Formation of Covalent Hybrids from Amino-Terminal and Carboxy-Terminal Fragments of Two Ovomucoid Third Domains†

Maciej Wieczorek and Michael Laskowski, Jr.*

ABSTRACT: Turkey ovomucoid third domain and Gambel's quail ovomucoid third domain were converted to their modified forms by specific hydrolysis of the Leu[18]–Glu[19] reactive site peptide bond. The modified inhibitors were reduced, yielding in each case two chains, the NH_2-terminal 1–18 and COOH-terminal 19–56, which could be separated by gel exclusion chromatography under reducing conditions. The chains were then converted to mixed disulfides with glutathione. The NH_2-terminal peptide of turkey domain was mixed with COOH peptide of Gambel's quail domain and allowed to form interchain disulfide bridges to produce "Turbel" hybrid. The hybrid was obtained in 20% yield and was active as a proteinase inhibitor. This modified hybrid could be purified to virtual homogeneity by ion-exchange chromatography. The reactive site peptide bond was then enzymatically synthesized yielding virgin "Turbel" hybrid. The amino acid sequence of this hybrid was in exact accord with expectations. We have shown further that the reactive site hydrolysis, disulfide reduction, reoxidation, and reactive site bond resynthesis cycle do not affect K_{assoc} for chymotrypsin. Thus, if the results described here are general, we have a useful method for generating many avian ovomucoid third domain variants.

Our laboratory is primarily engaged in determining the sequence to reactivity algorithm for avian ovomucoid third domain (Laskowski, 1980; Laskowski et al., 1981; Empie & Laskowski, 1982). In order to achieve this goal, we need a large number of ovomucoid third domain variants. At the present stage, the variants we have utilized were all natural proteinase inhibitors; however, it is quite clear that the number of natural variants that can be obtained will not be sufficient to complete the algorithm. Other methods of generating variants are clearly needed.

Among the various available methods of generating variants, one was already extensively applied to protein proteinase inhibitors, although not to avian ovomucoid third domains. This is enzymatic semisynthesis (Sealock & Laskowski, 1969; Laskowski, 1978). In this technique the reactive site peptide bond is specifically hydrolyzed by a cognate serine proteinase, and the newly formed COOH-terminal residue, P_1 [Schecter & Berger notation (1967)], is enzymatically removed and either enzymatically (Sealock & Laskowski, 1969; Jering & Tschesche, 1976) or chemically (Odani & Ono, 1980; Wenzel & Tschesche, 1981) replaced. After the replacement is made, the hydrolyzed reactive site peptide bond is enzymatically resynthesized. (Sometimes the replacement of P_1 and resynthesis of the peptide bond occur in the same step, virtually concurrently.) An analogous system of reactions has also been developed for the newly formed amino-terminal residue, P_1', of modified inhibitor (Kowalski & Laskowski, 1976; Odani & Ikenaka, 1978). These approaches have been of great value in the understanding of the inhibitory specificity, and indeed it is the ability to carry out these replacements and to study their consequences that encouraged us to seek the sequence to reactivity algorithm. We are therefore applying these methods to avian ovomucoid third domains (H. Wojciechowska and M. Laskowski, Jr., unpublished results). However, thus far described enzymatic semisyntheses of inhibitors are limited to residues P_1 and P_1', i.e., the residues immediately adjacent to the reactive site. Recently, Empie & Laskowski (1982) have shown that residues which are sequentially quite distant from the reactive site may exert great influence on the enzyme–inhibitor interaction, provided that they make a contact with the enzyme in the enzyme–inhibitor complex. Thus, we need a technique which would allow us to vary these residues as well. Formation of covalent hybrids from NH_2-terminal fragment of one protein and COOH-terminal fragment of another closely related protein is just such a technique (Harbury, 1978 and many earlier references listed therein). In this technique, cytochromes *c* from two different species were CNBr cleaved at Met[65] to generate two sets of fragments: NH_2 terminal (1–65) which terminates in homoserine lactone and COOH terminal (66–104). Then, the NH_2-terminal fragment of one species is mixed with the COOH terminal of another. The two chains not only recombine but also allow for the synthesis of a peptide bond between Hse[65] of the NH_2-terminal fragment and the amino-terminal residue (66) of the COOH-terminal fragment. Such an approach clearly allows one to study the effect of residues quite distant from the split peptide bond, provided that the needed variant peptides are available.

There is a major difference between the approach of Harbury and co-workers and the one we have adopted here. Small protein proteinase inhibitors generally, although not always (Laskowski & Kato, 1980), contain disulfide bridges. Therefore, once the reactive site peptide bond is hydrolyzed, the disulfide bridges must be ruptured in order to separate the fragments. More importantly, the disulfide bridges must be re-formed once the fragments are mixed. This step is not one that is advocated by current biochemistry tests. Reoxidation of completely reduced two-chain proteins is far more difficult than that of single-chain proteins and therefore it is relatively rarely attempted. It is customary to talk about almost negligible yields in reoxidation of insulin (a two-chain protein) in contrast to the excellent yields on reoxidation of proinsulin (a single-chain protein) (Steiner & Clark, 1968). However, a great deal of work on reoxidation of insulin was done, and now relatively good yields are obtained. For example, Gattner et al. (1981) report 13–15% yield on reoxidation. We were particularly impressed by the recent success of Duda & Light (1982) who obtained about 30% yields on reoxidation of two-chain Thr-neochymotrypsinogen. On the other hand,

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success in two-chain protein reoxidation is still far from a foregone conclusion. Pancreatic trypsin inhibitor (Kunitz) appears to be a disulfide-containing analogue of the already described cytochrome *c* system. In both, a single methionyl can be converted to homoseryl lactone by CNBr treatment with cleavage of peptide bond, and in both, the peptide bond can be re-formed (the new bond now involves the Hse residue) by incubation at mildly alkaline pH values (Dyckes et al., 1978). The two-chain form of pancreatic trypsin inhibitor (Kunitz) was reduced but could not be reoxidized (Dyckes et al., 1978).

