Articles

Probing Intermolecular Main Chain Hydrogen Bonding in Serine Proteinase−Protein Inhibitor Complexes: Chemical Synthesis of Backbone-Engineered Turkey Ovomucoid Third Domain†

Wuyuan Lu,*,‡ M. A. Qasim,§ Michael Laskowski, Jr.,§ and Stephen B. H. Kent‡

The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, and Chemistry Department, Purdue University, West Lafayette, Indiana 47907

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ABSTRACT: Intermolecular main chain H-bonding networks are frequently encountered at the interface of complexes of protein proteinase inhibitors and their cognate enzymes. Studies of X-ray crystal structures of many protein inhibitors complexed with serine proteinases have revealed that the amide NH group of the P1 residue in the inhibitor donates an H-bond to the carbonyl C═O group of Ser^{214} and Ser^{195} O^{γ} in the enzyme (Ser^{125} and Ser^{221} in subtilisins, respectively). To probe the energetic contribution of this backbone H-bond in the complexes of OMTKY3 with several serine proteinases, native chemical ligation was used for the total synthesis of a backbone-engineered analog of OMTKY3, in which the amide peptide bond between Thr^{17} (P2) and Leu^{18} (P1) was replaced by an ester bond, i.e., −CONH− to −COO−. This chemical "mutation" effectively eliminated the backbone H-bond donated by the NH group of Leu^{18}. By measuring association equilibrium constants for synthetic wild-type OMTKY3 and the backbone-engineered ester analog interacting with a panel of six serine proteinases, we have determined that the P1 NH → O substitution weakens the binding of OMTKY3 to its cognate enzymes by an average of 15-fold, i.e., 1.5 ± 0.3 kcal/mol. These results place a quantitative value on the contribution of the intermolecular backbone H-bond in enzyme−inhibitor recognition.

H-bonding has long been the subject of debate with regard to its precise role in protein folding and protein stability as well as in protein recognition (Kauzmann, 1959; Dill, 1990; Honig & Yang, 1995; Myers & Pace, 1996). Despite tremendous progress made in the past decade, the energetic significance of H-bonds in proteins continues to remain poorly understood largely due to the complexity resulting from entropic and enthalpic contributions to the H-bond formation (Fersht, 1987; Dill, 1990; Connelly et al., 1994).

The vast majority of H-bonds in proteins are formed between the backbone amide NH group (as a donor) and the backbone carbonyl C═O group (as an acceptor) (Pauling et al., 1951; Baker & Hubbard, 1984; Stickle et al., 1992). Not surprisingly, backbone−backbone H-bonding networks are often observed at the interface of protein complexes and are believed to be important in determining the specificity of protein recognition (Jencks, 1969; Fersht, 1985). Thus, studying *backbone* H-bonding appears to be essential in understanding the energetic contributions of H-bonds to protein folding and protein−protein interactions. Nonetheless, this major group of H-bonds has so far received little attention, presumably because of the lack of the experimental means necessary to directly address their role.

Site-directed mutagenesis is commonly used for studying side chain H-bonding in proteins (Fersht et al., 1985; Alber et al., 1987; Shirley et al., 1992). However, removal of a backbone H-bond can only be achieved by proline substitution. Such mutation invariably causes additional structural perturbation, which can complicate the subsequent assessment of the free energy change associated with removal of the backbone H-bond. A recently developed technique of *in vitro* translation (Mendel et al., 1995) allows many noncoded amino acids to be incorporated into proteins and therefore holds great promise for protein backbone engineering. Even so, systematic removal of backbone H-bonds in proteins still remains a challenging task.

Total chemical synthesis (Muir & Kent, 1993) possesses a unique ability and versatility in incorporating into proteins

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* To whom correspondence should be addressed. Phone: (619) 784-7075. FAX: (619) 784-7319. E-mail: wuyuan@chemical.ligation.com.

‡ The Scripps Research Institute.

§ Purdue University.

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[1] Abbreviations: OMTKY3, turkey ovomucoid third domain; BPTI, bovine pancreatic trypsin inhibitor; CHYM, bovine α-chymotrypsin; PPE, porcine pancreatic elastase; HLE, human leukocyte elastase; SCARL, subtilisin Carlsberg; SGPA, *Streptomyces griseus* proteinase A; SGPB, *S. griseus* proteinase B; SPPS, solid-phase peptide synthesis; RP-HPLC, reversed-phase high-pressure liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; Boc, *tert*-butyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEA, *N*,*N*-diisopropylethylamine; HBOT, 1-hydroxybenzotriazole; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; DCM, dichloromethane; DMAP, 4-(dimethylamino)-pyridine; NEM, *N*-ethylmorpholine; MBHA, 4-methylbenzhydrylamine; PAM, 4-(hydroxymethyl)phenylacetamidomethyl; TFA, trifluoroacetic acid; ACN, acetonitrile; Ape, α-aminopentanoic acid.

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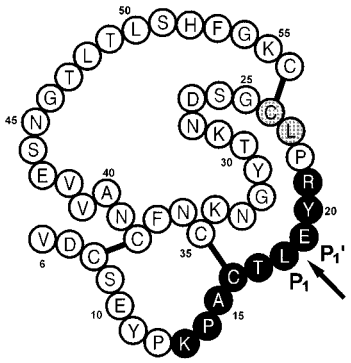

FIGURE 1: Amino acid sequence of (6−56)OMTKY3. The residues in black are involved in direct contact with cognate enzymes. The arrow indicates the reactive site scissile peptide bond; the shaded residues Leu[23]−Cys[24] represent the site for native chemical ligation. In this work, the P1 residue Leu[18] was replaced by L-leucic acid to incorporate an ester bond between Thr[17] and Leu[18].

unnatural amino acids and a variety of other novel structural and functional modifications in both side chains and the backbone of the polypeptide. Introduction of N-alkylated amino acids is a straightforward way of removing backbone H-bonds in synthetic proteins (Rajarathnam et al., 1994). However, due to the fact that extra alkyl groups can sometimes cause unnecessary structural and thermodynamic perturbation, a better approach would be to replace the peptide amide by an ester, i.e., −CONH− to −COO−. Ester bonds were routinely incorporated into small peptides (Arad & Goodman, 1990) and enzyme substrates (Bramson et al., 1985; Thomas et al., 1987). Nonetheless, reports on ester bond analogs of proteins made by total chemical synthesis are extremely scarce in the literature. In one case, semisynthesis was used to replace a peptide bond in bovine pancreatic trypsin inhibitor (BPTI)[1] by an ester bond; binding of the resultant BPTI analog to trypsin was reduced by 25-fold, i.e., 1.9 kcal/mol (Groeger et al., 1994). However, backbone engineering by semisynthesis is generally limited to a few special positions in the protein. Recent advances in chemical synthesis (Muir, 1995), particularly the development of the native chemical ligation technique (Dawson et al., 1994), have made possible direct incorporation of ester bonds into proteins by total chemical synthesis.

