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COVALENT HYBRIDS OF OVOMUCOID THIRD DOMAINS MADE FROM ONE SYNTHETIC AND ONE NATURAL PEPTIDE CHAIN

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We have obtained two semisynthetic covalent hybrids (Wieczorek, M. & Laskowski, M., Jr., (1983) Biochemistry 22, 2630-2636) of turkey ovomucoid third domain by coupling the natural 19-56 peptide fragment with crude, synthetic peptides 1-18 and 6-18, respectively. We have reformed all of the disulfide bridges and then we have enzymatically synthesized the 18-19 peptide bond. The enzyme-inhibitor association constants for interaction with five different serine proteinases were the same for the semisynthetic proteins 1-56 and 6-56 and for natural proteins 1-56 and 4-56. Further, the semisynthetic 1-56 and natural 1-56 proteins were indistinguishable in analytical ion exchange and reverse-phase chromatography. This work shows that 1) making the covalent hybrids from synthetic and natural material is a facile and efficient method for preparing variants for highly quantitative sequence to reactivity studies, 2) the first five NH_2-terminal residues of avian ovomucoid third domains have no effect on inhibitory activity, and 3) it is sufficient and convenient to prepare 6-56 proteins rather than 1-56 for inhibitory activity studies.

In order to continue our work on the sequence to reactivity algorithm for serine proteinase inhibitors in general, and for avian ovomucoid third domains in particular (1,2,3), we need a large supply of variants of these inhibitors. This requirement was originally met by isolation and sequencing of natural, avian ovomucoids (4) but now we need new variants whose sequences can be specified in advance. This can be done either by genetic engineering or by synthetic or semisynthetic methods. In fact, a semisynthetic method (5) has already been developed and seven new variants (4,6) were produced by its use. In this method the reactive site peptide bond is specifically hydrolyzed to form (after reduction of the disulfide bridges) NH_2-terminal [1-18][b] and COOH-terminal [19-56] fragments. Then the NH_2-terminal fragment from one avian

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[2] Numbers enclosed in brackets indicate amino acid residues.

Abbreviations:

OMTKY, turkey ovomucoid; OMTKY3, turkey ovomucoid third domain; OMTKY3*, OMTKY3 with the reactive site peptide bond hydrolyzed; des-Leu^1-Ala^2-Ala^3 OMTKY3, a truncated form of OMTKY3 whose three NH_2-terminal amino acid residues (Leu^1-Ala^2-Ala^3) were enzymatically removed; Syn_{18}OMTKY3, semisynthetic OMTKY3 with the NH_2-terminal, 1-18 fragment chemically synthesized; Syn_{13}OMTKY3, des-Leu^1-Ala^2-Ala^3-Val^4-Ser^5 Syn_{18} OMTKY3; SGPA and SGPB, Streptomyces griseus proteases A and B, respectively; DTT, dithiothreitol; K_a^{obs}, observed association equilibrium constant.

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species is mixed with the COOH-terminal fragment from another and allowed to combine to form a two-chain inhibitor. The reactive site peptide bond in this inhibitor is then enzymatically closed. Unfortunately, the yields in these reactions are rather low and we were limited to the use of readily available ovomucoids. A large increase in generality can be obtained by synthesizing the shorter (NH_2-terminal) of the two peptide chains and combining it with the longer (COOH-terminal) chain of a readily available species (in this case turkey). Here we describe this procedure and show that it should be fairly routine.

In the earlier work on sequence to reactivity algorithm (2,3) we were bothered by the variation of the NH_2-terminal length of the ovomucoid third domains (4). We were convinced on the basis of good evidence that the first five NH_2-terminal residues have essentially no influence on the enzyme-inhibitor association, but a formal proof was lacking. In this paper we provide the proof. Incidentally, we also show that the 6-18 peptide can be used in reoxidation just as well as the 1-18 peptide, thus reducing the synthetic problem in variant production to the synthesis of tridecapeptides.

