Primary Structure of Human $\alpha_2$-Macroglobulin

COMPLETE DISULFIDE BRIDGE ASSIGNMENT AND LOCALIZATION OF TWO INTERCHAIN BRIDGES IN THE DIMERIC PROTEINASE BINDING UNIT*

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The disulfide bridge pattern of human $\alpha_2$-macroglobulin ($\alpha_2$M) given earlier (Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Lønnblad, P. B., Magnusson, S., and Petersen, T. E. (1984) J. Biol. Chem. 259, 8318-8327) has been revised by showing that Cys$^{308}$ and Cys$^{408}$ in one subunit are bridged to Cys$^{308}$ and Cys$^{408}$, respectively, in the adjacent subunit of the proteinase binding dimer. Thus, the $\alpha_2$M-dimer contains two interchain disulfide bridges, and the individual subunits are arranged in an antiparallel fashion. These results are the outcome of partial reduction experiments, where reduction of methanamine-treated $\alpha_2$M with 1-8 mM mercaptoethanesulfonate at pH 8.0 resulted in the appearance of 2.6 mol of SH-groups per mol of free subunit. Apart from reduction of the two interchain bridges, the intrachain bridges Cys$^{28}$-Cys$^{276}$, Cys$^{372}$-Cys$^{448}$, Cys$^{788}$-Cys$^{828}$, and Cys$^{624}$-Cys$^{660}$ are reduced to a minor extent under these conditions. The disulfide bridge pattern of $\alpha_2$M has been completed by showing that the $\alpha_2$M subunit contains 11 intrachain bridges, including a bridge connecting Cys$^{447}$ with Cys$^{480}$.

The primary structure of the human plasma glycoprotein $\alpha_2$M has been determined recently by methods of protein chemistry (1-5). The identical 180-kDa subunits of $\alpha_2$M contain 1451 residues and are pairwise disulfide-bridged to 360-kDa dimers (6, 7). These are further noncovalently associated to the tetrameric $\alpha_2$M of 720 kDa. $\alpha_2$M forms stable complexes with a variety of proteinases from all four classes, EC 3.4.21-24, and complex formation is initiated by specific limited proteolysis in the so-called "bait" region, residues 681-686 (5). $\alpha_2$M-proteinase complexes are rapidly cleared from the circulation by receptor-mediated endocytosis, primarily by hepatocytes and Kupffer cells of the liver. The disulfide-bridged 360-kDa dimers of $\alpha_2$M constitute its functional proteinase binding units (8-11). The localization of 11 disulfide bridges in $\alpha_2$M (5) was unambiguously determined by the

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‡The abbreviations used are: $\alpha_2$M, $\alpha_2$-macroglobulin; $\alpha_2$M-MA, $\alpha_2$-macroglobulin treated with methanamine and iodoacetic acid; MES, mercaptoethanesulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DETE, dithioerythritol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); HPLC, high performance liquid chromatography; FTIR, phenylthiohydantoin; TFA, trifluoroacetic acid; RT, room temperature.

MATERIALS AND METHODS

RESULTS

Partial Reduction of $\alpha_2$M-MA with MES—No information on the localization of interchain bridges in $\alpha_2$M can be obtained from analysis of small disulfide-bridged peptides, since these could represent either intra- or interchain bridges. As seen from the alignment of the CNBr fragments shown in Fig. 1, none of the disulfide bridges within CB2, CB6, CB18, and CB21 can be interchain bridges, since this would be revealed by a characteristic change in size when examined by nonreducing and reducing SDS-PAGE. Under nonreducing conditions, the size of CB2 was estimated at 18 kDa, while those of CB6 + CB8, CB18 + CB23 + CB26, and CB21 were 20, 40, and 28 kDa, respectively (1). Rather than investigating conditions where sufficiently large fragments could be generated from the $\alpha_2$M-dimer so that the assignment of interchain bridges could be based on SDS-PAGE analysis, partial reduction of $\alpha_2$M was investigated instead. Pilot studies using 1 mM dithioerythritol at pH 8.0 indicated reduction of about six to eight disulfide bridges for nearly complete disappearance of the 360-kDa disulfide-bridged dimer of native $\alpha_2$M (not shown), compatible with earlier results (9, 21).