OMTKY3 (Figure 1) is a potent protein inhibitor of many serine proteinases that have a neutral S1 specificity pocket (Empie & Laskowski, 1982). Systematic site-directed mutagenesis studies at the P1 position of OMTKY3 (Lu et al., 1996a) have revealed that proline is the most deleterious residue at the P1 position of OMTKY3 for most of the enzymes studied. In contrast, the introduction of a P1 residue with an aliphatic side chain of similar size to Pro, namely, L-α-aminopentanoic acid (Ape) (Bigler et al., 1993), *enhanced* the binding by an average of −8 kcal/mol compared with the P1 Pro (Lu et al., 1996a). One obvious consequence of introducing proline at P1 is deletion of a H-bond donated by the amide NH group of the P1 residue in the inhibitor to the carbonyl C═O group of Ser[214] and Ser[195] O^{γ} in the enzyme (Figure 2). This H-bond has been found to be highly conserved in most serine proteinase−protein inhibitor complexes crystallized (Read & James, 1986; Bode & Huber, 1992; Huang et al., 1995).

How important energetically is this particular backbone H-bond? It is unreasonable to assume that the −8 kcal/mol from the P1 Pro → Ape substitution is entirely due to deletion of the backbone H-bond. In order to address this issue, we have applied the native chemical ligation method to the synthesis of a backbone-modified analog of OMTKY3, where the amide peptide bond between Thr[17] (P2) and Leu[18] (P1) was replaced by an ester bond, i.e., −CONH− to −COO−. Here we report the total chemical synthesis of (6−56)Thr[17](COO)Leu[18]OMTKY3 and the results of thermodynamic studies of the ester-containing inhibitor interacting with a panel of six serine proteinases: bovine α-chymotrypsin (CHYM); subtilisin Carlsberg (SCARL); human

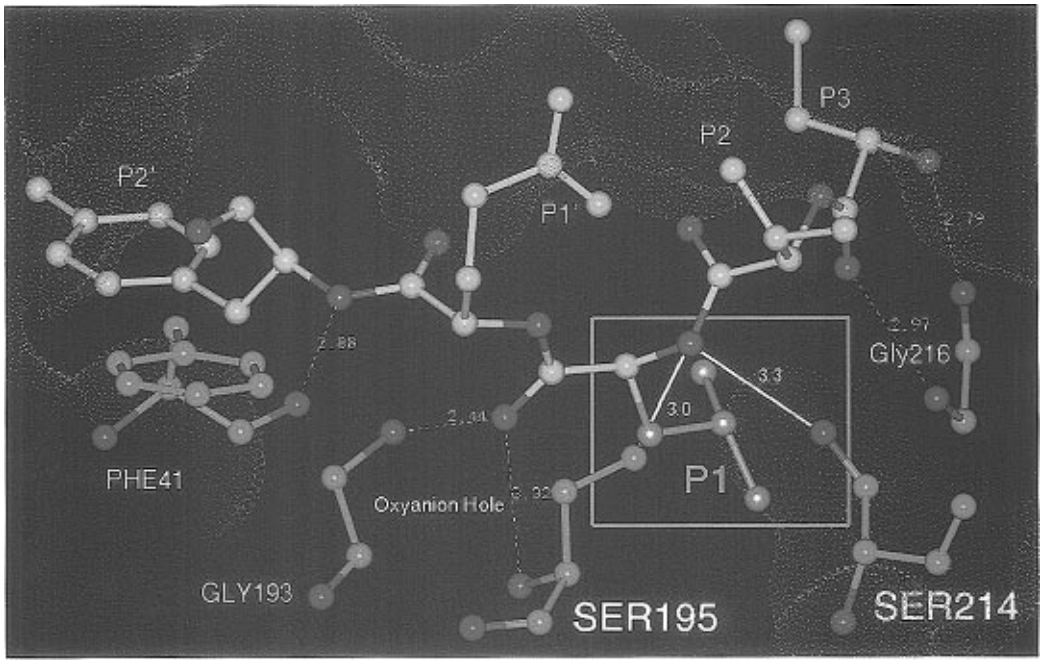

FIGURE 2: Close-up view of the intermolecular backbone H-bonding at the interface of CHYM-OMTKY3 (Fujinaga et al., 1987). The residues of the inhibitor (P3−P2′, in Schechter and Berger notation) and of the enzyme (in chymotrypsinogen numbering) are depicted in green and in pink, respectively. The main chain N atoms are in blue and the carbonyl O atoms are in red. The backbone H-bond donated by the P1 NH group in the inhibitor to Ser[214] O and Ser[195] O^{γ} in the enzyme is highlighted in the box.

leukocyte elastase (HLE); porcine pancreatic elastase (PPE); *Streptomyces griseus* proteinases A and B (SGPA and SGPB).

EXPERIMENTAL PROCEDURES

Materials. Boc-L-amino acids and HBTU were purchased from Novabiochem; Boc-Cys-(4-CH_3Bzl)OCH_2-PAM-resin and DIEA were obtained from Applied Biosystems. Aminomethyl-resin was prepared in this laboratory by Dr. Lynne Canne according to published procedures (Mitchell et al., 1978). L-leucic acid [(*S*)-2-hydroxyisocaproic acid], benzyl bromide, benzyl mercaptan, and thiophenol were purchased from Aldrich Chemical Co. CHYM was purchased from Worthington Biochemical Co.; HLE was purchased from Elastin Products Co., Inc.; PPE was a generous gift from the late Dr. M. Laskowski, Sr.; SCARL was obtained from Sigma Chemical Co. SGPA and SGPB were purified from Pronase (Sigma). All of the chromogenic substrates were purchased from Bachem Bioscience Inc.

