RAT α_1-ANTITRYPSIN. PRELIMINARY CHARACTERISATION OF THE IN VITRO mRNA TRANSLATION PRODUCT

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1. Introduction

The glycoprotein α_1-antitrypsin (α_1-AT) is the major inhibitor of proteolytic enzymes in human plasma [1]. It has been purified from several species including man [2] and rat [3]. The molecular weight of rat α_1-AT is about 50 000. α_1AT Deficiency is associated with pulmonary disease (emphysema) in man and in some cases with liver disease. In patients with α_1-AT deficiency there is an accumulation of α_1-AT in the endoplasmic reticulum due to defective secretion of the protein [4] with frequent distension of the cisternal space by globules of precipitated α_1-AT. Agarose electrophoresis of human intrahepatic α_1-AT reveals fractions with a more cathodal mobility as well as one identical to plasma α_1-AT [5]. The cathodal fractions possibly correspond to complex formation with intrahepatic proteolytic enzymes, the presence of a propeptide similar to that reported for albumin, or incompletely glycosylated protein species. These observations suggest that a study of the biosynthesis of α_1-AT may be of interest with respect to the pathogenesis of liver disease associated with α_1-AT deficiency. In this paper cell-free translation of rat liver mRNA and isolation of the α_1-AT translation product are reported as is the partial amino acid sequence of its signal or prepeptide.

2. Materials and methods

Tritiated amino acids and [^{35}S]methionine of the highest available specific activity were obtained from the Radiochemical Centre, Amersham. Oligo (dT)-cellulose (type 2) was from Collaborative Research Inc., and Enhance® from New England Nuclear. Chemicals for the sequenator were obtained from Rathburn Chemicals. Guanidinium thiocyanate (purum) was from Fluka. Protein A Sepharose was from Pharmacia. All other chemicals were reagent grade.

Rat α_1-AT was purified using a modification of the procedure described for human α_1-AT [2, to be published]. The purified protein migrated as a single band on SDS–PAGE in both reduced and unreduced form (fig.1), and was quantitatively complexed by added

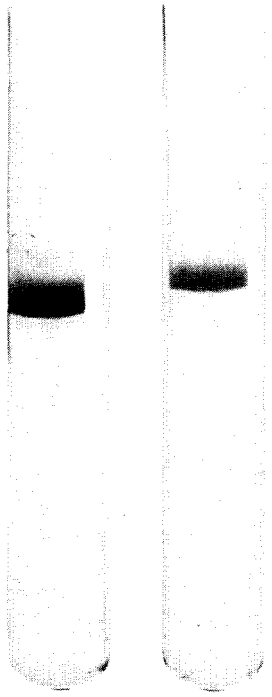

Fig.1. SDS–PAGE (disc) of purified rat plasma α_1-AT. About 10 μg was applied to each gel. Left, sample reduced with 2-mercaptoethanol prior to electrophoresis; right, unreduced sample.

Abbreviations: α_1-AT, α_1-antitrypsin; SDS, sodium dodecyl sulphate; PTH, phenylthiohydantoin; PAGE, polyacrylamide gel electrophoresis

trypsin. Rabbits were immunized with α_1-AT emulsified with Freund's complete adjuvant. The immunoglobulin fraction of the antiserum was eluted in 50 mM Tris buffer, pH 7.2, from a DEAE-Sephadex A-50 column.

Messenger RNA from rat liver was prepared according to the method of Chirgwin et al. [6], using 50% instead of 75% ethanol in the first precipitation step. Poly(A)-containing RNA was obtained by chromatography on oligo(dT)-cellulose, again using the conditions of Chirgwin et al. [6]. RNA was quantitated using $E^{1\%}_{1\,cm}$ at 260 nm of 200.

In vitro translation of RNA was performed in a rabbit reticulocyte lysate treated with staphylococcal nuclease according to Pelham and Jackson [7]. Incubations were for 90 min at 35°C. Total incorporation of isotope into proteins was estimated by liquid scintillation measurements after trichloroacetic acid precipitation of a small aliquot of the incubate on filter paper. Translation products were also analysed by SDS–PAGE using 10–15% gradient slab gels. Unstained gels were dried and autoradiography using fluorographic enhancement [8] was performed at −70°C for 2–5 days.

Immunoprecipitation of the α_1-AT translation product was performed in 1% NP-40 buffer according to Dobberstein et al. [9]. α_1-AT was eluted from the protein A Sepharose with 1% SDS. Sperm whale myoglobin (300 nmol) was added as carrier and proteins were precipitated by the addition of five volumes of acetone containing 0.1 M HCl. After incubation overnight at 22°C and centrifugation, the precipitate was dissolved in 0.4 ml formic acid. It was then subjected to automated Edman degradations in a Beckman 890 C sequenator using the 1 M quadrol program as described previously [10]. The degradation products were evaporated to dryness, dissolved in methanol and the radioactivity was measured in a Packard liquid scintillation spectrometer. Rat plasma α_1-AT was degraded by the same procedure and PTH-derivatives were identified by high pressure liquid chromatography [11].