Portions of this paper (including "Materials and Methods," "Experimental," part of "Results," Figs. 3-12, and Tables 1-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2126, cite the authors, and include a check or money order for $6.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Since the interchain bridges presumably are among the most solvent-accessible and hence most easily reduced bridges, their selective reduction was investigated using mercaetoethanesulfonate (MES). This reagent is strongly solvated and has a higher redox potential than that of dithioerythritol (22), and might yield a more selective reduction than dithioerythritol. Analytical time course experiments using 6 mM MES at pH 8.0 indicated that for native α2M there was a progressive reduction of interchain bridges, whereas for α2M-MA, no further reduction appeared to take place after about 10 min as judged from SDS-PAGE under nonreducing conditions (not shown). Accordingly, the reduction of α2M-MA with varying amounts of MES was investigated. Fig. 2A shows the appearance of the 180-kDa subunit as a result of incubating α2M-MA with increasing amounts of MES (0.4-32 mM) for 30 min at room temperature. In Fig. 2B, the generation of partially reduced α2M-MA subunits is related to the total amount of subunit present. In parallel, the amount of SH-groups present in partially reduced α2M-MA was determined by DTNB titration after removal of excess reductant by gel chromatography and related to the amount of subunit present. The results demonstrated a close correlation between the SH-groups appearing and the extent of generation of partially reduced subunit. In the range of 1-8 mM MES, the stoichiometry between SH-groups appearing and partially reduced subunits was close to 2.6. At higher levels of MES, excessive reduction took place, but even at the highest concentration of MES used (32 mM), only about 83% of the subunits had their interchain bridges reduced. Under the same conditions, the degree of reduction of disulfide bridges in native α2M was higher (Fig. 2B), but even at a point where about 5.5 SH-groups had appeared per subunit (at 14 mM MES), 50% of the α2M dimers still had their interchain bridges intact as judged from nonreducing SDS-PAGE (not shown). Hence, at low concentrations of MES, the generation of the partially reduced subunits of α2M-MA is dependent on the reduction of only two interchain bridges in the 360-kDa dimer, accompanied by a minor extent of reduction of other (intrachain) disulfide bridges.

Localization of the Interchain Disulfide Bridges in the α2M Dimer—On a preparative scale, α2M-MA was reduced with 4 mM MES for 10 min and the SH-groups appearing labeled by reaction with tritium-labeled iodoacetic acid. After exhaustive reduction with dithioerythritol and carboxymethylation using unlabeled iodoacetic acid in the presence of guanidinium chloride, chymotryptic peptides were generated and separated on a DEAE-Sephacel column (Fig. 3, Miniprint). Four pools containing the major part of the radiolabel were further separated by ion-exchange and reverse-phase HPLC (Figs. 4, A-D and 5, A-E, Miniprint). The incorporation of tritium label in these (impure) peptides was estimated at 0.2-2.2 × 10^5 cpm/nmol as opposed to 10-12 × 10^5 cpm/nmol for the major peptides CT1-CT4. This result indicates that the intrachain bridges Cys258-Cys376, Cys372-Cys346, Cys364-Cys408 and, in particular, Cys386-Cys408 are relatively exposed to solvent.

Completion of the Disulfide Bridge Assignment of α2M—Since Cys255 and Cys438 are engaged in interchain bridge formation, Cys447 and Cys540 would be expected to form an intrachain bridge. These residues are located in CB9 and CB11, respectively, and a pool containing the medium sized CNBr fragments from α2M was isolated (Figs. 8 and 9, Miniprint). After reverse-phase HPLC on a Vydac C4 column (Fig. 10A, Miniprint), the expected fragment set was recovered pure, as judged from amino acid analysis and sequence determination (Tables 2 and 3, Miniprint). This strongly indicated that CB9 was disulfide-bridged to CB11, which is further