Methods. Methylchymotrypsin was prepared according to Ryan and Feeney (1975) and was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the coupling procedures provided by the manufacturer. Analytical RP-HPLC was performed on an Applied Biosystems 140B solvent delivery system equipped with an Hewlett−Packard Series 1050 detector, using a Vydac C18 narrow-bore column (5 μm, 2.1 × 150 mm). Preparative RP-HPLC was carried out on a Waters Delta Prep 4000 system using a Vydac C18 column (15−20 μm, 50 × 250 mm). Solvent A for HPLC was water containing 0.1% TFA; solvent B was 90% ACN containing 0.09% TFA. Analytical cation-exchange chromatography was performed on a Pharmacia FPLC system using a Mono-S HR5/5 column. Buffer A was 50 mM NaOAc, pH 5.0; buffer B was buffer A plus 1 M NaCl. Mass spectrometry analysis was carried out on a PE Sciex API-III quadrupole ESI mass spectrometer and a Ciphergen prototype MALDI mass spectrometer.

Thermal denaturation studies were carried out in 50 mM KOAc, pH 4.3, on a Perkin−Elmer LS50 spectrofluorometer equipped with an automated temperature control unit. Fluorescence change induced by the rupture of the Tyr^{31}•••Asp^{27} H-bond in OMTKY3 upon heating was monitored at an excitation wavelength of 275 nm and an emission wavelength of 305 nm, with constant stirring of about 100 μg sample in a 3-mL stoppered cuvette. Heating, integrating, data sampling, and processing were fully automated. Melting temperatures (T_m) were obtained by nonlinear least squares analysis using Enzfitter with an accuracy of ±1 °C, and data were then normalized on the basis of fraction of denaturation.

The inhibitor−enzyme association equilibrium constants were measured on a Hewlett-Packard HP8450A spectrophotometer by a modified version of the Green and Work method (Green & Work, 1953; Empie & Laskowski, 1982). The experiment was carried out at 21 ± 2 °C at pH 7.0 in 0.1 M Bis-Tris buffer containing 0.02 M $CaCl_2$ and 0.005% Triton X-100. For most enzymes, the dynamic range of K_a values is from 10^3 to 10^{12} with an accuracy of ±20%.

Synthesis of (24−56)OMTKY3. (24−56)OMTKY3 was manually synthesized (Figure 3) in stepwise fashion using the published *in situ* neutralization/HBTU activation protocol for Boc chemistry (Schnölzer et al., 1992). All amino acids were of the L-configuration. The following side chain protection was used: Arg(tosyl); Asn(xanthyl); Asp(OcHxl); Cys(4MeBzl); Glu(OcHxl); His(DNP); Lys(2ClZ); Ser(Bzl); Thr(Bzl); Tyr(BrZ). Boc-amino acids (2.2 mmol) were activated by HBTU in the presence of DIEA and were coupled onto 0.2 mmol of preloaded Boc-Cys-(4-CH_3Bzl)-OCH_2-PAM-resin. TFA (100%) was used for removal of N^{α}-Boc groups; DMF was used for flow washes throughout the entire synthesis. Coupling yields were quantitated by ninhydrin assay for residual free amine (Sarin et al., 1981). After the chain assembly was complete, crude peptides were deprotected and cleaved from the resin by treatment with anhydrous HF in the presence of 5% *p*-cresol/*p*-thiocresol (1:1) for 1 h at 0 °C. Following the purification on preparative C18 RP-HPLC by running a gradient of 25−40% B over 60 min at 30 mL/min, the fractions of correct mass were pooled and lyophilized. The molecular weight was ascertained by ESI-MS (observed mass 3513.3 ± 0.6 Da; calculated 3512.9 Da, average isotope composition). The typical yield of the purified (24−56)OMTKY3 in the reduced form was 0.05 mmol on a 0.2 mmol scale synthesis.

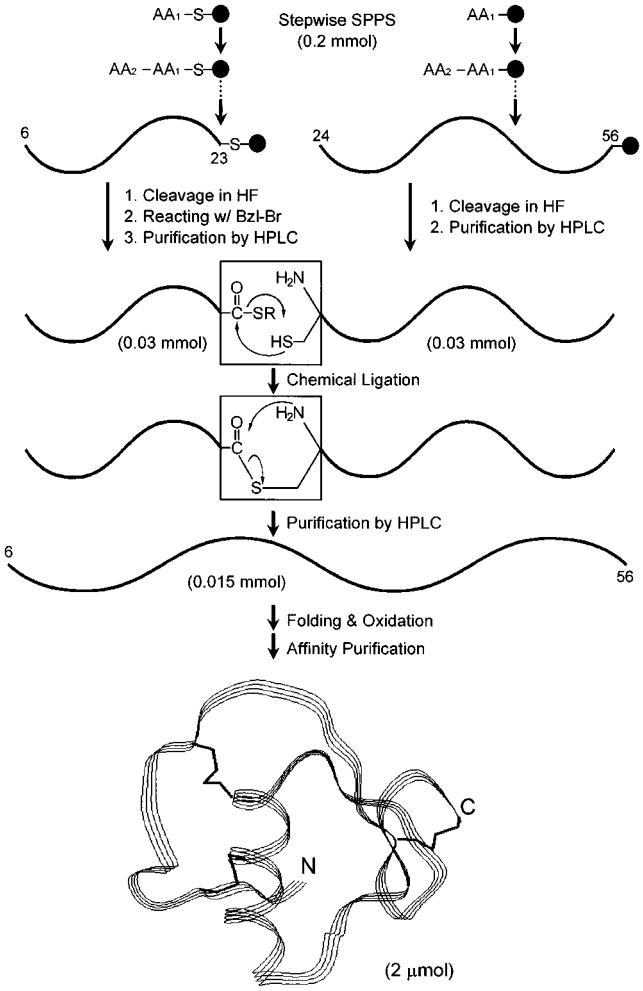

FIGURE 3: Strategies for the total synthesis of (6−56)Thr^{17}-(COO)Leu^{18}OMTKY3.

