Synthesis and catabolism of rabbit α_1 -antitrypsins F and S

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The metabolic relationship between the two major forms of rabbit α_1 -antitrypsin, F and S, was investigated by using labelling techniques *in vivo* and *in vitro*. After the injection of [^{14}C]leucine, the S/F specific-radioactivity ratio showed characteristic changes with time: at 1 h, the ratio was high (1.2–1.4), but by later times (5–7 h) it decreased to a value of approx. 1.1. Two different techniques were used to purify α_1 -antitrypsin for labelling with iodine. The half-lives of the differentially labelled and simultaneously injected F- and S-forms were 68.1 (± 7.6 s.d.) and 55.3 (± 8.1 s.d.) h respectively. Combined electrophoretic and γ -spectrometric studies provided no evidence for metabolic interconversion of the α_1 -antitrypsin forms in the circulation. These observations suggest that rabbit α_1 -antitrypsins F and S are, despite their close chemical composition and immunological identity, metabolically independent proteins. Therefore the possibility is raised that α_1 -antitrypsin synthesis in rabbits is controlled by two autosomal genes or two sets of such genes.

Rabbit plasma contains two chemically closely related forms of α_1 -antitrypsin. On electrophoresis in alkaline polyacrylamide gels, the inhibitor migrates as two broad bands, designated α_1 -antitrypsins F (fast) and S (slow). The presence of several subcomponents can be observed in each band under optimal loading conditions. The two forms are immunologically identical and their carbohydrate content appears to be the same, but small differences have been established between their amino-acid compositions and molecular weights (Koj *et al.*, 1978).

These earlier findings pose the question as to the metabolic relationship between the two forms: are α_1 -antitrypsins F and S independent products of protein synthesis, or does one form derive from the other by some postsecretional modification? Investigations pertinent to this problem are reported here.

Experimental

Materials

Na^{125}I , Na^{131}I and L-[4,5- ^3H]leucine were obtained from New England Nuclear Corp., Boston, MA, U.S.A. DL-[carboxy- ^{14}C]leucine was from I.B.J., Swierk, Poland; DEAE-Sephadex A-50,

Sephacrose 6B and Sepharose–concanavalin A were from Pharmacia, Uppsala, Sweden; CNBr was from Eastman–Kodak Co., Rochester, NY, U.S.A.; 2-mercaptoethanol, 1-*O*-methyl α -D-glucopyranoside, L-cysteine and Nbs₂ were from Sigma, St. Louis, MO, U.S.A. Monospecific guinea-pig antisera to rabbit α_1 -antitrypsin and antithrombin III, and polyvalent goat serum to rabbit plasma proteins were a gift from Dr. J. Gaudie, McMaster University.

Methods

Preparation of α_1 -antitrypsins F and S for labelling with iodine. Two different procedures were used, which will be referred to as Methods A and B. In each case, the starting material was citrated rabbit plasma.

Method A, consisting of salting out with $(\text{NH}_4)_2\text{SO}_4$, ion-exchange chromatography on DEAE-Sephadex at pH 8.8 and 6.5, and affinity chromatography on Sepharose–Cibacron Blue, has already been described (Koj *et al.*, 1978). Method B was adapted from a technique described by Laurell *et al.*, (1977) for the purification of human α_1 -antitrypsin utilizing thiol–disulphide interchange. Plasma was saturated at 50% (w/v) with solid $(\text{NH}_4)_2\text{SO}_4$ at 4°C, stirred for 30 min and centrifuged at 10000g and 4°C for 20 min. The supernatant was collected and dialysed exhaustively at 4°C against 0.1 M-Tris/HCl, pH 7.5, containing

Abbreviation used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

1 mM each of MgCl_2 , CaCl_2 and MnCl_2 . A column (2.5 cm $\times$ 12 cm) of Sepharose–concanavalin A was equilibrated with this buffer, and the dialysed supernatant (approx. 150 ml) was passed through it at a flow rate of approx. 36 ml/h. Subsequently the column was washed with the equilibrating buffer until the A_{280} of the effluent was less than 0.02, and then eluted by 0.1 M-Tris/HCl, pH 7.5, containing 0.2 M-1-*O*-methyl α -D-glucopyranoside. The eluted protein was dialysed overnight at 4°C against 0.1 M-Tris/HCl, pH 8.1, containing 0.01 M-disodium EDTA and then concentrated to 15–20 ml by ultrafiltration in an Amicon chamber. After further dialysis (4°C, overnight) against the Tris/EDTA buffer (see above), the preparation was divided into 5 ml portions and kept at –40°C until chromatography on Sepharose–cysteine.

