Volume 216, number 1, 79–82 FEB 04703 May 1987

Disruption of the Lys-290–Glu-342 salt bridge in human α_1-antitrypsin does not prevent its synthesis and secretion

R.C. Foreman

Department of Pharmaceutical Chemistry, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, England

Received 5 March 1987

The object of this work was to test the hypothesis that failure to secrete the Z variant of human α_1-antitrypsin is related to the loss of a particular structural feature, the Lys-290 to Glu-342 salt bridge. Oligonucleotide-directed mutagenesis was used to disrupt the salt bridge by substituting a glutamic acid for lysine at residue 290. RNA transcripts prepared from this mutant DNA and from the normal cDNA were both able to direct the synthesis of protein in a cell-free system and after injection into *Xenopus* oocytes. Furthermore, the constructed mutant α_1-antitrypsin was secreted as readily as the normal inhibitor.

α_1-Antitrypsin; Z variant; Site-directed mutagenesis; α_1-Antitrypsin secretion; (*Xenopus* oocyte)

1. INTRODUCTION

α_1-Antitrypsin (AAT) is the principal protease inhibitor found in human serum. The main function of the protein appears to be the inhibition of neutrophil elastase and thereby the prevention of proteolytic damage to the lung [1–3]. The naturally occurring Z variant of the inhibitor is synthesised in hepatocytes but its secretion from these cells is inefficient compared to that of the normal, M variant [4,5]. A point mutation changes glutamic acid at residue 342 in the normal protein to lysine in the Z variant and this single substitution is responsible for the secretory defect [6,7].

The failure to secrete Z α_1-antitrypsin is not confined to the hepatocyte. *Xenopus* oocytes injected with liver mRNAs from normal and PiZZ individuals synthesise α_1-antitrypsin but the Z protein accumulates intracellularly while the M variant is readily secreted [8,9]. How a single amino acid substitution produces such a radical change in protein transport is still far from clear however, crystallographic studies [10] have suggested that Glu-342 forms a salt bridge with Lys-290 in the normal inhibitor. The loss of this feature in the Z variant is thought to have an effect on the folding of the newly synthesised protein and prevent its subsequent passage along the secretory pathway. To study this possibility I have used site-directed mutagenesis to disrupt the salt bridge from 'the other side' by replacing the lysine at 290 with glutamic acid. Messenger RNAs coding for normal and Lys-290–Glu mutant AATs were injected into *Xenopus* oocytes to measure the effect of this mutation on the secretion of the inhibitor.

Correspondence address: R.C. Foreman, Department of Pharmaceutical Chemistry, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, England

2. MATERIALS AND METHODS

All DNA and RNA modifying enzymes were from BRL (Bethesda Research Laboratories) or New England Biolabs. The oligonucleotide adaptor was from Pharmacia. Radiolabelled compounds and the reticulocyte lysate translation kit were from Amersham. M 13 vectors and bacterial strains for site-directed mutagenesis were from

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

Anglian Biotechnology. Other reagents were Analar grade or better and were supplied by Sigma. General methods and the use of restriction enzymes have been described elsewhere [11].

Oligonucleotides for mutagenesis and sequencing were prepared on a Pharmacia gene assembler and purified by polyacrylamide gel electrophoresis and UV shadowing or by HPLC on a Whatman Particel 10 SAS column. Oligonucleotide-directed mutagenesis was undertaken essentially as described [12,13]. Putative mutants were sequenced using the dideoxynucleotide chain termination method [14].

The vector SP64T [15] was cut with *Bgl*II at a site between the 5′- and 3′-flanking regions and a *Pst*I adaptor inserted at this point. The *Pst*I site in the polylinker was removed before ligation of the AAT sequences into the vector. Constructs were linearised by cleavage with *Xba*I and transcribed with SP6 polymerase according to the manufacturer's instructions except for the addition of 0.5 mM $m^7G(5')ppp(5')G$ to the reaction mix [16].