Synthesis of (6−23)Thr^{17}(COO)Leu^{18}αCOSH. The thioacid peptide (6−23)Thr^{17}(COO)Leu^{18}αCOSH was also synthesized manually (Figure 3) by stepwise SPPS methods using the same protocol as used for Boc chemistry discussed above. The synthesis was performed on 0.2 mmol of Boc-Leu-(thioester linker)-aminomethyl-resin prepared according to Canne et al. (1995). The coupling of leucic acid and the following Boc-Thr was conducted using a modified version

of HBOT/DIC and DMAP/DIC chemistry (Bramson et al., 1985). Excess L-leucic acid (2.2 mmol) was coupled to the growing peptide chain in DMF/DCM (1:1) in the presence of 2.2 mmol of DIC, 2.5 mmol of HOBT, and 0.8 mmol of NEM. The reaction proceeded to completion in 30 min as judged by standard ninhydrin assay. The coupling of Boc-Thr (2.2 mmol) was carried out for 60 min in DMF/DCM (1:1) in the presence of 2.2 mmol of DIC, 0.8 mmol of NEM, and catalytic amount of DMAP (less than 0.04 mmol). These reaction conditions were based on model studies (below).

Formation of an ester bond is slower than that of an amide bond due to the fact that the hydroxyl group is less reactive as a nucleophile than is an amine. Conditions that favor esterification often cause racemization, which may become progressively worse as the peptide chain grows on resin. In the synthesis of the N-terminal segment (6−23)Thr^{17}(COO)-Leu^{18}αCOSH, the primary concerns were coupling efficiency and possible racemization. To address these issues, a five-residue model peptide encompassing the reactive site of the inhibitor, H-Cys-Thr-(COO)Leu-Glu-Tyr-OH, was made on MBHA resin. The cleaved product was analyzed by analytical RP-HPLC and ESI-MS. Neither termination nor deletion products were detected, indicating that the esterification reaction was indeed highly efficient under the conditions used. In order to investigate possible racemization, racemic leucic acid [(±)-2-hydroxyisocaproic acid] was incorporated into the model peptide, and the two resultant diastereomers (1:1) were separated to baseline on C18 RP-HPLC. By comparison of the chromatograms, it was concluded that racemization in the synthesis of the model peptide using (−)-L-leucic acid was not detected (<1%).

After the chain assembly was complete, peptides were deprotected and cleaved in HF containing 5% anisole for 1 h at 0 °C. The crude peptide was dissolved in 40% B HPLC solvent and then lyophilized. The fully unprotected thioacid peptide, (6−23)Thr^{17}(COO)Leu^{18}αCOSH, was dissolved, at 6−7 mg/mL, in 0.1 M acetate and 6 M GuHCl, pH 4.0, followed by the addition of benzyl bromide (4 μL/mL). The reaction was allowed to proceed with stirring for 30 min, and the reaction mixture was then loaded onto preparative C18 RP-HPLC (30−55% B over 60 min at 30 mL/min). The resultant benzyl thioester peptide, (6−23)Thr^{17}(COO)-Leu^{18}αCOSBzl, was pooled and lyophilized (yield 0.03 mmol on a 0.2 mmol scale synthesis). The observed mass of the product was 2191.7 ± 0.4 Da (calculated monoisotopic mass 2190.0 Da; average isotopic mass 2191.4 Da).

Native Chemical Ligation. The ligation of (6−23)Thr^{17}-(COO)Leu^{18}αCOSBzl and (24−56)OMTKY3 was carried out according to the principles described in Dawson et al. (1994, 1996) and Lu et al. (1996b). In brief, equal molar amounts of (6−23)Thr^{17}(COO)Leu^{18}αCOSBzl and (24−56)-OMTKY3 were dissolved, at a total concentration of about 8 mg/mL, in 0.1 M phosphate buffer containing 6 M GuHCl, pH 7.5, to which 1% benzyl mercaptan and 3% thiophenol (v/v) had been added. The ligation reaction was allowed to proceed to completion with stirring for 36 h (Figure 4) before being loaded onto preparative C18 RP-HPLC using 25−40% B over 60 min at 30 mL/min. The desired fractions were pooled and lyophilized for protein folding (yield 0.015 mmol on a 0.2 mmol scale synthesis). It is worth noting that the ester bond seemed to be very stable during the entire period of ligation at pH 7.5.

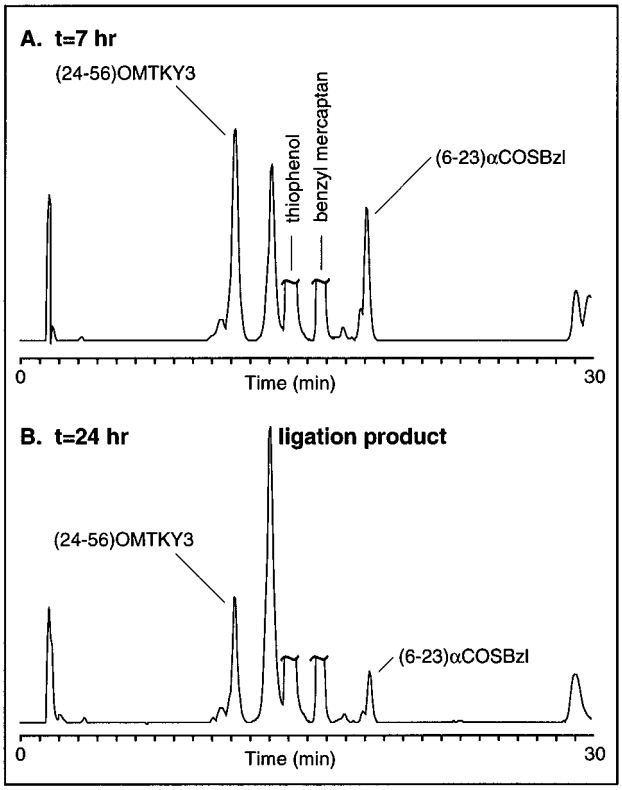

FIGURE 4: Native chemical ligation of (6−23)Thr^{17}(COO)-Leu^{18}αCOSBzl and (24−56)OMTKY3 (for details, see Experimental Procedures). The ligation reaction was monitored by injecting 1 μL of aliquot onto a narrow-bore C18 RP-HPLC running a gradient of 20−55% B at a flow rate of 300 μL/min over 24 min. The reaction went to completion in 36 h.