It is the purpose of this paper to show that formation of hybrids from avian ovomucoid third domains based upon reactive site peptide bond hydrolysis, disulfide rupture, reoxidation of the two-chain modified inhibitor, and resynthesis of the peptide bond is possible in reasonable yield. To this end we have produced a covalent hybrid by combining the NH_2-terminal part of turkey ovomucoid third domain with COOH-terminal part of Gambel's quail ovomucoid third domain. This particular hybrid was chosen as convenient for describing the technique and proving the final structure.

Experimental Procedures

Materials

Avian eggs were obtained from domestic game breeders. Bovine α-chymotrypsin was obtained from Worthington Biochemical Corp. Subtilisin Carlsberg was purchased from Sigma Chemical Co. Proteinase K was from Boehringer Mannheim. *Staphylococcus aureus* V-8 proteinase was a product of Miles Laboratories, Ltd. Aspergillopeptidase B from *Aspergillus oryzae* and *Streptomyces griseus* protease B were generous gifts from Dr. G. Kalnitsky and from Drs. M. N. G. James and L. Smillie, respectively. Micropack TSK G2000SW column for gel-exclusion HPLC[1] was purchased from Varian Instrument Group.

Organic reagents were obtained from the following sources: the chromogenic turnover substrate *N*-succinylglycylglycyl-L-phenylalanine *p*-nitroanilide, Vega Biochemicals; dithiothreitol, Calbiochem-Behring Corp.; glutathione disulfide and cysteine hydrochloride monohydrate, Sigma Chemical Co.; guanidine hydrochloride (sequanal grade), Pierce Chemical Co. All other chemicals were reagent grade.

Methods

Preparation of Turkey (OMTKY3) and Gambel's Quail (OMGMQ3) Ovomucoid Third Domains. Ovomucoids were prepared by a modified procedure of Lineweaver & Murray (1947) as described by Bogard et al. (1980). Ovomucoid third domains were generated by limited proteolysis with staphylococcal proteinase (Kato et al., 1977, 1978) and isolated by molecular sieving chromatography in 5% formic acid (Kato et al., 1978; Bogard et al., 1980). Further purification of the carbohydrate-free inhibitor was performed by DEAE-Sepharose CL-6B chromatography on a 0–0.1 M linear sodium chloride gradient in 0.04 M Tris-HCl buffer, pH 8.8.

Preparation of Modified Inhibitors, OMTKY3 and OMGMQ3*.* Both modified inhibitors (with the Leu^{18}–Glu^{19} reactive site peptide bond hydrolyzed) were obtained by treatment of the virgin inhibitors with α-chymotrypsin or *Streptomyces griseus* proteinase B at low pH, followed by isolation of the product on DEAE-Sepharose CL-6B as described above (W. Ardelt and M. Laskowski, Jr., unpublished results).

Reduction of Modified Inhibitors and Preparation of Mixed Disulfide Derivatives of Inhibitor Chains. Ovomucoid third domains were routinely reduced with DTT (molar ratio 50:1 per disulfide bridge of protein) in 6 M guanidine hydrochloride and 0.2 M Tris-HCl, pH 8.7, at 25 °C for 18 h, under a nitrogen atmosphere (Creighton, 1974) yielding two peptides. The fully reduced peptides were separated by Bio-Gel P-10 chromatography in 6 M guanidine hydrochloride and 0.014 M HCl, desalted on Sephadex G-10 in 0.15 M acetic acid, and freeze-dried.

The reduced peptides (2–4 mg/mL) were treated with 0.2 M GSSG in 6 M guanidine hydrochloride and 0.2 M Tris at pH 9.0 under nitrogen at room temperature for 15 h (Duda & Light, 1982). The peptide fraction was separated on Sephadex G-25 with 0.15 M acetic acid, followed by lyophilization.

Enzyme Activity Measurements. Chymotrypsin, subtilisin, and proteinase K assays were carried out with 10^{-7}–10^{-6} M enzyme and *N*-succinylglycylglycyl-L-phenylalanine *p*-nitroanilide (8×10^{-5} M) as substrate (Shinar & Gertler, 1979). The production of *p*-nitroaniline was measured at 410 nm on a Cary 118 spectrophotometer. The buffer was 0.1 M Tris-HCl and 0.02 M $CaCl_2$, pH 8.3. In some cases, 0.005% Triton X-100 was included (Empie & Laskowski, 1982).

Determination of Operational Concentration for Enzymes and Inhibitors. Relative concentrations of an enzyme and inhibitor were determined by a tritration method. The enzyme was titrated by an inhibitor. Residual activity was measured as described for enzyme activity measurements.

Determination of Relative Association Constants. Association constants (K_{assoc}) of inhibitors with bovine α-chymotrypsin were determined by equilibrium competition method according to Empie & Laskowski (1982) with some modifications. In an experiment described by Empie & Laskowski (1982), all components of an equilibrium mixture (free inhibitors and their complexes with α-chymotrypsin) were analyzed by gel-exclusion HPLC. The peaks corresponding to the components of the mixture were not separated very well, and integration of the individual peaks was difficult. In our modified procedure, after equilibrium was achieved, free inhibitors were first separated from their complexes by methylchymotrypsin affinity chromatography (Ryan & Feeney, 1975), and then, only free inhibitors were analyzed by HPLC. Two peaks corresponding to glycosylated and nonglycosylated inhibitors were resolved very well, and difficulties with their integration were completely eliminated.