3. Results and discussion

α_1-AT is an acute-phase reactant protein with 2–3 fold increases in plasma concentration due to an increased rate of synthesis after tissue injury. We therefore treated rats with subcutaneous injections of turpentine 24 h prior to sacrifice to increase the intrahepatic concentration of α_1-AT mRNA. As rat livers are rich in ribonuclease, we adopted the method of Chirgwin et al. in which tissue homogenisation is performed in guanidinium thiocyanate containing *N*-lauroyl sarcosine and 2-mercaptoethanol which ensures rapid and complete protein denaturation. With this method 0.1–0.5 mg of polyadenylated RNA was obtained per gram of liver. The E_{260} to E_{280} ratio of the final product was greater than 2.07.

The rat liver mRNA effectively stimulated protein synthesis in the reticulocyte lysate. Optimal stimulation was obtained with approximately 40 μg poly(A)-RNA per ml. The antiserum against α_1-AT was monospecific and well suited for immunoprecipitation of α_1-AT from the translation mixtures (fig.2). The addition of anti-α_1-AT to the translation did not significantly increase the incorporation of labelled amino acids into pre α_1-AT in contrast to what has been

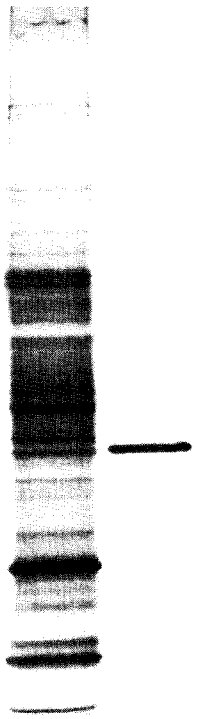

Fig.2. Fluorogram of SDS–PAGE of peptides obtained by cell-free synthesis in a rabbit reticulocyte lysate using rat liver mRNA (left). The product immunoprecipitated with a monospecific antiserum against rat α_1-AT is shown to the right.

reported for some other proteins whose translation products have had low solubility [12]. Addition of increasing amounts of purified rat plasma α_1-AT to the incubates prior to immunoprecipitation successively displaced the labelled α_1-AT from the antibodies. The apparent molecular weight of the reduced and alkylated translation product was 45 000 as judged from the SDS–PAGE whereas that of reduced rat plasma α_1-AT was approximately 52 000. The difference in molecular weights is explained by the total lack of carbohydrate in the translation product.

For sequence analysis of the in vitro translation products incubations of 0.25–1.0 ml were used. Translations with [^{3}H]leucine and [^{3}H]isoleucine have been performed. SDS–PAGE of the immunoprecipitated [^{3}H]leucine translation product showed that 88% of the radioactivity was in the α_1-AT band. This step was therefore judged unnecessary and the washed immunoprecipitates were eluted in 1% SDS, directly precipitated with acetone containing 0.1 M HCl after addition of carrier myoglobin, dissolved in formic acid and subjected to sequenator degradation. The results of the degradations are shown in fig.3. The background radioactivity was fairly high in the leucine degradation but low in the isoleucine degradation. As calculated from the sequenator run of [^{3}H]-leucine labelled α_1-AT an approximate repetitive yield of 93.5% was obtained. Assignments of isoleucine in position 4 and leucine in positions 8,9,10,11,14,17 and possibly 22 could be made. Analysis of the PTH derivatives of plasma α_1-AT after sequenator degradation gave the amino-terminal sequence: Glu, Asp, Ala, Gln, Glu, Arg, Asp, Arg. Since there are no leucine or isoleucine residues in the amino-terminal part of plasma α_1-AT, no alignment in an overlapping segment has been possible. The length of the signal peptide thus remains unknown.

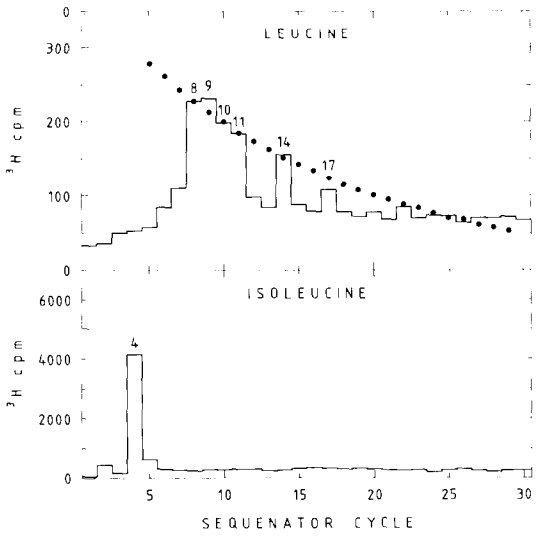

Fig.3. Automated Edman degradation of rat α_1-AT synthetized in the rabbit reticulocyte lysate with [^{3}H]leucine and [^{3}H]isoleucine. The α_1-AT was immunoprecipitated as described in section 2. The numbers over the histogram indicate the positions where identification of the radioactive amino acid was regarded positive. The dotted line is an hypothetical curve for a degradation with 93.5% repetitive yield.

The results suggest that the α_1-AT pre-peptide contains clusters of hydrophobic residues in similarity to other pre-peptides sequenced [13]. Like both bovine and rat pre-proalbumin pre α_1-AT has a block of four adjacent leucine residues [14]. A closer comparison with other prepeptides must await the competition of the sequence of the α_1-AT pre-peptide.

Acknowledgements

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