Protein Folding and Purification. (6−56)Thr^{17}(COO)Leu^{18}-OMTKY3 in the reduced form was dissolved at 1.2 mg/mL in 0.6 M Tris−HCl, 6 M GuHCl, and 6 mM EDTA, pH 7.5. Protein refolding was initiated by a rapid 6-fold dilution with water. The solution was gently stirred in an open air container for 8 h before being loaded onto a methylchymotrypsin−Sepharose affinity column equilibrated at pH 7.5. After being washed with 0.2 M NaCl, active (6−56)-Thr^{17}(COO)Leu^{18}OMTKY3 was eluted with 0.01 M HCl and 0.2 M NaCl. The bound fraction was then desalted on a Sephadex G-25 column and lyophilized for further characterization.

An ester bond, in general, is more susceptible than an amide bond to attack by nucleophiles such as hydroxyl ion. Therefore, the stability of the ester-containing protein in aqueous solution was a major concern. It was found that *unfolded* (6−56)Thr^{17}(COO)Leu^{18}OMTKY3, when suspended in Tris buffer of pH 8.7, underwent rapid hydrolysis ($t_{1/2} \approx 4$ h), even though it was very stable at acidic pH. For this reason, protein refolding should, in principle, be performed at low pH so that hydrolysis can be kept at a minimal level. However, low pH significantly slowed the refolding process presumably because of protonation of the sulfhydryl group of Cys. A compromise was reached at pH 7.5. Even so, after purification on a methylchymotrypsin affinity column, the final yield (defined as the weight ratio of lyophilized active protein after purification and desalting to the starting unfolded form) of (6−56)Thr^{17}(COO)Leu^{18}-OMTKY3 was only about 15%. Nonetheless, the folded ester-containing protein is fully active and stable at neutral

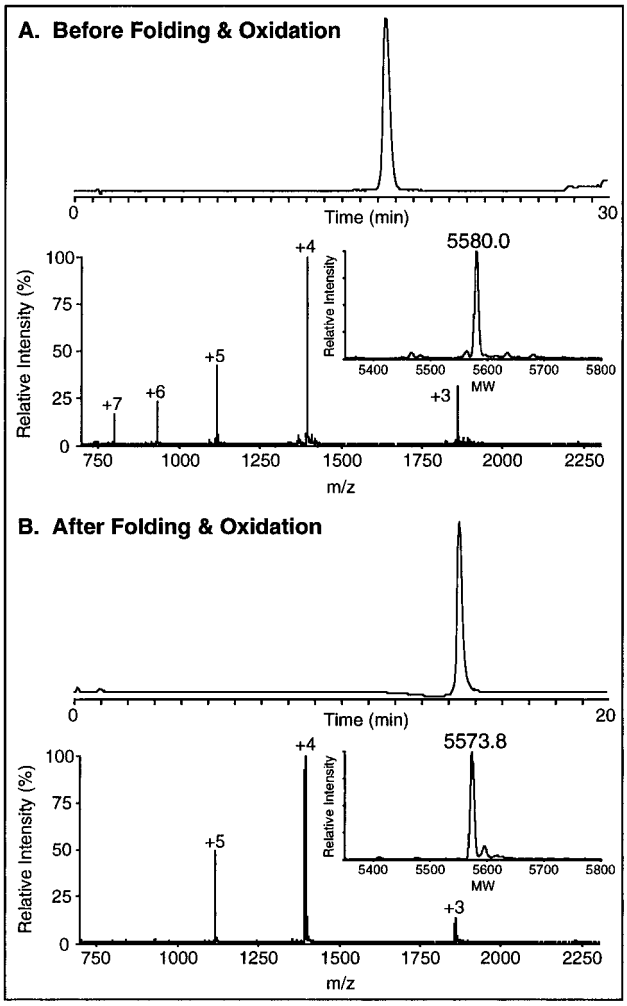

FIGURE 5: (A) Purified unfolded ligation product (6−56)-Thr[17](COO)Leu[18]OMTKY3 on a narrow-bore C18 RP-HPLC running a gradient of 20−40% B at a flow rate of 300 μL/min over 24 min. The observed mass of 5580.0 ± 0.9 Da (calculated average isotope mass: 5580.3 Da) was determined by ESI-MS. (B) Folded and affinity-purified final product (6−56)Thr[17]-(COO)Leu[18]OMTKY3 on a Mono-S cation-exchange FPLC running a gradient of 0−75% B at a flow rate of 1 mL/min over 15 min. The observed mass of 5573.8 ± 0.8 Da (calculated average isotope mass: 5574.3 Da) is 6 Da lower than that of the unfolded reduced form, indicating that three disulfides have formed.

pH, and no appreciable hydrolysis was detected 24 h after being suspended in Bis-Tris buffer at pH 7.0.

RESULTS

The synthetic P1 ester OMTKY3 was characterized by electrospray mass spectrometry, analytical cation−exchange chromatography, disulfide mapping, and thermal denaturation studies. ESI-MS analysis of the product before and after folding and oxidation is presented in Figure 5. The observed masses of the reduced form (5580.0 ± 0.9 Da) and the oxidized form (5573.8 ± 0.8 Da) were within experimental error of the calculated masses based on average isotope composition (5580.3 and 5574.3 Da, respectively). The mass difference of 6 Da between reduced and oxidized forms was consistent with the formation of three disulfides. Previously, synthetic wild-type (6−56)OMTKY3 was prepared by both stepwise SPPS and native chemical ligation and was found to be structurally as well as functionally identical to its recombinant counterpart (Lu et al., 1996b). Interestingly,