The preparation and micro-injection of *Xenopus* oocytes was as described by Colman [17]. Oocytes were injected with ~20 ng of RNA or an equal volume of sterile water then cultured in unlabelled Barth's saline for 18 h. Unhealthy oocytes were discarded and the remaining oocytes cultured in Barth's saline containing 0.8 $mCi \cdot ml^{-1}$ [^{35}S]methionine for 24 h. Oocyte extracts and incubation media were immunoprecipitated as previously described [8].

Translation assays and immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis on 10% gels followed by fluorography [8,18].

3. RESULTS AND DISCUSSION

To examine the function of the Lys-290–Glu-342 salt bridge the lysine residue was replaced with a glutamic acid to ensure the complete disruption of the salt bridge and to approximate the charge repulsion found in the Z protein. To do this a 17 base oligonucleotide was synthesised with a single mismatch at the first base of the Lys-290 codon (AAA to GAA). A full length cDNA for α_1-antitrypsin [19], was cloned in an M13 mp8 vector and the desired mutant generated as described by Winter et al. [12]. The mutant gene was sequenced to ensure the desired mutation was in place. It was then excised and inserted into the 'translation vector' to give the construct shown in fig.1. This vector is a modification of that described by Krieg and Melton [15]. The 5′-flanking region from *Xenopus* globin mRNA contributes a capping site and ribosome binding site to allow the transcription of readily translatable AAT mRNA. To permit correct alignment of the AAT cDNA in relation to the flanking sequences a *Pst*I site has been inserted downstream from the 5′-flanking region and a similar site deleted from the polylinker sequence. In vitro transcripts were synthesised in the presence of $m^7G(5')ppp(5')$ to produce capped RNAs for subsequent protein synthesis [16].

Translation of normal and mutant coding RNAs in a reticulocyte lysate cell-free system labelled with [^{35}S]methionine showed that both species directed the production of protein (fig.2). In both cases strong bands of equal intensity were produced (relative molecular mass 46 kDa) which corresponded in size to the translation product from total human liver mRNA immunoprecipitated with antiserum raised against plasma AAT. The

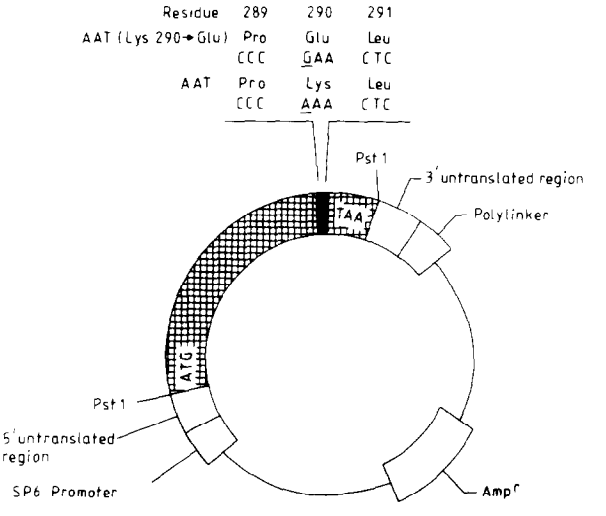

Fig.1. Structure of the SP6 translation vector and sequences of AAT and the Lys-290–Glu mutant DNAs. The cross-hatched zone indicates mature α_1-antitrypsin coding sequences and the inner dark region represents the area for site-directed mutagenesis. RNA synthesis starts at the SP6 promoter and proceeds clockwise.