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**Fig. 1.** Schematic representation of the α2M subunit showing the location of 11 intrachain disulfide bridges and 2 interchain disulfide bridges. The figure differs from that shown in Ref. 5 by assigning an intrachain bridge to Cys447-Cys461 and interchain bridges to Cys395-Cys408 and Cys438-Cys540. The size and alignment of the 26 CNBr fragments, the position of the bait region, and the reactive β-cysteinyl-g-glutamyl thiol ester are indicated. Positions of carbohydrate groups are shown by filled diamonds. The inset shows the antiparallel alignment of the subunits within the dimeric proteinase binding unit (only interchain bridges are indicated).
bridged to CB17 (2, 5). Following reduction, $^{14}$C carboxymethylation, and rechromatography on the same column (Fig. 1B, Miniprint), CB9, CB11, and CB17 were isolated. While amino acid analysis (Table 2, Miniprint) clearly showed that the fragments indeed were CB9, CB11, and CB17, CB17 was recovered in a very low yield as also seen before (1, 2). For unknown reasons, the NH$_2$ termini of these fragments had become blocked during isolation, and no verification could be made by sequence analysis. The bridge connecting Cys$^{447}$ and Cys$^{448}$ was finally proven by analyzing a chymotryptic digest of CB9 + CB11 + CB17. The peptides were separated by reverse-phase HPLC, and the relevant bridge peptide(s) were identified following reduction and $^3$H carboxymethylation (Fig. 11, A and B, Miniprint). The results of amino acid analysis and sequence determination of the peptides representing the bridges Cys$^{447}$-Cys$^{448}$ (CT5 and CT6) and Cys$^{472}$-Cys$^{473}$ (CT7 + CT8) are shown in Table 4 and Fig. 12 (Miniprint), respectively.

**DISCUSSION**

In continuation of earlier investigations on the primary structure of human $\alpha_2$M (1–5), we have now completed the determination of its disulfide bridge pattern. The subunit of $\alpha_2$M contains 11 intrachain bridges, including a bridge connecting Cys$^{447}$ and the recently recognized Cys$^{456}$ (12, 13). The subunits of the proteinase binding $\alpha_2$M-dimer are held together by two interchain bridges. These bridges connect Cys$^{355}$ and Cys$^{464}$ in one subunit with Cys$^{406}$ and Cys$^{424}$, respectively, in the adjacent subunit. Cys$^{355}$ and Cys$^{464}$ were known from previous studies to be disulfide-bridged (2, 5), and the assignment of these half-cystine residues as being involved in interchain formation was the outcome of partial reduction experiments. Although not investigated in detail, a minor extent of reduction in $\alpha_2$M-MA of the following intrachain bridges could be demonstrated: Cys$^{228}$-Cys$^{276}$, Cys$^{372}$-Cys$^{473}$, Cys$^{508}$, Cys$^{826}$, and Cys$^{821}$-Cys$^{869}$. Of these bridges, Cys$^{508}$-Cys$^{826}$ was most easily reduced.

The use of $\alpha_2$M-MA in these experiments was important for localizing the interchain bridges since these bridges were more easily reduced in $\alpha_2$M-MA than in native $\alpha_2$M (21). In addition, reduction of interchain bridges in $\alpha_2$M-MA appeared to be complete in about 10 min at 6 mM MES, while a time-dependent reduction of interchain bridges in native $\alpha_2$M was seen, accompanied by additional reduction of other bridges (Fig. 2B). Thus, the conformational change accompanying thiol ester cleavage by methylamine results in an increased accessibility of the interchain bridges. While HPLC gel chromatography and nondenaturing PAGE revealed the presence of free subunits, dimers, and intact tetramers upon reduction of native $\alpha_2$M with MES, as also found earlier with N-acetylcyesteine (6), partially reduced $\alpha_2$M-MA contained only tetramers (not shown). Thus, $\alpha_2$M-MA forms a very stable tetramer even when the interchain bridges have been reduced (9). Although extensive reduction of native $\alpha_2$M results in subunits which readily form insoluble aggregates and thus presumably are denatured (6), reduction of only four to eight disulfide bridges per dimer does not change the tertiary structure of the subunit of native $\alpha_2$M to any great extent (9, 21). In one case, active tetramers were recovered upon reoxidation (9), while noncovalently associated active dimers were obtained in another case (21). In both instances, the internal $\beta$-cysteine-$\gamma$-glutamyl thiol esters were largely intact.

