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CARBOXY TERMINAL FRAGMENT OF HUMAN α-1-ANTITRYPSIN FROM HYDROXYLAMINE CLEAVAGE: HOMOLOGY WITH ANTITHROMBIN III

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Summary: Human α-1-antitrypsin (AT) was reacted with hydroxylamine at pH 9.0 giving cleavage at an Asn-Gly bond. A fragment of molecular weight 8,500 was released and this was isolated and sequenced. The fragment had the same carboxy terminal amino acid sequence as intact AT. The 80 residue polypeptide contained the Z variant mutation site and a portion of sequence identical to that found by others for the reactive site, inferring the presence in AT of two active sites. This sequence combined with previously published work gives a continuous sequence of 152 amino acid residues from the carboxy terminal end of the AT molecule, including the mutation site of the S variant. The sequence shows strong homology with human antithrombin III.

Human α-1-antitrypsin (AT) also known as α-1-proteinase inhibitor, is a plasma protein that protects tissue from destruction by endogenous serine proteases. Genetic variants may be associated with decreased plasma levels with a consequent predisposition to the destructive lung disease, emphysema. A severe deficiency occurs due to the common Z variant allele which as well as lung disease is also associated with liver disease, due to the accumulation of the Z AT in the liver cells. It appears that a partial block occurs during the synthesis of Z AT at the level of transition from rough to smooth endoplasmic reticulum, some AT going on to be secreted, other material accumulating within the cell (1). The reason for this secretory blockage of the Z protein is not known.

The molecular defect of the Z AT has been shown to be due to replacement of a glutamic acid by a lysine at position 100 of one of the largest cyanogen bromide polypeptides, the 'C-fragment' (2,3,4,5). It was considered desirable to position this fragment and hence the Z site in terms of the whole molecule.

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Methods

Human AT was isolated from plasma as previously described (6). Cleavage of AT by hydroxylamine followed a method modified from that of Bornstein and Balian (7). Twenty ml of 2 M hydroxylamine, 2 M guanidine, 0.2 M sodium carbonate, pH 9.0, was prepared by dissolving the first two reagents in a minimum volume of water adjusting the pH to 7 with KOH pellets and then dissolving the sodium carbonate. The pH was adjusted to 9.0 with 5 M KOH and the volume made up to 20 ml with water. 100 mg of AT was dissolved in this solution and incubated at 45^{o} for 5 h. Insoluble material was removed by centrifugation and the supernatant was desalted on Sephadex G-15 using 1% acetic acid as the eluent. The fraction that eluted in the void volume was freeze-dried and chromatographed on Sephadex G-75, with monitoring at both 280 nm and 230 nm. The peak that eluted within a volume of 2.2-2.6 void volumes was pooled and freeze-dried.

The molecular weight of the fragment was determined by SDS polyacrylamide gel electrophoresis with a 10% acrylamide concentration. The amino acid composition was determined on a Technicon NC-1 amino acid analyser following hydrolysis in 6 M HCl at 110^{o} for 24 and 72 h.

Standard methods previously described (5,6) were used for the digestion of the fragment with trypsin and *S. aureus V8* protease; and for peptide mapping. Sequencing was carried out by the dansyl-Edman technique (8) with amide assignation from peptide and degradation-peptide electrophoretic mobilities at pH 6.4. Cyanogen bromide, CNBr, cleavage of 5 mg of fragment in 200 µl of 70% formic acid was carried out with the addition of 15 mg of CNBr for 18 h at room temperature.

Results

SDS polyacrylamide gel electrophoresis of the products following digestion of AT with hydroxylamine is shown in Figure 1. The three major bands are, unreacted AT, a small fragment calculated to be about 8,500 daltons, and a fragment with a molecular weight near 44,000, some 8,000 daltons less than unreacted AT.

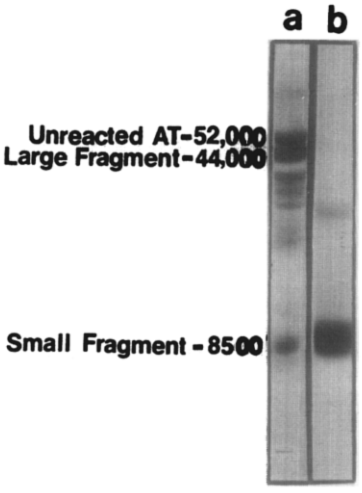

Figure 1. SDS acrylamide gel electrophoresis of (a) α-1-antitrypsin after reaction with hydroxylamine and (b) of the purified 8,500 dalton fragment from (a).