Materials and Methods

Peptides for Semisynthesis. The two synthetic peptides (crude forms), 18-mer [1-18] and 13-mer [6-18] (see Fig. 1) were purchased from BioSearch, Inc. The synthesis was carried out automatically on a BioSearch SAM Two Peptide Synthesizer using N,N'-diisopropylcarbodiimide. Each peptide was cleaved from the resin by treatment with HF and anisole. The COOH-terminal natural fragment [19-56], of OMTKY3 was prepared as previously described (5).

When needed, the peptides were reduced with DTT (5,7), converted to mixed disulfides with glutathione (5,8) and stored at -15°C.

Turkey ovomucoid third domain, OMTKY3 [1-56], was prepared as described elsewhere (4).

Des-Leu^1-Ala^2-Ala^3 OMTKY3 [4-56] was generated by limited proteolysis of OMTKY with pepsin according to Kato et al. (9) and purified as described in (4).

Enzymes and substrates. Bovine α-chymotrypsin and pepsin were purchased from Worthington Biochemical Corp. Subtilisin Carlsberg was obtained from Sigma Chemical Company. Streptomyces griseus proteinase A and B were prepared from pronase (Sigma Chemical Co.) in our laboratory by an extensive modification of Jurasek et al. (10). Porcine elastase I was a gift from the late Dr. M. Laskowski, Sr.

The chromogenic turnover substrates, N-succinylglycylglycyl-L-phenylalanine p-nitroanilide, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine p-nitroanilide, and N-succinyl-L-alanyl-L-alanyl-L-alanine p-nitroanilide were products of Vega Biochemical. N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-norvaline p-nitroanilide was purchased from BACHEM Fine Chemicals.

Sequence determination. Automated amino acid sequence determination was performed on a Beckman 890C sequencer. PTH-amino acids were then identified by reverse-phase high-performance liquid chromatography (11).

Amino acid analysis. Protein and peptide samples were hydrolyzed in constant boiling HCl at 110°C for 24 h. The hydrolysates were dried under reduced pressure over sodium hydroxide. The amino acid analyses were performed on a Durrum D-500 amino acid analyzer. A computer integer fit program adopted from Hoy et al. (12) was used to calculate the results.

Enzyme-inhibitor equilibrium constants (K_a^{obs}) were measured as described by Empie & Laskowski (2) and Park (3). The substrates used are listed above. The enzyme-inhibitor reactions were performed at 22°C in a buffer containing 0.1M Tris-HCl, 0.02M $CaCl_2$, and 0.005% w/v Triton X-100, pH 8.3.

Reverse-phase HPLC was performed according to Lin et al. (13) on a Waters μBondapak C_{18} column with a binary gradient (water, trifluoroacetic acid, acetonitrile).

Analytical ion-exchange chromatography (FPLC) was performed as described in (4).

Results and Discussion

The two semisynthetic protein proteinase inhibitors were prepared by recombination of synthetic and natural peptides using our procedure described in (5). The amino acid sequences of the synthetic peptides correspond to the NH_2-terminal sequence of OMTKY3 (see Fig. 1). The natural peptide [19-56] (COOH-terminal fragment of the semisynthetic inhibitors) was generated from OMTKY3. The two synthetic peptides, 18-mer [1-18] and 13-mer [6-18] were purchased from BioSearch, Inc. as crude (not purified after deprotection) products. Each of these peptides was reduced with DTT and partially purified by Bio-Gel P-10 chromatography in 6 M guanidine HCl and 0.01 M HCl (5). In some cases, the crude material was first dissolved in 0.05 M $NH_4(HCO_3)$, washed with ethyl acetate to remove traces of organic impurities and lyophilized. The main fraction (about 75% of the applied material) obtained after Bio-Gel P-10 chromatography was collected and the reduced peptide was converted to mixed disulfide with glutathione (5,8). The synthetic peptides were not purified further by reverse-phase HPLC, but they were directly employed in the recombination experiment to generate semisynthetic inhibitor. The mixed disulfide derivatives of the synthetic (NH_2-terminal) and of natural (COOH-terminal) peptides were mixed and refolded at 4°C, pH 8.6 with 5 mM cysteine as the disulfide interchange catalyst (5,8). In the refolding mixture, the NH_2-terminal peptide was 200 μM and the COOH-terminal was 100 μM. This generated about 25 μM inhibitor from the 1-18 peptide and 20 μM inhibitor from 6-18 peptide as judged by assay with subtilisin. Subtilisin