To prepare this conjugate, Sepharose 6B was activated with CNBr (Porath *et al.*, 1973) and was allowed to react with L-cysteine at pH 9.0. Unbound ligand was removed as described by Laurell *et al.* (1977). The quantity of bound thiol, determined by the method of Brocklehurst *et al.* (1973), was in the range 0.13–0.16 $\mu\text{mol/g}$ of moist gel. The gel was poured into a jacketed LKB column at 37°C, reduced by washing with 200 ml of 0.02 M-2-mercaptoethanol in 0.1 M-Tris/HCl, pH 8.1, containing 0.01 M-disodium EDTA, and activated by washing overnight with 100 ml of Nbs_2 (1 mg/ml of the Tris/EDTA buffer) by using a recirculating arrangement. Excess Nbs_2 was removed by washing with 0.1 M-Tris/HCl, pH 8.1, containing 0.01 M-disodium EDTA. The dialysed protein solution was warmed to 37°C and loaded on the Sepharose–cysteine column at a flow rate of approx. 13 ml/h. Unbound proteins were removed by washing with 0.1 M-Tris/HCl, pH 8.1, containing 0.01 M-disodium EDTA, then bound proteins were eluted by 0.02 M-mercaptoethanol dissolved in the Tris/EDTA buffer. The eluate was concentrated by pressure dialysis against 0.01 M-Tris/HCl, pH 8.0, and decolorized at the same time by placing 1 g of activated charcoal in the outer chamber.

From examination by immunoelectrophoresis (Scheidegger, 1955), the product obtained by either Method A or B was pure α_1 -antitrypsin. The F- and S-forms were finally separated by preparative polyacrylamide-gel electrophoresis as described previously (Koj *et al.*, 1978), except that the gel was pre-electrophoresed for 1 h at 20 mA and left overnight. Approx. 10 mg of protein in 1.5 ml was loaded for each run. Fractions were monitored at 280 nm and, if required, by analytical electrophoresis (Clarke, 1964). Material containing the pure F- and S-forms was pooled separately and concentrated by pressure dialysis against 0.01 M-Tris/HCl, pH 8.0. Samples (approx. 0.5 mg) of each form were labelled at pH 8.0 and ^{125}I and ^{131}I

respectively by using the ICl technique (McFarlane, 1958). The number of iodine atoms substituted per molecule of protein ranged between 0.1 and 0.5.

Studies with iodine-labelled α_1 -antitrypsins. Half-lives of the F- and S-proteins were compared in New Zealand White rabbits of 2.5–4.0 kg body wt. A mixture of both proteins (0.2–0.3 mg each), labelled with different isotopes of iodine, was injected intravenously. Blood samples (11–14) were collected over a period of approx. 160 h for the measurement of plasma-protein-bound radioactivities as described elsewhere for transferrin turnover (Regoeczi *et al.*, 1974). Values obtained were plotted on a logarithmic scale against time on a linear scale. The terminal slope of the plasma-radioactivity curve was determined by curve peeling (Jacquez, 1972) and its half-life was calculated by standard semilogarithmic regression analysis on a Wang model-720C computer.

The possibility of interconversion *in vivo* between the F- and S-forms was examined in three rabbits. A mixed injection of ^{131}I -labelled α_1 -antitrypsin F and ^{125}I -labelled S (25–40 μCi of each) was given and blood samples were taken 5 min, 1 h, 3 h and 5 h after the injection. Portions (2 ml) of the heparinized plasma were chromatographed on Sepharose–concanavalin A under the same conditions as those given above for the preparation of α_1 -antitrypsin for iodination by Method B. The desorbed radioactivity peak material was freeze-dried, redissolved in 2 ml of water and dialysed overnight against the electrophoresis buffer at 4°C. Analytical polyacrylamide-gel electrophoresis (Clarke, 1964) was carried out with each sample in triplicate. After staining with Amido Black and destaining, the two α_1 -antitrypsin bands were excised and their radioactivity was measured in a Packard model-5986 multichannel analyser. Results were evaluated by analysis of the variances.