Amino Acid Analysis. Protein and peptide samples were hydrolyzed in constant boiling hydrochloric acid at 110 °C for 24 and 48 h. The hydrolysates were dried at room temperature in vacuo over sodium hydroxide pellets. The amino acid analyses were performed on a Durrum D-500 amino acid

[1] Abbreviations: OMTKY3, turkey ovomucoid third domain (sugar free); $OMTKY3_{CHO}$, turkey ovomucoid third domain sugar containing; OMGMQ3, Gambel's quail ovomucoid third domain; A peptide, NH_2-terminal peptide (1–18) obtained from OMTKY3 or OMGMQ3; B peptide, COOH-terminal peptide (19–56) obtained from OMTKY3 or OMGMQ3; $A\text{-}(SH)_2$, reduced form of peptide A; $B\text{-}(SH)_4$, reduced form of peptide B; $A\text{-}(SSG)_2$ and $B\text{-}(SSG)_4$, glutathione-mixed disulfide derivatives of peptides A and B, respectively; A-(SS), disulfide form of peptide A alone; "Turbel", hybrid inhibitor containing A peptide from OMTKY3 and B peptide from OMGMQ3; "Gamkey", hybrid inhibitor containing A peptide from OMGMQ3 and B peptide from OMTKY3; SGPB, *Streptomyces griseus* protease B; GSSG, glutathione disulfide; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CM, carboxymethyl; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; K_{assoc}, association equilibrium constant; K_{hyd}, peptide bond hydrolysis equilibrium constant. Asterisk indicates that the reactive site peptide bond of an inhibitor is hydrolyzed.

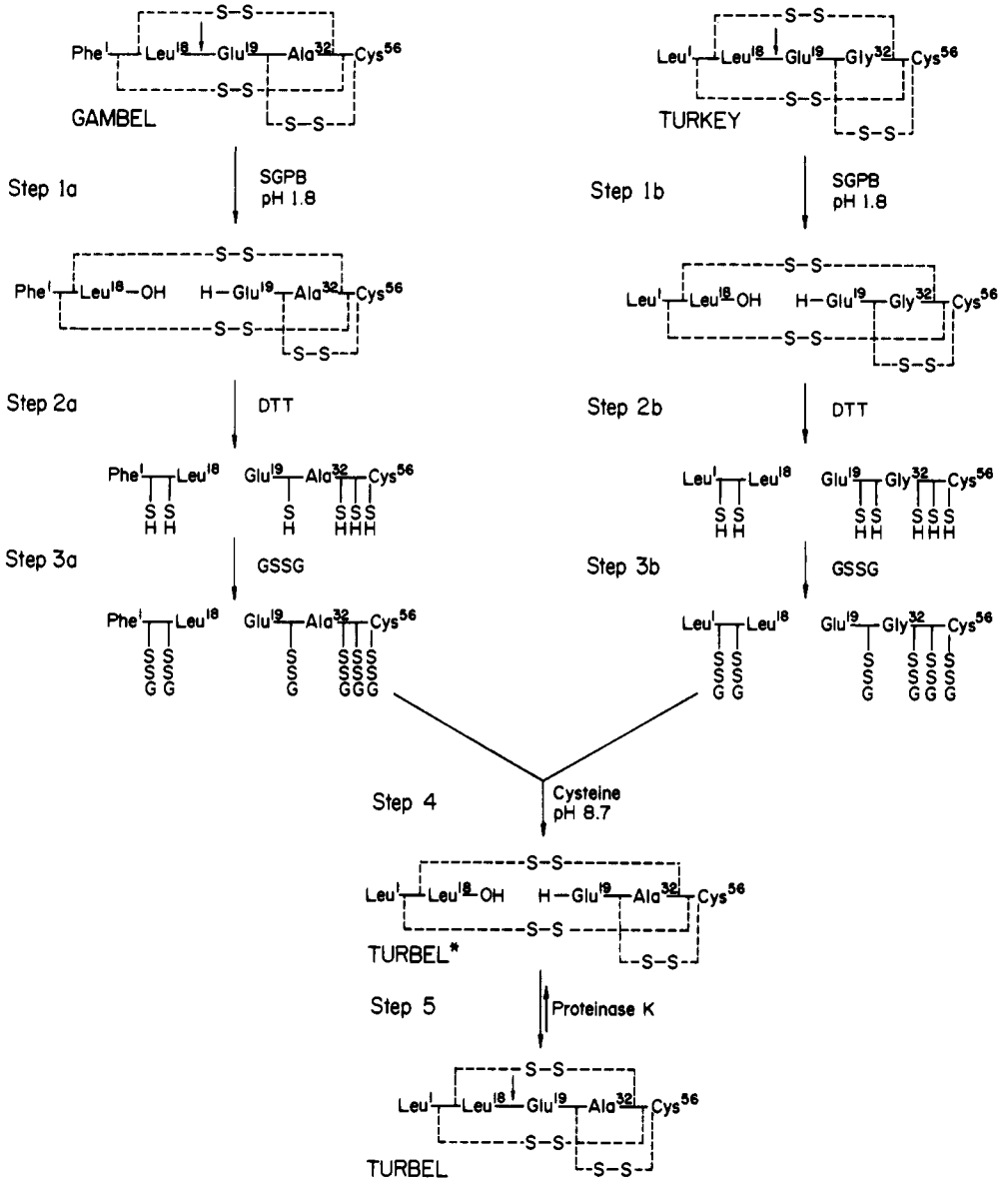

FIGURE 1: Schematic representation of the chemical and enzymatic reactions involved in the preparation of hybrid inhibitor, "Turbel". Ovomucoid third domains from Gambel's quail (step 1a) and turkey (step 1b) were converted by SGPB into modified forms (the reactive site peptide bond hydrolyzed) at pH 1.8. The modified inhibitors were then fully reduced by DTT, producing two peptides (residues 1–18 and 19–56) (steps 2a and 2b). The reduced peptides were separated by Bio-Gel P-10 chromatography (Figure 2) and converted into glutathione-(G-) mixed disulfide derivatives (steps 3a and 3b). NH_2-terminal peptide from turkey (residues 1–18) and COOH-terminal peptide from Gambel (residues 19–56) were combined in the presence of a catalytical amount of cysteine, producing hybrid inhibitor, "Turbel*" (step 4). After the purification procedure (Figures 3 and 4), the reactive site peptide bond of modified "Turbel*" was enzymatically synthesized with proteinase K, and the virgin hybrid inhibitor was finally obtained (step 5). The reactive site peptide bond (Leu^{18}–Glu^{19}) of the virgin inhibitors is indicated by (↓).

analyzer. A computer integer fit program adopted from Hoy et al. (1974) was used to calculate the results.