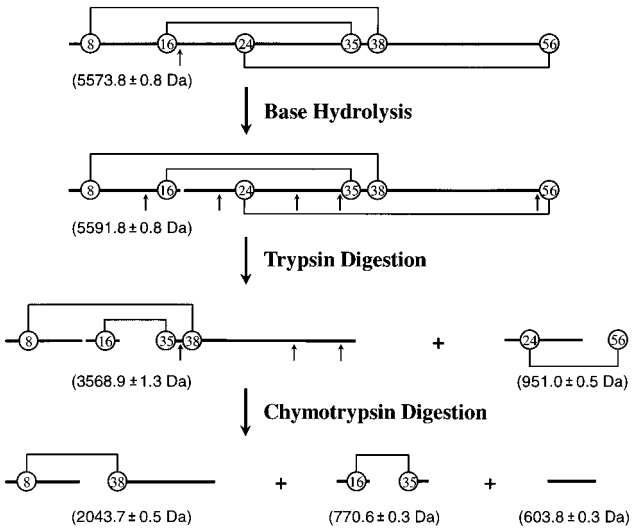

FIGURE 6: Strategies for the determination of disulfide pairing in (6−56)Thr[17](COO)Leu[18]OMTKY3.

as was the case with both synthetic and recombinant wild-type (6−56)OMTKY3 (Lu et al., 1996b), the ester OMTKY3 also showed a broad front-tailing peak on analytical C18 RP-HPLC (data not shown). However, the corresponding reduced form as well as the base-hydrolyzed oxidized form of (6−56)Thr[17](COO)Leu[18]OMTKY3 both gave very sharp peaks. These observations were attributed to partial denaturation of highly stable OMTKY3 in the acidic HPLC solvent (Lu et al., 1996b). In order to demonstrate the chromatographic purity of the ester analog, Mono-S cation-exchange FPLC was used to analyze the material (Figure 5).

The correct pairing of three disulfides in the folded ester-containing inhibitor was ascertained using limited proteolysis combined with analyses of cleaved peptide fragments by both MALDI-MS and ESI-MS (Figure 6). Wild-type OMTKY3, like many other protein proteinase inhibitors, is proteolysis resistant under mild conditions (unpublished results). The ester analog, however, after cleavage of the base-labile ester bond, could be easily digested by trypsin and chymotrypsin. Digestion with trypsin generated on RP-HPLC a major component of 3568.9 ± 1.3 Da and a minor component of 951.0 ± 0.5 Da, which corresponded to the fragment (6−17)-(35−55) held together by two disulfides (Cys8, Cys16, Cys35, and Cys38; calculated average isotope mass 3569.0 Da) and the fragment (22−29)-(56) interlinked by Cys24 and Cys56 (calculated monoisotopic mass 951.4 Da, average mass 952.1 Da), respectively. Further digestion of the major component with chymotrypsin yielded three major fragments whose masses were found to be 2043.7 ± 0.5, 770.6 ± 0.3, and 603.8 ± 0.3 Da. They were identified as (6−13)-(38−48) (calculated average isotope mass 2044.3 Da; single isotope mass 2042.9 Da), (14−17)-(35−37) (calculated average isotope mass 770.9 Da; single isotope mass 770.3 Da), and fragment 49−53 (calculated average isotope mass 603.7 Da; single isotope mass 603.3 Da), respectively. The topology of the three disulfides, Cys24-Cys56, Cys8-Cys38, and Cys16-Cys35, was therefore found to be identical to that in the native OMTKY3, suggesting that the ester analog was correctly folded.

A strong H-bond between Asp[27] $O^{\delta 2}$ and Tyr[31] O^{η} (O···H−O, 2.45 Å) is almost totally conserved among natural ovomucoid third domain variants isolated from egg whites

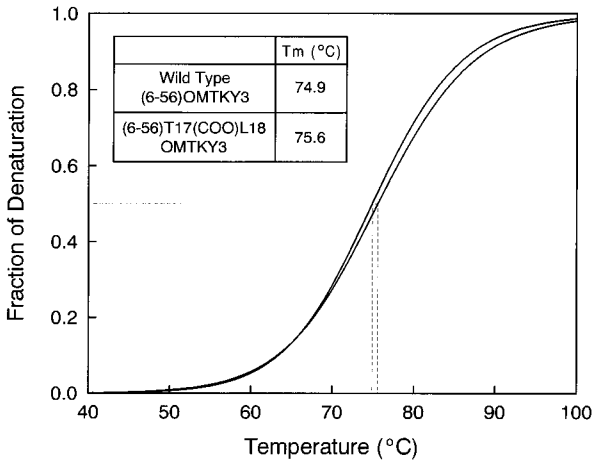

FIGURE 7: Thermal denaturation of (6−56)Thr[17](COO)Leu[18]-OMTKY3 and synthetic wild-type (6−56)OMTKY3 at pH 4.3.

Table 1: K_a Values Measured at 21 ± 2 °C, pH 7.0

enzymes	OMTKY3 (ester analog)	OMTKY3 (wild type)	K_a ratio (amide/ester)	$\Delta\Delta G°$ (kcal/mol)
CHYM	4.4×10^9	6.8×10^{10}	15	1.6
PPE	1.1×10^9	3.2×10^{10}	29	2.0
SCARL	1.0×10^9	9.3×10^9	9	1.3
SGPA	8.8×10^9	1.2×10^{11}	14	1.5
SGPB	1.1×10^9	2.0×10^{10}	18	1.7
HLE	6.3×10^8	2.4×10^9	4	0.8

(Apostol et al., 1993). The rupture of this H-bond upon heating gives rise to a characteristic fluorescence signal which can be used for monitoring melting transition and determining melting temperatures (T_m). The melting behavior of the P1 ester OMTKY3 and the synthetic wild type were compared after normalization as fraction denaturation curves; the results were identical with regard to their melting transition curves and T_m values (Figure 7), which again strongly indicates that the ester-containing polypeptide chain folded correctly.

Association equilibrium constants (K_a's) were measured for (6−56)Thr[17](COO)Leu[18]OMTKY3 and wild type (6−56)OMTKY3 interacting with a panel of serine proteinases comprising CHYM, PPE, HLE, SCARL, SGPA, and SGPB and are given in Table 1. $\Delta\Delta G^{\text{amide}\rightarrow\text{ester}}$ values that reflect an increase in the free energy change were calculated according to $\Delta\Delta G^{\text{amide}\rightarrow\text{ester}} = RT \ln(K_a^{\text{amide}}/K_a^{\text{ester}})$. The results indicated that the P1 NH → O substitution weakens the binding of OMTKY3 to its cognate enzymes by an average of 15-fold, i.e., 1.5 ± 0.3 kcal/mol.