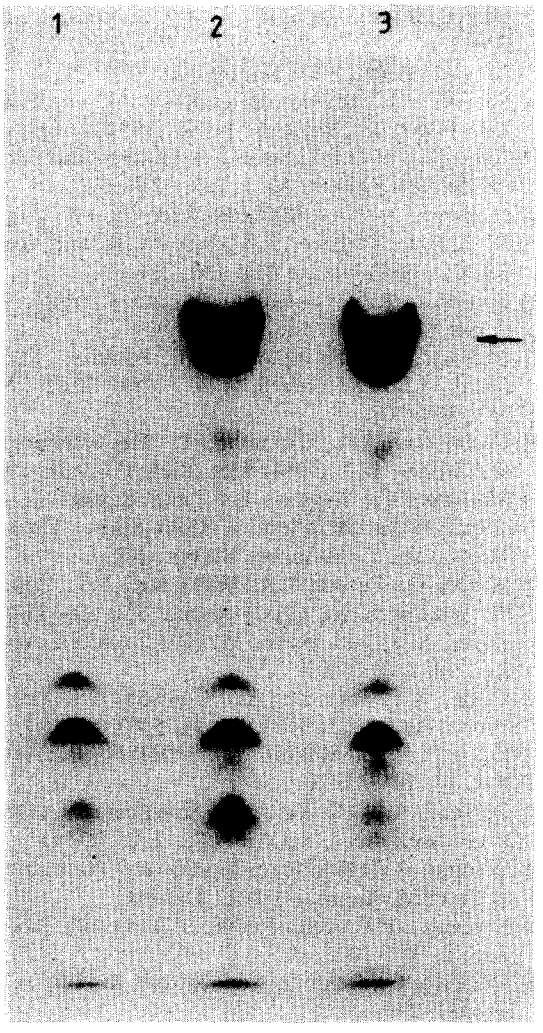

Fig.2. The translation of normal and Lys-290 to Glu transcripts in a reticulocyte lysate cell-free translation system. Lanes: 1, control without added mRNA; 2, normal AAT RNA; 3, Lys-290 to Glu RNA. The arrow indicates the position of immunoprecipitated AAT from translation of total human liver poly(A^+) RNA.

presence of full length polypeptides after cell-free translation indicates the absence of incomplete transcripts, a point that might be obscured by the post-translational modifications undertaken by the oocyte [8,20].

To investigate the effect of the Lys-290 to glutamate mutation on the secretion of AAT, normal and mutant RNAs were injected into oocytes from *Xenopus laevis* (fig.3). Both transcripts produced an intracellular species with a molecular mass of 54 kDa corresponding to the partially glycosylated precursor and a secreted AAT with a molecular mass of 58 kDa, equivalent to the fully glycosylated protein. Secreted bands were of equal intensity showing that normal and mutant proteins were efficiently transported by the oocyte secretory apparatus. Translation of normal human liver mRNA in the same system supports the view that both SP6 transcripts direct the synthesis of protein that is exported as readily as the authentic liver species. This is in contrast to the aberrant secretion of inhibitor synthesised by liver mRNA from a patient homozygous for the Z gene, also shown in fig.3.

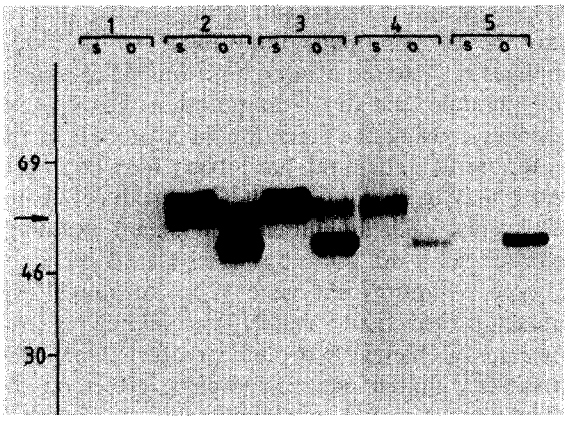

Fig.3. Injection of normal and mutant coding transcripts into *Xenopus* oocytes. S, secreted material; O, species from oocyte extracts. Lanes: 1, uninjected oocytes; 2, injection of normal AAT transcript; 3, Lys-290 to Glu RNA; 4, injection of normal (PiMM) human liver poly(A^+) RNA; 5, poly(A^+) RNA from a Z homozygote.

Crystallographic studies on the structure of AAT are complicated by difficulties in crystallising the intact protein but measurements using a proteolytically modified inhibitor have demonstrated the proximity of Lys-290 and Glu-342 [10]. The possibility of ionic interaction is confirmed by protein modelling using FRODO graphics (R.C.F. and M.J. Sternberg, unpublished). But the present work makes it plain that the absence of the salt bridge is not responsible for the failure to secrete the Z variant since the Lys-290–Glu mutant AAT described here also lacks the salt link and displays a charge repulsion similar to the Z protein but does not accumulate intracellularly.