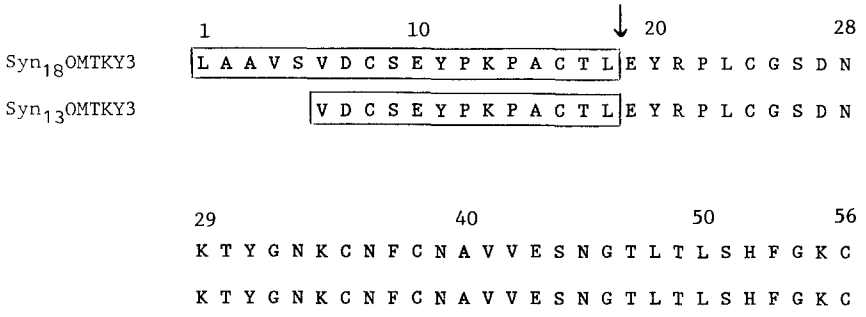

Fig. 1. Amino acid sequences of two semisynthetic inhibitors: Syn_{18}OMTKY3 and Syn_{13}OMTKY3. The peptide fragments [1-18 and 6-18] shown in boxes were chemically synthesized. The remaining COOH-terminal fragment [19-56], identical in both sequences, is natural and was generated from OMTKY3 (5). ↓ indicates the reactive site peptide bond. This bond can be specifically hydrolyzed and synthesized by serine proteinases (14).

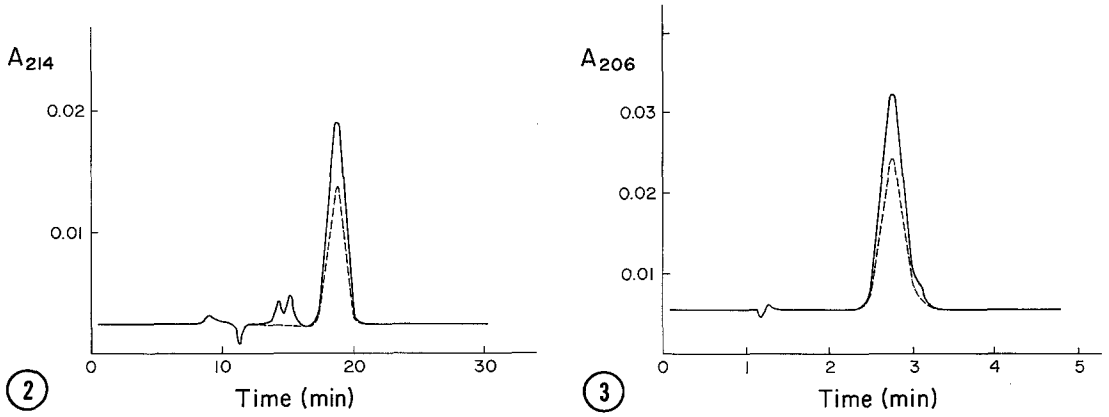

Fig. 2. Reverse-phase HPLC analysis of Syn_{18}OMTKY3.
——— Syn_{18}OMTKY3 (4 μg)
- - - OMTKY3 (natural) (3 μg)
μ-Bondapak C18 (Waters Associates) column
Mobile phase was acetonitrile-H_2O gradient (26%→30%) with 0.1% TFA additive. HPLC chromatogram of Syn_{13}OMTKY3 is virtually the same as the chromatogram of Syn_{18}OMTKY3. The retention time of Syn_{13}OMTKY3 is shorter, as expected, since this variant does not contain the NH_2-terminal hydrophobic pentapeptide.