Sequence Determination. Amino acid sequence determination of the isolated hybrid was carried out on a Beckman Model 830C sequenator employing a 90-min Quadrol program and Sequemat converter. The amino acid phenylthiohydantoin derivatives were then analyzed, by reverse-phase high-performance liquid chromatography (Lottspeich, 1980).

Results

Reoxidation of Virgin Inhibitor. In order to be quite certain that virgin turkey ovomucoid third domain [not only entire turkey ovomucoid (Sjoberg & Feeney, 1968)] can be readily reoxidized with regain of activity, these experiments were repeated with the purified domain. The activity regain was 90 ± 10%. The result is not surprising since it has been shown that chicken ovomucoid third domain can be successfully reoxidized and that the native conformation as judged by circular dichroism is fully recovered (Matsuda et al., 1981).

Reoxidation of Modified Inhibitor. One of the central requirements of this work is the successful reoxidation of modified (reactive site peptide bond hydrolyzed) ovomucoid third domains. This is strikingly different from the reoxidation of virgin ovomucoid third domains, since reoxidation of reduced modified inhibitor involves the combination of two separate peptide chains—a process commonly considered unlikely to occur in high yield. Thus, before attempting to produce covalent hybrids from different ovomucoid molecules, we performed tests on reoxidation of a mixture made up from the two separated peptide chains of modified turkey ovomucoid. The reduced chains were separated by Bio-Gel P-10 chromatography in 6 M guanidine hydrochloride, pH 1.8 (Figure 2). This yields the NH_2-terminal A peptide, residues 1–18, and the COOH-terminal B peptide, residues 19–56 (Figure 1). The identity of the separated chains was confirmed by amino acid analyses, and complete agreement with expected

composition was obtained. Subsequent to separation, the reduced (-SH) chains were converted to mixed disulfides with glutathione:[2] A-$(SSG)_2$ and B-$(SSG)_4$ (Duda & Light, 1982), where G denotes glutathione (Figure 1). The conversion to mixed disulfides was made after rather than before separation as the unconverted, reduced chains were easier to separate. Regeneration of the modified inhibitor was accomplished by mixing A-$(SSG)_2$ and B-$(SSG)_4$ under nitrogen in a solution of 0.1 M Tris-HCl, pH 8.6, 0.2 M KCl, 1 mM EDTA (Creighton, 1974), and 5–10 mM cysteine (Odorzynski & Light, 1979). The reaction was allowed to proceed at room temperature for about 20 h. A small precipitate, which is generally formed during the reaction, was removed by centrifugation. The supernatant was desalted on a Sephadex G-25 column and lyophilized. The lyophilized material was assayed for its ability to inhibit subtilisin. The yield strongly depends upon the concentration of A-$(SSG)_2$ and B-$(SSG)_4$. The optimal molar yield (with respect to the limiting peptide chain) was 15–25% when the sum of the concentrations of the two peptides was 200–450 μM and when A-$(SSG)_2$ was in slight molar excess. Use of peptide concentrations such that the sum was greater than 450 μM resulted in extensive precipitation of peptide B-$(SSG)_4$ and therefore was not pursued further, except that large excesses of peptide A-$(SSG)_2$ could increase the yield with respect to B-$(SSG)_4$, if the concentration of B-$(SSG)_4$ was low (less than 100 μM). Once the partial success of reoxidation was established by inhibitory activity assay, we transferred our attention to the more detailed study of hybrid ovomucoid third domains. Therefore, the reoxidized modified turkey ovomucoid third domain was not purified.

Formation of Hybrids from Turkey and Gambel's Quail Ovomucoid Third Domains. Schematic representation of the chemical and enzymatic reactions involved in the preparation of the hybrid inhibitor "Turbel"—the NH_2 terminus of turkey and the COOH terminus of Gambel's quail ovomucoid third domain—is shown in Figure 1. This particular hybrid was chosen for the initial study because of the following reasons: (a) Turkey ovomucoid third domain was readily available in our laboratory, and more importantly, extensive information about the conversion of this domain from virgin to modified and from modified to virgin and on its reactions with numerous serine proteinases is available (W. Ardelt and M. Laskowski, Jr., unpublished results). (b) Gambel's quail ovomucoid third domain, which was also available in moderate amounts, differs from turkey ovomucoid third domain at two sequence positions. One of these differences, Leu^1 in turkey and Phe^1 in Gambel's quail, lies in the NH_2-terminal A peptide, and the other, Gly^{32} in turkey and Ala^{32} in Gambel's quail, lies in the COOH-terminal B peptide (see Figure 1). The presence of differences both in the A peptide and in the B peptide is a prerequisite of constructing two hybrids, in our case, "Turbel" (arbitrarily chosen for the detailed study) and "Gamkey", neither of which corresponds in sequence to one of the starting species. Thus, obtaining a material with the predicted sequence of "Turbel" cannot be explained by small contamination with the original starting material as might be the case if we ended with reoxidation of turkey ovomucoid third domain or of Gambel's quail ovomucoid third domain alone. (c) It has been shown