The stretches around Cys$^{355}$ and Cys$^{464}$ are strikingly hydrophilic: $-$Arg$^{247}$-Lys-Tyr-Ser-Ala-Ser-Asp-Cys-His-Gly-Glu-Asp-Ser-Gln- and $-$Lys$^{453}$-Asp-Arg-Ser-Pro-Cys-Tyr-Gly-Tyr-Gln-Trp-, respectively. These sequences probably constitute segments of chain reversal in discrete $\beta$-barrel-type domains, each approximately 100–200 residues in length (23). This could expose the interchain bridges to solvent, compatible with their facile reaction with the highly solvated redundant MES. The $\alpha_2$M subunit evidently contains sets of internal disulfide bridges having widely differing solvent accessibility as probed by partial reduction, but no information on their role in maintaining the native structure of the $\alpha_2$M subunit has yet been obtained.

The individual chains of the dimeric unit of $\alpha_2$M are arranged in an antiparallel fashion (Fig. 1, inset). This orientation would be compatible with that proposed in a recent hypothetical model of $\alpha_2$M primarily based on electron microscopy and x-ray scattering studies (24). In this model, the proteinase binding disulfide-bridged $\alpha_2$M-dimer is regarded as a lobed basket- or mesh-like structure, capable of accommodating one proteinase molecule within its lumen. Whether the partially reduced noncovalently associated dimers re-
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Ported in Ref. 21 represent the disulfide-bridged dimers of $\alpha_2M$, or whether they represent dimers assembled from subunits not covalently linked in the $\alpha_2M$ tetramer is presently unclear.

In view of the common evolutionary origin of $\alpha_2M$ and the complement proteins C3 and C4 (25), it is likely that the $\alpha_2M$ subunit contains a number of functional domains shared with C3 and C4. However, the disulfide bridge pattern of those proteins, which circulate as monomers or perhaps as noncovalently associated two subunits necessary for formation of the functional dimeric proteinase binding unit. In this respect, murinoglobulin (26) and rat $\alpha_1$ inhibitor III (27) form an exception since they circulate as monomers or perhaps as noncovalently associated dimers. Following cleavage of only one bait region in $\alpha_2M$, the subunits express co-operativity with regard to activation of the internal thiol esters, so that two SH-groups appear to be binding of one proteinase molecule under conditions of low proteolytic activity (11). The ensuing conformational change, which results in irreversible binding and effective steric shielding of the activating proteinase, also appears to be a property of the covalently associated dimer.

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fractions having the highest radioactivity were combined and the absorbance at 280 nm determined in a Beckman DU-1 spectrophotometer. After adjusting the absorbance at 417 nm to zero with the sample coulter and the reference coulter containing 1.0 ml buffer A each received 25 μl 1.0 M Tris (dissolved in buffer A), and pH at 417 nm was determined. The content of the αv-groups in partially reduced ανM-MA was related to the amount of proteins using ε 1 cm, 1 cm, 5.4 ml = 13,000 M-1 cm-1. For DTNB (19) and 0.2, 200 mg = 9.0 for μM (210 μM - 80 μM). The results are shown in Table 2 (main text). Similar experiments were performed with native ανM (28,29,29 - main text).

Preparation of partially reduced ανM-MA. ανM-MA (100 mg in 20 ml buffer A) was made 4 M in M2-Cys and allowed to react for 10 min before being dialyzed into 0.1 M M2-Cys, pH 8.2, to give chromatographic columns of Sephadex G-25 F 35 X 2.5 cm. The flow rate was 400 ml so that partially reduced ανM-MA was separated from excess reductant in less than 45 ml. Using DTNB this preparation was found to contain 1.76 μmol of αν-disulfide groups per mg ανM-MA. The free αν-groups were labeled by reacting with Nε-Nε'-carbodiimide of hexamethylenediamine (57 μM/ml) for 9 × 10-5 min at pH 8.2 and the mixture again subjected to Sephadex G-25 F gel chromatography in buffer A (column size: 6 × 40 cm). The recovery of ανM-MA carbodiimide ανM-MA was 88.9% mg in 2.2 M buffer A.