DISCUSSION

We have demonstrated that substituting an O atom for the P1 NH group in OMTKY3 weakens the binding of OMTKY3 to the six serine proteinases by an average of 1.5 ± 0.3 kcal/mol. In order to understand the physical meaning of this experimentally determined value, we need some detailed analysis of the following equations to elucidate factors influencing the differential binding. (Note that only one H-bond acceptor, the carbonyl C═O group of Ser[214], is represented in the following equations. However, the simplified treatment will affect neither qualitative nor quantitative conclusions to be made below.)

$$[E{>}C{=}O\cdots H_2O] + [I{>}N{-}H\cdots H_2O] = [E{>}C{=}O\cdots H{-}N{<}I] + 2H_2O \qquad \Delta G_1 \quad (1)$$

$$[E{>}C{=}O\cdots H_2O] + [I{>}O\cdots H_2O] = [E{>}C{=}O\cdots O{<}I] + 2H_2O \qquad \Delta G_2 \quad (2)$$

In eqs 1 and 2 (E, enzyme; I, inhibitor), ΔG_1 and ΔG_2 are the apparent free energy changes associated with the binding to the enzyme of wild-type OMTKY3 and its P1 ester analog, respectively. $\Delta\Delta G^{\text{amide}\rightarrow\text{ester}}$ can be therefore defined as

$$\Delta\Delta G^{\text{amide}\rightarrow\text{ester}} = \Delta G_2 - \Delta G_1 = \Delta G^{O\cdots O} + (\Delta G^{I>NH}{}_{\text{solvation}} - \Delta G^{I>O}{}_{\text{solvation}}) - \Delta G^{\text{H-bond}}{}_{(<0)} \quad (3)$$

Apparently, the decrease in binding for the ester analog could, in principle, not only result from the loss of the H-bond but also be influenced by the van der Waals and electrostatic interaction between the two O atoms in the H-bond-depleted complex, $\Delta G^{O\cdots O}$, as well as the differential solvation energy of the two inhibitors in the free form, $[\Delta G^{I>NH}{}_{\text{solvation}} - \Delta G^{I>O}{}_{\text{solvation}}]$. It has been suggested by calculations that an amide bond has a higher solvation energy than does an ester bond (Bash et al., 1987). However, there have been no documented experimental data to substantiate the difference. While it is difficult to directly assess the magnitude of $\Delta G^{O\cdots O}$ and $[\Delta G^{I>NH}{}_{\text{solvation}} - \Delta G^{I>O}{}_{\text{solvation}}]$ in eq 3, experimental evidence suggested that it is negligible. In an elegant study, Groeger et al. (1994) substituted through semisynthesis both an O atom and a methylene group (−CH_2−) for the P2′ amide NH in BPTI, which is H-bonded to the carbonyl C═O group of Phe[41] in trypsin (Huber et al., 1974). They found that both substitutions decreased the binding of the backbone-engineered BPTI to trypsin by 25-fold, or 1.9 kcal/mol, which reflected an intrinsic energy of the H-bond between the P2′ NH and the Phe[41] C═O in the BPTI−trypsin complex. Clearly, if the contribution of $\Delta G^{O\cdots O}$ and $[\Delta G^{I>NH}{}_{\text{solvation}} - \Delta G^{I>O}{}_{\text{solvation}}]$ were to be significant, the $\Delta\Delta G$ value would be different when a methylene group is used to replace the amide NH group.

In OMTKY3, the experimentally measured $\Delta\Delta G^{\text{amide}\rightarrow\text{ester}}$ value, 1.5 ± 0.3 kcal/mol, is in good accord with many results reported by others in the literature. Fersht et al. (1985) concluded from mutation studies of tyrosyl-tRNA synthetase that removal of the partner of an uncharged donor/acceptor increases the free energy by 0.5−1.8 kcal/mol. Shirley et al. (1992) determined that most intramolecular H-bonds in ribonuclease T1 contribute 1.3 ± 0.6 kcal/mol to the stability of the protein. It is conceivable that 1.5 ± 0.3 kcal/mol associated with the substitution of an ester for the P2−P1 amide peptide bond in OMTKY3 is a realistic measure of the loss of the backbone H-bond donated by the P1 residue of the inhibitor to the enzyme.

Interestingly, Bartlett and Marlowe (1987) reported that replacement of a backbone phosphonamidate NH group, which is H-bonded to the carbonyl C═O group of Ala[113] in thermolysin (3.0 Å), by an O atom in a series of phosphonamidate inhibitors of thermolysin reduced the binding by 4.0 kcal/mol. The X-ray crystal structure revealed that the resultant phosphonate O atom occupies essentially the same position as did the phosphonamidate NH (Tronrud et al., 1987). The reduction of inhibitory activity was attributed solely to the loss of the backbone−backbone H-bond (Bartlett

& Marlowe, 1987). It is not unlikely that the unusually large free energy change could have been caused by a significant entropy loss during complexation, because replacement of phosphonamide by phosphonate in the small three-residue peptide inhibitor would certainly constitute great flexibility in the backbone.

In summary, the backbone H-bond donated by the P1 residue is energetically significant. It provided an average of -1.5 ± 0.3 kcal/mol to the free energy change of the formation of six enzyme−inhibitor complexes. However, the magnitude of the contribution is far less than enough to account for the huge deleterious effect of Pro substitution at the P1 position of OMTKY3. The structure of Pro^{18}-OMTKY3 in the complex of SGPB has been solved recently (Huang & James, personal communication). The side chain of P1 proline was found to be unable to fit into the S1 pocket. Consequently, two additional backbone H-bonds at the interface of the complex (the P2′ NH to the carbonyl C═O of Arg^{41} and the NH of Ser^{195} to the P1 carbonyl C═O) lengthened by 0.5 Å. In addition, an intramolecular side chain H-bond between Thr^{17} and Glu^{19} of OMTKY3, which is believed to be very important in maintaining the rigidity of the binding loop (Read & James, 1986), stretched by 0.5 Å. It is conceivable that the steric noncomplementarity of P1 Pro and the subsequent weakening of intermolecular as well as intramolecular H-bonds are important factors in determining the extremely poor binding of Pro at the P1 position. More ester analogs that specifically delete other intermolecular backbone H-bonds in the complexes of OMTKY3 with different serine proteinases will ultimately give rise to a better answer.