Twelve mg of the purified small fragment (Fig. 1) was obtained from 100 mg of AT. The N-terminal sequence of the fragment confirmed that it was a single polypeptide fragment with the initial 8 residue sequence: Gly-Ala-Asx-Leu-Ser-Gly-Val-Thr. This portion of sequence was recognized as being identical to residues 73-80 from the CNBr 'C-fragment' (4). Tryptic peptide maps were produced giving 10 major spots three of which were identical by amino acid composition and limited sequence results to those derived from the CNBr 'C-fragment'. The sequence of the remaining tryptic peptides was determined and peptide overlaps established from *S. aureus* digest peptides and CNBr fragmentation peptides. The combined results are shown in Figure 2. The sequence of the fragment was in agreement with the residue composition from the total amino acid analysis and its calculated molecular weight 8,618 was in agreement with the estimated value of 8,500. There was partial tryptic cleavage at Met 44, giving both peptides 30-44 and 45-51 as well as peptide 30-51. Peptide mobilities, both chromatographic and electrophoretic, excluded the presence of a carbohydrate sidechain in the fragment.

Discussion

Reaction of AT with hydroxylamine gives cleavage at primarily a single site with 60% yields of the 8,500 dalton carboxy-terminal peptide. The

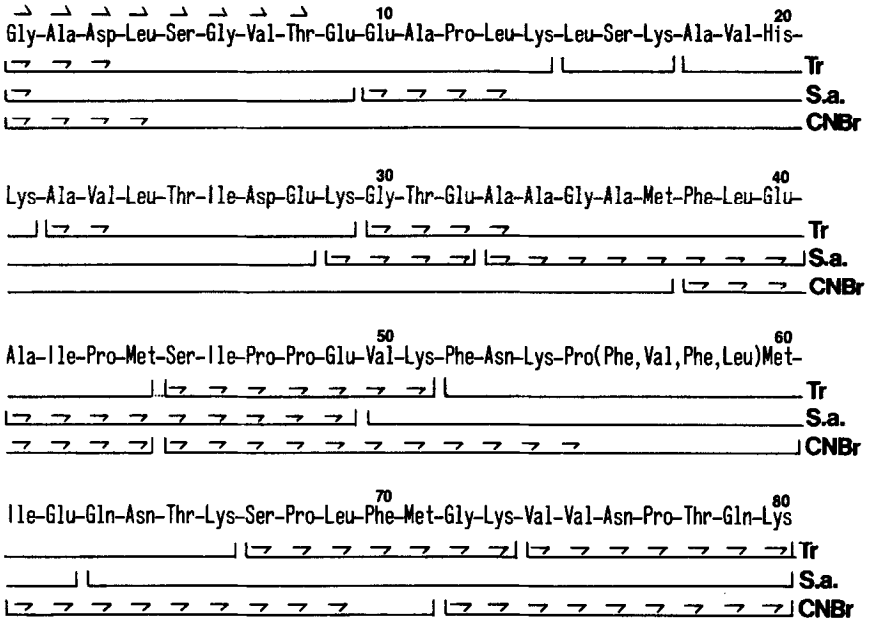

Figure 2. Sequence of the carboxy terminal fragment released by hydroxylamine cleavage of α-1-antitrypsin. Residues 1-37 overlap with the previously determined CNBr 'C-fragment'. Positions assigned by dansyl-Edman steps on subpeptides ⟶ and the whole fragment ⟶. Cleavages: Tr, trypsin; S.a, *S. aureus* protease; CNBr, cyanogen bromide.

placing of this 8,500 dalton fragment at the C-terminus is as expected from the molecular weight data and is confirmed by the identity in sequence of the last nine residues of the fragment with the sequence obtained for the C-terminus of the whole molecule by Travis and Johnson (9). The sequence of the fragment, as shown in Figure 2, has been determined by dansyl-Edman sequencing with the exception of residues 56-59 and the overlap 60-61. The amino acid analysis of the CNBr subpeptide (residues 45-60) showed 2 phenylalanines, 1 valine and 1 leucine unaccounted for in the partial sequence 45-55 of the subpeptide. These have been provisionally placed as Phe,Val,Phe,Leu, by homology with an identical sequence to residues 39-61 obtained by Johnson and Travis (10). The overlap 60-61 was inferred by exclusion.