Chromatographic aliquots of reduced, carbodiimide-ανM-MA, 320 μl, was to 72 ml of the preparation described above was added 2.4 μmol of L-valine chloride to 4 M, and all remaining disulfide bridges were reduced with 9 ml DTE for 1 × 10-3 at pH 8.5. After reaction with 20 ml unlabeled JH25D40, for 1.0 × 10-3 and labeled ανM-MA was recovered by gel chromatography on a column of Sephadex G-25 F (1 × 40 cm) equilibrated and eluted with 0.1 M M2-Cys, pH 8.2, at a flow rate of 400 ml/h. The pooled material (5 μl in 212 ml) was digested with 0.4 μmol of CNBr (for 2 h R.T.), and digestion was stopped by adding 125 μl 0.4 M tris(hydroxymethyl)aminomethane (Tris) and freezing. The chromatographic aliquot of ανM-MA was completely solved in 22 ml 10 M M2-Cys, pH 8.2, and was separated by strong ion-exchange chromatography on a column of DEAE-Sephadex in 0.1 M M2-Cys, containing buffers (21) (3). On the basis of the radioactivity profile eight pools were collected and freeze-dried. To obtain an impression of the complexity of each pool and the distribution of radioactivity one-branched product was subjected to reverse phase HPLC on a column of Nucleosil 100 C18, and a number of radioactive fractions examined by amino acid and sequence analysis (not shown). Except for pool 7, no pure radioiodinated peptides could be recovered from the major peaks of radioactivity (not shown). Pools 5, 6, 7, and 8, containing about 95% of the amount of labeled peptide to the DEAE-Sephadex column, were further separated by strong ion-exchange HPLC on a column of Nucleosil 100 C18, using a gradient of NH4HCO3 (21), and the separation was monitored by recording the absorbance at 250 nm (21) and by measuring the radioactivity in 20 or 0.1 ml samples from each fraction (individual area). The fractions obtained (horizontal bars) were pooled and further analyzed (Figs. 4D-6). Fractions 1 and 2 were pooled into A, and the remaining A fractions were pooled into B. The fractions obtained from the DEAE-Sephadex column were pooled into C, and the remaining C fractions were pooled into D and E. The fractions from the DEAE-Sephadex column were pooled into F and G, and the remaining F and G fractions were pooled into H and I, respectively. Approximately 250 μl of 0.1 M M2-Cys, 0.1 M Tris, pH 8.5 containing 0.1 ml DTE. After reduction for 20 min 20 μl [14C]JH25D40 and unlabeled JH25D40 were added to a final concentration of 2 μM. The reduced and radiolabeled fragments (C12, C13), and C131 were separated by reverse phase HPLC on the same column of RDA A and analyzed (Fig. 12B). These fragments could also be separated on a column of Nucleosil 100 C18 (not shown). Their amino acid composition is given in Table 2. About 30 cm [14C]JH25D40 was dissolved in 3 ml 0.1 M Tris, mixed with 1.0 ml 0.2 M M2-Cys, and digested with 10 μg of Lys C for 2 h at room temperature. After freezing the digest was washed with 1.0 ml 0.1 M Tris, 1.0 ml 0.1 M Tris, and 0.1 ml 0.1 M Tris and separated on a Nucleosil 100 C18 column (Fig. 13A). The remaining 0.1 μl was dried, redissolved in 50 μl 0.1 M M2-Cys, 0.1 M Tris, pH 8.5, reduced with DTE and radiolabeled with unlabeled JH25D40, and separated on the column again.

By difference analysis the fractions labeled E and F in Fig. 11A were judged to contain disulfide bridged peptides, and these fractions were dried and reconstituted in I M M2-Cys, pH 8.5 containing 0.1 ml DTE. After reduction for 20 min 20 μl [14C]JH25D40 and unlabeled JH25D40 were added by addition of 25 μl [14C]JH25D40 followed by excess of unlabeled JH25D40. The separation of the reduced, carbodiimide-ανM-MA corresponds to the G/F-C2 bridge on the Nucleosil 100 C18 column is shown in Fig. 11B. The motives of the peptide set representing the disulfide bridge G/F-C2 are denoted CTS and CTE. The peptide set representing the bridge G/F-C1 is denoted CTZ-CYF (the net was not separated). Their amino acid compositions are shown in Table 4, and results of sequence analysis are shown in Fig. 12.