Fig. 3. Anion-exchange chromatography of Syn_{18}OMTKY3.
——— Syn_{18}OMTKY3 (1.5 μg)
- - - OMTKY3 (natural) (1 μg)
Mono Q (Pharmacia) column
Chromatogram was obtained isocratically. Mobile phase was 0.02 M NH_4Cl and 0.015 M NaCl, pH 9.0. Chromatogram of Syn_{13}OMTKY3 is virtually the same as the chromatogram of Syn_{18}OMTKY3.

was used as it reacts rapidly with inhibitors whose reactive site peptide bond [18-19] is hydrolyzed while other enzymes react slowly (14). These yields are comparable to the previous OMTKY3* reoxidation (5). The regenerated modified (the reactive site peptide bond split) inhibitor was purified by DEAE-Sepharose chromatography and the reactive site peptide bond was enzymatically synthesized employing the kinetically controlled dissociation method (5). Equimolar amounts of SGPA[3] and of modified inhibitor (≃ 0.5 mM) were allowed to incubate for 10-20 minutes at pH 7.5 in 0.1 M Tris buffer. The complex was then dissociated by dropping the pH of the solution to 1.1. The virgin semisynthetic inhibitor was purified by gel-exclusion chromatography on a Bio-Gel P-10, followed by ion-exchange chromatography on DEAE-Sepharose. The yield of peptide bond synthesis was about 50%.

Although the semisynthetic samples are not completely homogeneous, contaminations are small (Figs. 2 and 3). These contaminations can be removed by preparative reverse-phase

[3] Proteinase K was previously used for the enzymatic synthesis (5), however SGPA is more suitable in the present procedure due to a better control of inhibitor truncation (slower removal of the NH_2-terminal amino acid residues).

Table 1

The association equilibrium constants (K_a^{obs}) of natural and semisynthetic variants of OMTKY3

	K_a^{obs} (M^{-1})				
	Chymotrypsin	Elastase	Subtilisin	SGPA	SGPB
OMTKY3 (natural)	$1.8x10^{11}$	$4.2x10^{10}$	$4.3x10^{10}$	$2.8x10^{11}$	$5.6x10^{10}$
des-L-A-A OMTKY3 (natural)	$1.8x10^{11}$	$4.0x10^{10}$	$4.5x10^{10}$	$2.9x10^{11}$	$5.7x10^{10}$
Syn_{18}OMTKY3 (semisynthetic)	$1.6x10^{11}$	$3.7x10^{10}$	$3.8x10^{10}$	$2.9x10^{11}$	$5.5x10^{10}$
Syn_{13}OMTKY3 (semisynthetic)	$1.6x10^{11}$	$3.7x10^{10}$	$3.8x10^{10}$	$3.3x10^{11}$	$5.8x10^{10}$

The experimental error of K_a^{obs} measurements is ±15%.

chromatography but in our present experiment, the level of purity was sufficient to perform further analyses. Syn_{18}OMTKY3 and native OMTKY3, which have identical sequences, coelute exactly during reverse-phase and ion-exchange chromatography. The amino acid analyses agree well with those expected from the sequences of the proteins. In addition, the shorter variant was analyzed by sequencing of the first six NH_2-terminal residues. Only the expected sequence appeared.

It should be noted that we used crude rather than purified synthetic peptides in the reoxidation. Evidently a large purification was achieved in the refolding, the reactive site resynthesis steps, and in associated chromatographic isolations of products. Thus the synthetic procedure is no more complex than working with natural variants.

Table 1 is a comparison of association equilibrium constants of five different serine proteinases with the two semisynthetic variants and with the natural OMTKY3 and des-Leu^1-Ala^2-Ala^3 OMTKY3. As is seen there, all the association equilibrium constants are the same for each enzyme. This allows us to conclude that both automated chemical synthesis and the steps involved in semisynthetic reaction do not perturb the K_a^{obs} values and thus are suitable for quantitative work on the sequence to reactivity algorithm. An additional conclusion is that the first five residues of OMTKY3 (and by inference of other avian ovomucoid third domains) have no effect on K_a^{obs}. In our previous work (2,3) this was an assumption. This conclusion is strongly supported by inferences that can be made from X-ray crystallographic studies of

free inhibitors (15,16,17) and of enzyme-inhibitor complexes (18,19,20). In combination, these two conclusions suggest that in the future studies, the synthetic 6-18 peptide will suffice. Of the eleven contact positions between the inhibitor and SGPB (18), six are in the 6-18 peptide. Thus the ability to alter this peptide synthetically allows us to vary the majority of contact residues by replacing them not only with natural amino acid residues, but also with isotopically labelled or unnatural ones.

Acknowledgments

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