The sequence of the hydroxylamine fragment together with that of the CNBr 'C-fragment' gives a continuous sequence of 152 amino acid residues from the carboxy terminal end of the antitrypsin molecule. Residue

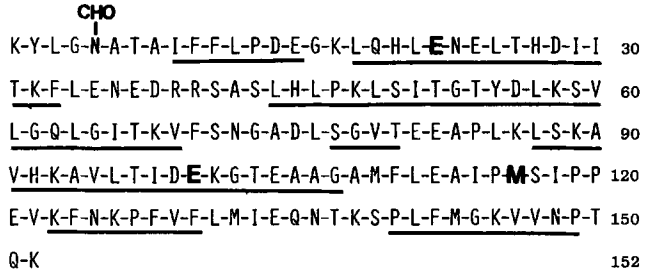

Figure 3. Carboxy terminal sequence of α-1-antitrypsin from combined hydroxylamine fragment and CNBr, 'C-fragment'. The sole carbohydrate attachment is at 5, S mutation at 22, Z mutation at 100, a reactive site at 116. Regions of conserved homology with antithrombin III are underlined.

positions in the ensuing discussion will refer to this combined sequence as in Figure 3. The overall fragment includes the mutation sites of the two common variants associated with deficiency, S and Z. The Z mutation is separated by 95 residues from the last carbohydrate side chain, at Asn 5, in the combined sequence (4). This means that the Z mutation cannot directly affect the initial addition of the carbohydrate core to the nascent polypeptide chain (11).

The newly determined sequence shows the unexpected presence of a putative active site at methionine 116. The sequence 111-133 is near-identical to that found by others (10,12) for an active site some 6,000 daltons from the N-terminus of the protein; whereas our sequence is some 4,000 daltons from the C-terminus. The observation of atypical tryptic cleavage at methionine 116 (Fig. 3) strengthens the belief that this is a C-terminal active site. The striking similarity of the sequence findings at the two widely separated sites provoked a careful review of the evidence presented in this paper. The evidence for the sequence of the C-terminus is firm, in particular the sequencing of the intact hydroxylamine fragment gave clear and single derivatives for each of eight Edman steps, excluding the possibility of peptide contamination. The conclusion is that α-1-antitrypsin may have two active sites, as in a number of plant protease inhibitors and the submandibular gland protease inhibitor of the dog (13).

Supportive evidence for the sequence was noted subsequent to its completion in the striking homology of the 152 residue fragment to the carboxy terminal sequence of human antithrombin III (14). This was confirmed by computer matching by Dr A. D. McLachlan, Laboratory of Molecular Biology, Cambridge; the areas of strongest homology being as underlined in Figure 3. This indicates a common origin for these two plasma proteins and the homologies provide support for the unexpected finding of sequence near the active site, common to that found by others near the amino terminus.

Acknowledgement

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References

1. Carrell, R. W., and Owen, M. C. (1979) Essays Med. Biochem. 4, 83-119. Biochemical Soc., London.
2. Yoshida, A., Lieberman, J., Gaidulis, L., and Ewing, C. (1976) Proc. Natl. Acad. Sci. 73, 1324-1328.
3. Jeppsson, J. O. (1976) FEBS Lett. 65, 195-197.
4. Owen, M. C., Lorier, M. A., and Carrell, R. W. (1978) FEBS Lett. 88, 234-236.
5. Shochat, D., Staples, S., Hargrove, K., Kozel, J. S., and Chan, S. K. (1978) J. Biol. Chem. 253, 5630-5634.
6. Owen, M. C., Carrell, R. W., and Brennan, S. O. (1976) Biochem. Biophys. Acta 453, 257-261.
7. Bornstein, P., and Balian, G. (1970) J. Biol. Chem. 245, 4854-4856.
8. Hartley, B. S. (1970) Biochem. J. 119, 805-822.
9. Travis, J., and Johnson, D. (1978) Biochem. Biophys. Res. Commun. 84, 219-224.
10. Johnson, D., and Travis, J. (1978) J. Biol. Chem. 253, 7142-7144.
11. Lodish, H. F., and Rothman, J. E. (1979) Sci. Am. 240, 38-53.
12. Kress, L. F., Kurecki, T., Chan, S. K., and Laskowski, M. Sr. (1979) J. Biol. Chem. 254, 5317-5320.
13. Dayhoff, M. O. (1978) Atlas of Protein Sequence and Structure, Vol. 5, Suppl. 3, pp. 131-143.
14. Petersen, T. E., Dudek-Wojciechowska, G., Sottrup-Jensen, L., and Magnusson, S. (1978) In: Atlas of Protein Sequence and Structure (ed. Dayhoff, M. O.) Vol. 5, Suppl. 3, p. 141.