Fig. 4. Separation of pools from the DEAE-Sephadex column (Figs. 1A) on a column of DEAE-U 150 to 300 μmol of ανM was equilibrated with 0.1 M M2-Cys and eluted at a gradient of NH4HCO3 (21). The separation was monitored by recording the absorbance at 250 nm (21) and by measuring the radioactivity in 20 or 0.1 ml samples from each fraction (vertical bars). The fractions obtained (horizontal bars) were pooled and further analyzed (Figs. 4D-6). Fractions 1 and 2 were pooled into A, and the remaining A fractions were pooled into B. Pool 4, same flow rate and fraction size as A, B, C, Pool 5, same flow rate and fraction size as A, B, C, Pool 6, same flow rate and fraction size as A, B, C. Two pools were collected 4 and 5, 6, 7, Pool 7, same flow rate and fraction size as 4 and 5. Fractions 1 and 2 were collected 4 and 5.

Fig. 5. Separation of the pools obtained from the DEAE-Sephadex column (Figs. 4D-6) on a column of Nucleosil 100 C18. The column (10 × 150 mm) was equilibrated with 15 μM Tris (15 ml/m) and eluted with a gradient of 150 μM Tris (21). The separation was performed at 1.0 ml/min at a flow rate of 2.0 ml/min, and were monitored by measuring the absorbance at 250 nm (21) (A). A Pool shown in Figs. 4D-6). The filled peak contained more than 95% of the applied radioactivity (CTZ). B Pool shown in Figs. 4D-6). The filled peak contained more than 95% of the applied radioactivity (CYS). C Pool shown in Figs. 4D-6). The filled peak contained nearly 100% of the applied radioactivity (CYS). D Pool shown in Figs. 4D-6). The filled peak contained nearly 100% of the applied radioactivity (CYS).

Fig. 6. Sequence of major tritium-labeled carbodiimide-ανM-MA peptides from partially reduced ανM-MA. The results of sequence analysis are shown in Fig. 11. The sequences of the peptide fragments are given in parentheses. The peptides obtained after digestion with chymotrypsin in all peptides was 28-32 ± 0.2 cm (mean).
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Table 1.

|  | C1 | C2 | C3 | C4 |
|---|---|---|---|---|
| Asx | 1.07(1) | 1.21(1) | 0.95(1) | 3.21(1) |
| Ser | 0.98(1) | 0.85(1) | 0.86(1) | 2.91(1) |
| Glx | 1.556(8) | 1.03(1) | 2.13(1) | 2.51(1) |
| Pro | 0.71(1) | 0.91(1) | 1.21(1) | 1.71(1) |
| Glu | 0.51(1) | 0.93(1) | 1.81(1) | 2.31(1) |
| Ala | 0.41(1) | 0.71(1) | 0.91(1) | 2.71(1) |
| Cys | 0.61(1) | 0.61(1) | 0.91(1) | 2.71(1) |
| Leu | 0.21(1) | 0.21(1) | 0.91(1) | 2.71(1) |
| Val | 0.21(1) | 0.21(1) | 0.91(1) | 2.71(1) |
| Tyr | 0.21(1) | 0.21(1) | 0.91(1) | 2.71(1) |
| Phe | 1.11(1) | 1.21(1) | 0.91(1) | 2.71(1) |
| Ile | 1.11(1) | 1.21(1) | 0.91(1) | 2.71(1) |

**RESULTS**

**Table 2.**

| Pool | CR3 | CR11 | CR17 |
|---|---|---|---|
| Asx | 14.3(1) | 2.7(1) | 0.9(1) |
| Ser | 11.2(1) | 2.7(1) | 0.9(1) |
| Glu | 16.1(1) | 2.7(1) | 0.9(1) |
| Gln | 20.2(1) | 2.7(1) | 0.9(1) |
| Pro | 11.2(1) | 2.7(1) | 0.9(1) |
| Gly | 11.2(1) | 2.7(1) | 0.9(1) |
| Cys | 0.41(1) | 0.41(1) | 0.41(1) |
| Leu | 0.51(1) | 0.51(1) | 0.51(1) |
| Val | 0.51(1) | 0.51(1) | 0.51(1) |
| Tyr | 0.51(1) | 0.51(1) | 0.51(1) |
| Phe | 1.11(1) | 1.11(1) | 1.11(1) |
| Ile | 1.11(1) | 1.11(1) | 1.11(1) |