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REFERENCES

Alber, T., Sun, D.-P., Wilson, K., Wozniak, J. A., Cook, S. P., & Matthews, B. W. (1987) *Nature 330*, 41−46.
Apostol, I., Giletto, A., Komiyama, T., Zhang, W., & Laskowski, M., Jr. (1993) *J. Protein Chem. 12*, 419−433.
Arad, O., & Goodman, M. (1990) *Biopolymer 39*, 1633−1649.
Baker, E. N., & Hubbard, R. E. (1984) *Prog. Biophys. Mol. Biol. 44*, 97−179.
Bartlett, P. A., & Marlowe, C. K. (1987) *Science 235*, 569−571.
Bash, P. A., Singh, U. C., Brown, F. K., Langridge, R., & Kollman, P. A. (1987) *Science 235*, 574−576.
Bigler, T., Lu, W., Park, S., Tashiro, M., Wieczorek, M., Wynn, R., & Laskowski, M., Jr. (1993) *Protein Sci. 2*, 786−799.
Bode, W., & Huber, R. (1992) *Eur. J. Biochem. 204*, 433−451.
Bramson, N. H., Thomas, N. E., & Kaiser, E. T. (1985) *J. Biol. Chem. 260*, 15452−15457.
Canne, L. E., Walker, S. M., & Kent, S. B. H. (1995) *Tetrahedron Lett. 36*, 1217−1220.
Connelly, P. R., Aldape, R. A., Bruzzese, F. J., Chambers, S. P., Fitzgibbon, M. J., Fleming, M. A., Itah, S., Livingston, D. J., Navia, M. A., Thomsom, J. A., & Wilson, K. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1964−1968.
Dawson, P. E., Muir, T. W., Clark-Lewis, I., & Kent, S. B. H. (1994) *Science* 266, 776−779.
Dawson, P. E., Churchill, M., Ghadiri, M. R., & Kent, S. B. H. (1996) *J. Am. Chem. Soc.* (submitted for publication).
Dill, K. A. (1990) *Biochemistry 29*, 7133−7155.
Empie, M. W., & Laskowski, M., Jr. (1982) *Biochemistry 21*, 2274−2284.
Fersht, A. R. (1985) *Enzyme Structure & Mechanism*, W. H. Freeman & Co., New York.
Fersht, A. R. (1987) *Trends Biochem. Sci. 12*, 301−304.
Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature 314*, 235−238.
Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol. 195*, 397−418.
Green, N. M., & Work, E. (1953) *Biochem. J. 54*, 347−352.
Groeger, C., Wenzel, H. R., & Tschesche, H. (1994) *Int. J. Pept. Protein Res. 44*, 166−172.
Honig, B., & Yang, A.-S. (1995) *Adv. Protein Chem. 46*, 27−58.
Huang, K., Lu, W., Anderson, S., Laskowski, M., Jr., & James, M. N. G. (1995) *Protein Sci. 4*, 1985−1997.
Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steigemann, W. (1974) *J. Mol. Biol. 89*, 73−101.
Jencks, W. P. (1969) *Catalysis in Chemistry & Enzymology*, Dover Publications, Inc., New York.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1−63.
Lu, W., Apostol, I., Qasim, M. A., Warne, N., Wynn, R., Zhang, W., Anderson, S., Chiang, Y.-W., Ogin, E., Rothberg, I., Ryan, K., & Laskowski, M., Jr. (1996a) *J. Mol. Biol.* (in press).
Lu, W., Qasim, M. A., & Kent, S. B. H. (1996b) *J. Am. Chem. Soc. 118*, 8518−8523.
Mendel, D., Cornish, V. W., & Schultz, P. G. (1995) *Annu. Rev. Biophys. Biomol. Struct. 24*, 435−462.
Mitchell, A. R., Kent, S. B. H., Engelhard, M., & Merrifield, R. B. (1978) *J. Org. Chem. 43*, 2845−2852.
Muir, T. W. (1995) *Structure 3*, 649−652.
Muir, T. W., & Kent, S. B. H. (1993) *Curr. Opin. Biotechnol. 4*, 420−427.
Myers, J. K., & Pace, C. N. (1996) *Biophys. J.* (in press).
Pauling, L.; Corey, R. B., & Branson, H. R. (1951) *Proc. Natl. Acad. Sci. U.S.A. 37*, 205−211.
Rajarathnam, K., Sykes, B. D., Kay, C. M., Dewald, B., Geiser, T., Baggiolini, M., & Clark-Lewis, I. (1994) *Science 264*, 90−92.
Read, R. J., & James, M. N. G. (1986) *Proteinase Inhibitors*, Vol. 7, pp 301−336, Elsevier Science Publishers, B.V.
Ryan, D. S., & Feeney, R. E. (1975) *J. Biol. Chem. 250*, 843−847.
Sarin, V. K., Kent, S. B. H., Tam, J. P., & Merrifield, R. B. (1981) *Anal. Biochem. 117*, 147−157.
Schechter, I., & Berger, M. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
Schnölzer, M., Alewood, P., Jones, A., Alewood, D., & Kent, S. B. H. (1992) *Int. J. Pept. Protein Res. 40*, 180−193.
Shirley, B. A., Stanssens, P., Hahn, U., & Pace, C. N. (1992) *Biochemistry 31*, 725−732.
Stickle, D. F., Presta, L. G., Dill, K. A., & Rose, G. D. (1992) *J. Mol. Biol. 226*, 1143−1159.
Thomas, N. E., Bramson, H. N., Miller, W. T., & Kaiser, E. T. (1987) *Biochemistry 26*, 4461−4466.
Tronrud, D. E., Holden, H. M., & Matthews, B. W. (1987) *Science 235*, 571−574.

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