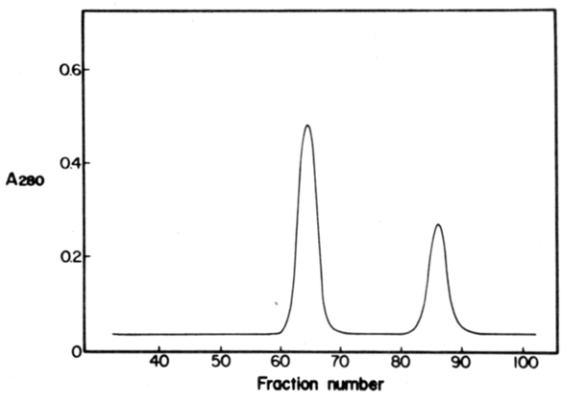

FIGURE 2: Bio-Gel P-10 separation of reduced peptides obtained from modified turkey ovomucoid third domain. The modified inhibitor (the reactive site peptide bond hydrolyzed) was fully reduced with DTT (pH 8.7). The two resulting peptides were applied on a Bio-Gel P-10 column equilibrated and eluted with 6 M guanidine hydrochloride, pH 1.8. The first peak represents COOH-terminal peptide (residues 19–56) and the second one, NH_2-terminal peptide (residues 1–18). A closely similar result was obtained with Gambel's quail ovomucoid third domain.

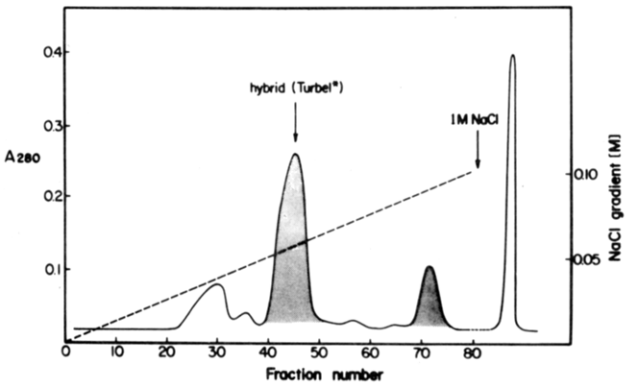

FIGURE 3: Partial purification of hybrid inhibitor, "Turbel*", by DEAE-Sepharose chromatography. The entire mixture of products after disulfide bridge formation was desalted on Sephadex G-25 and applied on a DEAE-Sepharose CL-6B column. The column was equilibrated with 0.04 M Tris-HCl buffer, pH 8.8, and eluted with 0–0.1 M linear gradient of NaCl in the same buffer. The peaks which showed inhibitory activity against subtilisin are shaded.

(Empie & Laskowski, 1982) that the interaction of Gambel's quail ovomucoid third domain with several proteinases is only slightly different from the interaction of turkey ovomucoid third domain with the same enzymes. Therefore, we expected that the methods developed for preparation of modified inhibitor, assay of the reoxidized product, isolation of products, and closure of the reactive site peptide bond developed for turkey ovomucoid third domain would be applicable to Gambel's quail ovomucoid third domain and to "Turbel" and "Gamkey" without modification. This presumption was found to be correct.

As is seen in Figure 1, the two ovomucoid third domains were enzymatically converted into their modified forms (the Leu^{18}–Glu^{19} reactive site peptide bond hydrolyzed) and then reduced by DTT in order to convert each of them into two peptide chains—the 1–18 or A-$(SH)_2$ peptide and the 19–56 or B-$(SH)_4$ peptide. The reduced peptides were separated (Figure 2) and converted to glutathione-mixed disulfide derivatives yielding in each case A-$(SSG)_2$ and B-$(SSG)_4$ (Figure 1; see also Methods). The formation of interchain disulfide bridges was performed by mixing equimolar quantities of A-$(SSG)_2$ derived from turkey ovomucoid third domain with B-$(SSG)_4$ derived from Gambel's quail ovomucoid third domain and allowing them to react under conditions described

[2] Amino acid analysis showed that all (two) cysteines in peptide A and 90% of all (four) cysteines in peptide B were converted into the mixed disulfide derivative. We assume that in the case of peptide B, B-$(SSG)_4$ is a main product of the reaction of reduced peptide B with oxidized glutathione (GSSG). The existence of other mixed disulfide components containing intra- or interchain disulfide bridges cannot be completely excluded.

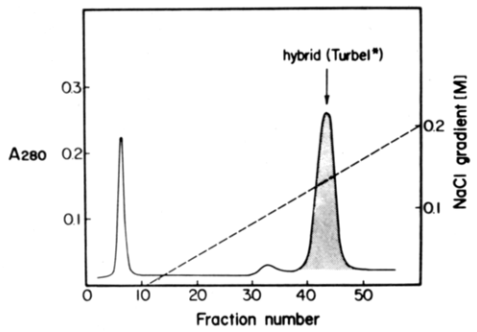

FIGURE 4: Purification of hybrid inhibitor, "Turbel*", by CM-Sepharose chromatography. The material recovered from DEAE-Sepharose chromatography (fractions 41–49) (Figure 3) was desalted and applied on a CM-Sepharose CL-6B column equilibrated with 0.05 M acetate buffer, pH 3.8. The column was developed with 0–0.2 M linear gradient of NaCl in the equilibrating buffer.

in a previous section. The newly generated inhibitory activity against subtilisin was approximately 25%. The entire material was subjected to DEAE-Sepharose ion-exchange chromatography (Figure 3). The first major peak (fractions 41–49) contains most of the inhibitory activity. Its elution position corresponds almost exactly to that of modified turkey ovomucoid. Surprisingly, another peak (fractions 69–75) also contains a significant (about 25% of the total) inhibitory activity. On the basis of its elution position, we assume that the material forming this peak is more negative than the desired hybrid. Its amino acid analysis exactly corresponds to that predicted for the hybrid. Since the amount of material was not large and the elution position somewhat anomalous, the material was not studied further.

The desired hybrid was finally purified by CM-Sepharose chromatography (Figure 4). In this process some contaminating peptide with the composition of A-(SS) was removed.