**Fig. 10.** Reverse phase HPLC separation of CR3 peptides from pool 1 [(Fig. 9). A column of Vydac C18 (250 μm) was used in methanol-water solvent systems containing 0.1% TFA. Flow rate was 2 ml/min and the column was operated at 60°C. The eluate was monitored by recording the absorbance at 220 nm and the fractions were collected. Absorbance at 220 nm (1 to 3). The fractions were desalted and subjected to enzymatic digestion. CNBr-CLE INTERNAL. The elution positions of the various peptides are indicated. 1 (CR1); 2 (CR11); 3 (CR17).
### Table 3. Sequencer analysis of Cys4-59-Cys7 1)

| Step | C9 | C1 | C87 |
|------|----|----|-----|
| 1    | Ser | Leu | Glv |
| 2    | Met | Phe | Tle |
| 3    | Phe | Gly | Thr |
| 4    | Gly | Cys | Ala |
| 5    | Cys | Met | Ala |
| 6    | Met | Ser | Gly |
| 7    | Ser | Met | Leu |
| 8    | Met | Phe | NFe |
| 9    | Thr | Phe | NFe |
| 10   | Ser | Phe | Val |
| 11   | Ala | Thr | Val |
| 12   | Val | Thr | Thr |
| 13   | Ser | Cys | NFe |
| 14   | Cys | Ser | Thr |
| 15   | Thr | Met | Thr |

1) Five small fragment set was used for deprotection. Residues identical to those of C9, C1, and C87, respectively, are underlined. Due to the overlapping of these derivatives in plate as broad peaks during reverse phase HPLC, NFe and NFe were not clearly identified except in the first three steps. The three sequences were obtained to nearly equimolar yield as judged from the yields in cases 1 (2.9 mm Phe-Cys) and 2.5 mm Phe-Cys, 3 (2.3 mm Phe-Cys and 1.9 mm Phe-Cys), and 4 (1.8 mm Phe-Cys and 1.9 mm Phe-Cys).

### Table 4. Amino acid composition of chymotryptic peptides derived from Cys4-59-Cys7 1)

| Peptide | Cys4 | Cys5 | Cys6 |
|---------|------|------|------|
| Asx     | 0.1(1)| 0.0(1)| 1.0(1) |
| Thr     | 1.0(1)| 0.0(1)| 1.0(1) |
| Ser     | 0.0(1)| 0.0(1)| 0.0(1) |
| Pro     | 0.0(1)| 0.0(1)| 0.0(1) |
| Glu     | 0.0(1)| 0.0(1)| 0.0(1) |
| Ala     | 1.1(1)| 2.1(1)| 1.1(1) |
| Cys     | 0.7(1)| 0.1(1)| 1.1(1) |
| Val     | 2.0(1)| 2.1(1)| 2.0(1) |
| His-Met | 0.0(1)| 0.0(1)| 0.0(1) |
| Lys     | 1.3(1)| 3.3(1)| 1.2(1) |
| Tyr     | 0.0(1)| 1.1(1)| 1.1(1) |
| Phe     | 1.1(1)| 1.1(1)| 1.1(1) |
| His     | 2.1(1)| 1.1(1)| 1.1(1) |
| Arg     | 3.3(1)| 3.3(1)| 3.3(1) |

1) Amount isolated (nmol): 3 3 6

### Position in the sequence

| Position | Cys4-59-Cys7 |
|----------|--------------|
| 442-457  | (536-549)    |
| 536-549  | (1060-1063)  |

1) The compositions are determined relative to the underlined residues, and the expected number of residues is given in brackets.
2) Determined as S-carboxymethyl cysteine.
3) Not determined.
4) The yield was approximately 15%, 45%, and 25%, respectively, for Cys4, Cys5, and Cys6-Cys7.