The disruption of this salt bridge in the Z protein may be of secondary importance to the local changes in structure around position 342 caused by the glutamic acid to lysine mutation. Putative sequences causing the retention of a protein in the endoplasmic reticulum have been identified [21] and it may be that the Z mutation produces or unmasks just such an intracellular signal. Recently another point mutation has been identified in the Z type α_1-antitrypsin gene [22]. Although this mutation (Val-213 to Ala-213) is unlikely to produce major changes in protein structure there is the possibility that it has a synergistic effect on the Glu-342 to Lys-342 mutation. This possibility is also open to investigation using site-directed mutagenesis techniques.

ACKNOWLEDGEMENTS

I thank Dr G. Ciliberto for the gift of AAT cDNA and Dr P. Krieg for the SP64T plasmid. I would also like to acknowledge the help of colleagues at University College, The School of Pharmacy and Professor Alan Malcolm and his colleagues at the Biochemistry Department Cross Hospital Medical School. This work was supported by grants from the Tobacco Advisory Council, the PF Trust and the Arthritis and Rheumatism Council.

REFERENCES

[1] Eriksson, S. (1964) Acta Med. Scand. 175, 197–205.
[2] Carrell, R.W. and Owen, M.C. (1979) Essays Med. Biochem. 4, 83–119.
[3] Carrell, R.W., Jeppsson, J.-O., Laurell, C.-B., Brennan, S.O., Owen, M.C., Vaughan, L. and Boswell, D.R. (1982) Nature 298, 329–334.
[4] Sharpe, H.L., Bridges, R.A., Krivit, W. and Frier, E.F. (1963) J. Clin. Lab. Invest. 15, 132–140.
[5] Jeppsson, J.-O., Larsson, C. and Eriksson, S. (1975) New Engl. J. Med. 293, 576–579.
[6] Jeppsson, J.-O. (1976) FEBS Lett. 65, 195–197.
[7] Kidd, V.J., Wallace, R.B., Itakura, K. and Woo, S.L.C. (1983) Nature 304, 230–234.
[8] Foreman, R.C., Judah, J.D. and Colman, A. (1984) FEBS Lett. 168, 84–88.
[9] Bathurst, I.C., Errington, D.M., Foreman, R.C., Judah, J.D. and Carrell, R.W. (1985) FEBS Lett. 183, 304–308.
[10] Loebermann, H., Tokuoka, R., Deisenhofer, J. and Huber, R.J. (1984) Mol. Biol. 177, 531–556.
[11] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[12] Winter, G., Fersht, A.R., Wilkinson, A.J., Zoller, M. and Smith, M. (1982) Nature 299, 756–758.
[13] Zoller, M.J. and Smith, M. (1982) Nucleic Acids Res. 10, 6487–6500.
[14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[15] Krieg, P.A. and Melton, D.A. (1984) Nucleic Acids Res. 12, 7057–7070.
[16] Darveau, A., Pelletier, J. and Sonenberg, N. (1985) Proc. Natl. Acad. Sci. USA 82, 2315–2319.
[17] Colman, A. (1984) Transcription and Translation, A Practical Approach, IRL, Oxford.
[18] Foreman, R.C. and Judah, J.D. (1982) Biosci. Rep. 2, 995–1002.
[19] Ciliberto, G., Dente, L. and Cortese, R. (1985) Cell 41, 531–540.
[20] Lane, C.D. (1983) Curr. Top. Dev. Biol. 18, 89–116.
[21] Munro, S. and Pelham, H.R.B. (1986) Cell 46, 291–300.
[22] Toshihiro, N., Satoh, K., Brantly, M.L., Ogushi, F., Fells, G.A., Courtney, M. and Crystal, R.G. (1986) J. Biol. Chem. 261, 15989–15994.