The purified hybrid was indistinguishable from modified turkey ovomucoid third domain by analytical DEAE chromatography (Ardelt & Laskowski, 1982) and by gel-exclusion chromatography (Bio-Gel P-10 and TSK G2000). This was an expected result since the only difference between the modified turkey ovomucoid third domain and the modified "Turbel" hybrid is the replacement of Gly^{32} (in turkey) by Ala^{32} (in "Turbel"). Such a change should not influence chromatographic behavior. The amino acid analysis of modified "Turbel" hybrid was in exact agreement with what was expected.

Enzymatic Synthesis of the Reactive Site Peptide Bond of the Hybrid. This laboratory has developed two major techniques for resynthesis of hydrolyzed peptide bonds in modified protein proteinase inhibitors—kinetically controlled dissociation of enzyme–inhibitor complex (Finkenstadt & Laskowski,.1967; Laskowski et al., 1976) and enzymatic achievement of modified $\rightleftarrows$ virgin inhibitor equilibrium near neutral pH (Estell et al., 1980). In the first technique, the modified inhibitor is allowed to react with roughly equimolar quantity of enzyme to form the enzyme–inhibitor complex. It is a major result of our work that this complex is the same chemical substance whether formed from virgin inhibitor or from modified inhibitor. Once formed, the complex is transferred to a medium where the rates of enzyme–inhibitor association are negligible (e.g., very low pH or concentrated guanidine hydrochloride solution). Under these conditions, the complex dissociates, and the yield of virgin inhibitor is governed not by equilibrium considerations but by relative rates of dissociation toward virgin and toward modified inhibitor. In most, but not all (W. Ardelt and M. Laskowski, Jr., unpublished results), cases thus far studied, the kinetically controlled dissociation conditions overwhelmingly favor the virgin inhibitor and thus offer a facile route for resynthesis of the peptide bond. There are two possible difficulties in this approach. For some enzyme–inhibitor pairs, the rate of reaction with modified inhibitor is very slow, and thus the complex may be difficult to form. The second problem is that once the kinetically controlled dissociation is affected, yielding predominantly virgin inhibitor, this virgin inhibitor may be converted to modified unless further contact between the enzyme and inhibitor is prevented.

For the kinetically controlled dissociation approach, proteinase K (Ebeling et al., 1974) was chosen since W. Ardelt and M. Laskowski, Jr. (unpublished results), found it suitable for this purpose both because it reacts relatively rapidly with modified inhibitors and because virgin inhibitor can be easily isolated from its dissociation products. Equimolar amounts of proteinase K and of modified "Turbel" hybrid (approximately 0.5 mM in 1 mL total volume) were allowed to incubate for 30 s at pH 6.5 in 0.1 M Tris–acetate and 0.02 M $CaCl_2$ in order to form the enzyme–inhibitor complex. The complex was then dissociated by addition of 0.2 mL of 1 M HCl, which lowered the pH of the solution to 1.0. The virgin inhibitor was then separated from the enzyme and from traces of modified inhibitor by gel-exclusion chromatography on Bio-Gel P-10 (pH 1.8), followed by ion-exchange chromatography on DEAE-Sepharose as described for preparation of OMTKY3* (see Methods). The yield of peptide bond synthesis was about 70%.

An alternative approach to resynthesis of the reactive site peptide bond in modified inhibitors was the addition of catalytic amounts of enzyme near to neutral pH in order to affect the modified $\rightleftarrows$ virgin inhibitor equilibrium. The potential disadvantages of this procedure are again two. One is that since K_{hyd} is typically only about unity, even the theoretical yield is only about 50%. The second is that most enzymes which are very strongly inhibited are also very slow in catalyzing the virgin to modified inhibitor equilibration. Exceptions are, however, known. Estell et al. (1980) found that *Dermasterias imbricata* trypsin-1 rapidly catalyzes the establishment of virgin $\rightleftarrows$ modified trypsin inhibitor equilibrium around neutral pH. More recently, W. Ardelt and M. Laskowski, Jr. (unpublished results), found that aspergillopeptidase B (Kalnitsky & Subramanian, 1964) behaves similarly with respect to turkey ovomucoid third domain. Therefore, aspergillopeptidase B was used for these studies.

The modified hybrid inhibitor was incubated with 3 mol % of aspergillopeptidase B at pH 6.0 (0.05 M Mes) for 45 h. The conversion was monitored by analytical ion-exchange chromatography (Ardelt & Laskowski, 1982). The final values were 65% virgin and 45% modified inhibitor. Virgin hybrid "Turbel" was isolated as described for kinetically controlled dissociation. The yield seems higher than expected (55%) from the results of W. Ardelt and M. Laskowski, Jr. (unpublished results), with turkey ovomucoid third domain at pH 6.0 in 0.50 M KCl and 0.05 M $CaCl_2$. We suspect that the difference is due to variance in ionic strengths between the experiments. Surprisingly, however, amino acid analysis and sequencing showed that the inhibitor obtained by this method was actually des-Leu^1-"Turbel" rather than "Turbel" itself. Evidently, the NH_2-terminal Leu was removed either by an aminopeptidase impurity present in our aspergillopeptidase B preparation or by aspergillopeptidase itself. Similar problems, removal of one or several residues from avian ovomucoid third domains, are now frequently encountered in our laboratory and are presently under study. A precedent for such reactions was first described by Omichi et al. (1980) in the subtilisin–SSI system.

The removal of the NH_2-terminal Leu was quite unfortunate since it negated part of our proof by sequencing (see below). Therefore, the proof by sequencing was done on virgin inhibitor obtained from kinetically controlled dissociation of proteinase K complex. On the other hand, des-Leu[1]-"Turbel" proved quite useful in comparison of K_{assoc} for association with that of α-chymotrypsin.

Proof of Structure by Sequencing. The semisynthetic virgin "Turbel" hybrid should show the following properties upon sequencing: (a) only one sequence should be apparent, because if the inhibitor were modified or heavily nicked at some other positions, several residues would be observed at each turn, (b) the NH_2-terminal residue should be only Leu (no trace of Phe) since the NH_2-terminal part comes from turkey and not from Gambel's quail, (c) residue 32 should be Ala and not Gly since the COOH-terminal peptide comes from Gambel's quail rather than turkey, and (d) all the remainder of the sequence should be as expected from Gambel's quail or turkey third domain sequences.

