which inhibition is improved by this cyclization. If the cysteines are sufficiently spaced, the inhibition remains almost unchanged, but all the other cyclic peptides show decreased inhibition. In fact, most positions obviously produce an unfavorable conformation and only a few lead to a cyclic inhibitor peptide with affinities comparable to the linear peptide (see Table II). Thus, the entropy term seems not to be a crucial parameter in this peptide inhibitor system, in contrast, *e.g.*, to a marked stabilization of antigenic peptide epitopes brought about by the introduction of a disulfide bridge (*19*).

The internal average error in the binding and inhibition experiments described here were in the same range as the internal average errors of an antibody/peptide antigen interaction on cellulose: between 6 and 52% (average 14%) with reference to the reproducibility of spot intensities within one substitutional matrix (*20*). The average error in the inhibition experiments was lower than in the binding experiments, as it is low for enzyme assays in general.

The favored method for finding a new peptide inhibitor for proteinases is certainly the phage display technique. However, for characterizing each single position within such a peptide inhibitor, and to bring about a possible increase in the interaction specificity, peptide synthesis on cellulose is the preferable method. Another advantage of peptide synthesis on cellulose is the possibility of using non-coded residues, such as *d*-amino acids, to increase the stability of inhibitory peptides against proteolytic attack.

If a detectable substrate for the protease is available, we recommend inhibition experiments using peptides cleaved from the support. Alternatively, simple binding experiments that generally give results similar to the inhibition data may be used, but the protease has to be labeled. Peptides of interest can then be synthesized on resin for further characterizations, such as K_i determination.

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