The sequence of "Turbel" was run, and all of these expectations were met. Since the material was run intact, the Cys residues were not detected, but all the appropriate positions were blank.

Interaction of "Turbel" Hybrid with α-Chymotrypsin. We feel that the data presented thus far are sufficient to establish with high probability that the chemical structure (linear sequence and pairing of disulfide bridges) are as would be expected for the "Turbel" hybrid. However, the possibility still exists that the conformation of "Turbel" differs from that of ovomucoid third domains which are isolated directly and not subject to the reactive site hydrolysis, disulfide reduction, oxidation, and reactive site synthesis cycle used here. This is not just an idle exercise since States et al. (1980) and Kosen et al. (1981) reported that reoxidized bovine pancreatic trypsin inhibitor has a slightly different conformation from the natural one unless it is heated to about 50 °C when the natural conformation is recovered. In deciding which of the possible tests for conformational identity or similarity might be the most appropriate and convenient, we realized that for our purposes all that is needed is to show that inhibitors subjected to our cycle interact with their cognate enzymes in an identical manner with the natural ones. The enzyme–inhibitor association constant K_{assoc} is a good quantitative measure of this. However, the equilibrium constant expected for the hybrid is not truly known except that Empie & Laskowski (1982) assumed that the first five residues of the inhibitor domain have no effect upon enzyme–inhibitor association. This problem can be circumvented since we have found that incubation with our preparation of aspergillopeptidase B quantitatively removes the first residue of the third domain. After such a removal, the sequences of "Turbel" and of Gambel's quail third domain are the same (see Figure 1). Now if the association equilibrium constants are also the same, we can state that the hybrid and the natural inhibitor are functionally equivalent. To this end, we have treated Gambel's quail ovomucoid third domain with aspergillopeptidase B under the conditions described for resynthesis of reactive site peptide bond in "Turbel". The virgin inhibitor component of this mixture was then isolated and shown to be des-Phe[1] Gambel's quail ovomucoid third domain (this conclusion is based on amino acid analysis and a five-residue sequence run). The relative constants with bovine α-chymotrypsin were then determined by competing the test domain against carbohydrate-containing turkey ovomucoid third domain and monitoring this competition by high-pressure liquid chromatography as described by Empie & Laskowski (1982) (some modifications of the method have been described under Experimental Procedures). The following results were obtained.

$$\frac{K_{assoc}(\text{des-Leu}^1\text{-"Turbel"})}{K_{assoc}(\text{OMTKY3}_{CHO})} = 3.8 \pm 0.8$$

$$\frac{K_{assoc}(\text{des-Phe}^1\text{-OMGMQ3})}{K_{assoc}(\text{OMTKY3}_{CHO})} = 3.7 \pm 0.8$$

$$\frac{K_{assoc}(\text{OMGMQ3})}{K_{assoc}(\text{OMTKY3}_{CHO})} = 3.3 \pm 0.6$$

$$\frac{K_{assoc}(\text{OMTKY3})}{K_{assoc}(\text{OMTKY3}_{CHO})} = 1.0 \pm 0.2$$

These results show clearly that the hybrid has the equilibrium constant that is expected from the natural inhibitor. They further serve to confirm the statements by Empie & Laskowski (1982) that neither carbohydrate attachment nor the NH_2-terminal residue has any effect on interaction with α-chymotrypsin.

Discussion

Our group has a clear need for avian ovomucoid third domain variants. The technique described here may go a long way toward satisfying this need since among the 36 different avian third domains, which we have sequenced until now, there are represented 18 different A peptides and 31 different B peptides. Thus, potentially we could produce $18 \times 31 = 558$ variants[3] *provided the technique is general.* The generality of the proposed technique is difficult to assess. We do not know as yet whether every A,B peptide combination will produce a hybrid inhibitor in satisfactory yield. Problems could arise from particularly limited solubility of some of the peptides or possibly from poor A and B complementarity. Even if the proper disulfide pairing is successful, the closure of the peptide bond in the resultant hybrid may prove difficult unless the hybrid is a relatively strong inhibitor of an available serine proteinase or unless a serine proteinase which is strictly specific for the reactive site and not another peptide bond is known. Thus, until we are truly aware of the technique's limitations, we will refrain from a lengthy discussion of its general application. It will certainly be of interest whether the technique can be applied to the formation of a covalent hybrid from A peptide of say the second domain with B peptide of the third, and so on.

The question of yield in the overall procedure is of considerable interest. Chemists are interested in yield for two reasons—one is that high yield is often taken as part of the proof that the synthesis was correctly carried out, and the other is efficiency. Here, the structure proof obtained by sequencing eliminates the need for high yield from the point of view of proof. From the point of view of efficiency, the relatively low yield obtained here is not especially bothersome in our application. In the case of "Turbel", the yield of proper disulfide bridge formation is about 20%. The final yield of the entire procedure, i.e., reactive site peptide bond hydrolysis, reduction, separation of peptide chains, oxidation, reactive site peptide bond synthesis, and numerous purification steps, is about 8%.

[3] Of these potential 558 variants, 36 represent the natural species we already have. Therefore, it may be more proper to talk about the potential for getting $558 - 36 = 522$ new variants. Note that this number increases dramatically as the number of available natural variants increases. This calculation is included here only to point out that a very large number of covalent hybrids is potentially available. We intend to produce only a relatively small number of these.

This means that, starting with 50 mg each of Gambel's quail and turkey ovomucoid third domains, we are able to prepare 4 mg of each of the two possible virgin hybrid inhibitors. This is quite a satisfactory amount for our purposes because in most cases only 3–5 mg of an inhibitor are necessary to perform all chemical and thermodynamic (K_{assoc}) studies. Preparation of 50–100 mg of starting material (ovomucoid third domain) is quite feasible for most avian species. One of the simple possibilities to increase the yield of hybrid production seems to be recycling of inactive material produced during disulfide bridge formation through reduction and subsequent reoxidation.

A very gratifying conclusion of this paper is the finding that the des-Leu[1]-"Turbel" hybrid which has the same sequence as des-Phe[1] Gambel's quail ovomucoid third domain has identical association constants with that of chymotrypsin. This finding proves that the numerous reactions involved in formation of "Turbel" are not deleterious and that if new hybrids are produced, they can be used with high confidence as equivalent to natural materials of the same sequence.

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Registry No. OMGMQ3, 85421-62-1; OMTKY3, 85421-58-5; OMGMQ3*, 85421-63-2; OMTKY3*, 85421-59-6; B-$(SH)_4$, 85421-56-3; A-$(SH)_2$, 85251-36-1; B-$(SSG)_4$, 85421-57-4; A-$(SSG)_2$, 85251-37-2; Turbel*, 85421-61-0; Turbel, 85421-60-9; chymotrypsin, 9004-07-3.

References

Ardelt, W., & Laskowski, M., Jr. (1982) *Anal. Biochem. 120*, 198–203.

Bogard, W. C., Jr., Kato, I., & Laskowski, M., Jr. (1980) *J. Biol. Chem. 255*, 6569–6574.

Creighton, T. E. (1974) *J. Mol. Biol. 87*, 563–577.

Duda, C. T., & Light, A. (1982) *J. Biol. Chem. 257*, 9866–9871.

Dyckes, D. F., Creighton, T. E., & Sheppard, R. C. (1978) *Int. J. Pept. Protein Res. 11*, 258–268.

Ebeling, W., Hennrich, N., Klockow, M., Metz, H., Orth, H. D., & Lang, H. (1974) *Eur. J. Biochem. 47*, 91–97.

Empie, M. W., & Laskowski, M., Jr. (1982) *Biochemistry 21*, 2274–2284.

Estell, D. A., Wilson, K. A., & Laskowski, M., Jr. (1980) *Biochemistry 19*, 131–137.

Finkenstadt, W. R., & Laskowski, M., Jr. (1967) *J. Biol. Chem. 212*, 771–773.

Gattner, H. G., Krail, G., Danho, W., Knorr, R., Wienke, H. J., Bullesbach, E. E., Schartmann, B., Brandenburg, D., & Zahn, H. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1043–1049.

Harbury, H. A. (1978) *Semisynth. Pept. Proteins, Pap. Int. Meet. Protein Semisynth., 1977*, 73–89.

Hoy, T. G., Ferdinand, W., & Harrison, P. M. (1974) *Int. J. Pept. Protein Res. 6*, 121–140.

Jering, H., & Tschesche, H. (1976) *Eur. J. Biochem. 61*, 453–463.

Kalnitsky, G., & Subramanian, A. R. (1964) *Biochemistry 3*, 1861–1867.

Kato, I., Kohr, W. J., & Laskowski, M., Jr. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 764.

Kato, I., Kohr, W. J., & Laskowski, M., Jr. (1978) *Proc. FEBS Meet. 47*, 197–206.

Kosen, P. A., Creighton, T. E., & Blout, R. E. (1981) *Biochemistry 20*, 5744–5754.

Kowalski, D., & Laskowski, M., Jr. (1976) *Biochemistry 15*, 1300–1309.

Laskowski, M., Jr. (1978) *Semisynth. Pept. Proteins, Pap. Int. Meet. Protein Semisynth., 1977*, 255–262.

Laskowski, M., Jr. (1980) *Biochem. Pharmacol. 29*, 2089–2094.

Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593–626.

Laskowski, M., Jr., Finkenstadt, W. R., Mattis, J. A., & McKee, R. E. (1976) *Protides Biol. Fluids 23*, 85–91.

Laskowski, M., Jr., Empie, M. W., Kato, I., Kohr, W. J., Ardelt, W., Bogard, W. C., Jr., Weber, E., Papamokos, E., Bode, W., & Huber, R. (1981) *32nd Mosbach Colloquium* (Eggerer, H., & Huber, R., Eds.) pp 136–152, Springer-Verlag, Berlin.

Lineweaver, H., & Murray, C. H. (1947) *J. Biol. Chem. 171*, 565–581.

Lottspeich, F. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1829–1834.

Matsuda, T., Watanabe, K., & Sato, Y. (1981) *FEBS Lett. 124*, 185–188.

Odani, S., & Ikenaka, T. (1978) *J. Biochem. (Tokyo) 84*, 1–9.

Odani, S., & Ono, T. (1980) *J. Biochem. (Tokyo) 88*, 1555–1558.

Odorzynski, T. W., & Light, A. (1979) *J. Biol. Chem. 254*, 4291–4295.

Omichi, K., Nagura, N., & Ikenaka, T. (1980) *J. Biochem. (Tokyo) 87*, 483–489.

Ryan, D. S., & Feeney, R. E. (1975) *J. Biol. Chem. 250*, 843–877.

Schecter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Sealock, R. W., & Laskowski, M., Jr. (1969) *Biochemistry 8*, 3703–3710.

Shinar, S., & Gertler, A. (1979) *Int. J. Pept. Protein Res. 13*, 218–222.

Sjoberg, L. B., & Feeney, R. E. (1968) *Biochim. Biophys. Acta 168*, 79–86.

States, D. J., Dobson, C. M., Karplus, M., & Creighton, T. E. (1980) *Nature (London) 286*, 630–632.

Steiner, D. B., & Clark, J. L. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 622–629.

Wenzel, H. R., & Tschesche, H. (1981) *Angew. Chem., Int. Ed. Engl. 20*, 295–296.

Wieczorek, M., & Laskowski, M., Jr. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 762.