Studies with radioactive amino-acid precursors. Incorporation of ^3H leucine into α_1 -antitrypsin as a function of time was studied comparatively with albumin, fibrinogen and antithrombin III. After the injection of the precursor (50 $\mu\text{Ci/kg}$) into a group of New Zealand White rabbits, blood samples (4 ml each) were collected into dry dipotassium EDTA (4 mg for 1 ml of blood), the first one at 30 min and then at approximately hourly intervals. Fibrinogen was isolated from 0.5 ml plasma samples as fibrin (Regoeczi, 1970) and albumin was purified by affinity chromatography on Sepharose–Cibacron Blue (Travis *et al.*, 1976) from the resulting serum. Antithrombin III and α_1 -antitrypsin were precipitated from separate plasma samples (0.5 ml each) by using monospecific antisera. Before the addition of the antisera, the samples were defibrinated by heat (56°C, 3 min), kept at 4°C overnight and clarified

by centrifugation. Antithrombin III was precipitated at slight antibody excess, α_1 -antitrypsin at antigen excess. (Complete precipitation of the latter was considered undesirable because of its abundance in serum.) Precipitates formed over 4 h at 22°C were collected by centrifugation and washed three times with cold 0.15 M-NaCl. For radioactivity counting, immunoprecipitates and fibrin were solubilized in NCS tissue solubilizer (The Radiochemical Centre, Amersham, Bucks., U.K.) and the excess NCS was neutralized with 50% (v/v) acetic acid. Radioactivity (d.p.m.) was calculated from internal standardization.

[^{14}C]Leucine specific activities of the F- and S-forms were compared in adult Sandylop rabbits. The dose (approx. 16 $\mu\text{Ci/kg}$) was made iso-osmotic with glucose and injected intravenously. Two blood samples (30 ml each) were obtained from each animal 1–7 h after the injection and α_1 -antitrypsin was isolated according to Method A (Koj *et al.*, 1978). After ascertaining protein purity by crossed immunoelectrophoresis (Weeke, 1973), the F- and S-forms were separated by preparative polyacrylamide-gel electrophoresis. Fractions containing the respective forms were pooled with a minimal overlap and specific radioactivities were determined from the pooled proteins.

Results

Purification of rabbit α_1 -antitrypsin by thiol-disulphide interchange yielded a product that gave a single precipitin line against a polyvalent antiserum directed against whole rabbit plasma. The product was electrophoretically indistinguishable from α_1 -antitrypsin isolated by a more complex procedure (Koj *et al.*, 1978). A preparation is shown in Fig. 1.

The general appearance of the plasma protein-bound-radioactivity curve of intravenously injected iodine-labelled α_1 -antitrypsin was as expected from observations with other plasma proteins (see the Discussion section). Significantly, however, the curves representing the differentially labelled F- and S-forms became divergent soon after the injection of the proteins (Fig. 2). Irrespective of the method used to prepare the α_1 -antitrypsin or of the iodine isotope attached to either form, the speed of disappearance of the S-form from the plasma always exceeded that of the F-form. Half-lives of the terminal slopes of the plasma curves of both α_1 -antitrypsins are compared for nine rabbits in Table 1.

In view of the above findings, studies with a radioactive precursor amino acid, [^{14}C]leucine, seemed desirable. Timing of the blood samples required some knowledge as to the appearance of newly synthesized α_1 -antitrypsin in the circulation. This information was procured from preliminary experiments with [^3H]leucine. The plasma ^3H radio-

Fig. 1. Electrophoresis of rabbit α_1 -antitrypsin, prepared by thiol-disulphide interchange, in 7.5% (w/v) polyacrylamide gel

(a) Whole α_1 -antitrypsin; (b) F-form; (c) S-form. Migration was from top to bottom, with anode at the bottom. The buffer was 5 mM-Tris/39 mM-glycine, pH 8.2. Runs were for 1 h at 4 mA per gel, and 40–60 μg of protein were loaded.

activity curve of whole (unfractionated) α_1 -antitrypsin from a group of rabbits contrasts with the corresponding curves for antithrombin III, fibrinogen and albumin in Fig. 3. As seen there, ^3H radioactivity rose steeply between 30 and 90 min in each of these proteins, with maximal values being reached by 2.5 h after the injection.

^{14}C specific radioactivities of α_1 -antitrypsins F and S were determined in two plasma samples from each of the four rabbits that had received [^{14}C]leucine. Results of these experiments (Table 2) showed that the ^{14}C specific activity of the S-protein was always higher than that of the F. S/F specific-radioactivity ratios for the earlier samples (1–3 h) averaged at 1.31 and those for the later samples

Fig. 2. Protein-bound radioactivities in the plasma of a 2.8-kg rabbit that received a mixed injection of ^{125}I -labelled α_1 -antitrypsin F (●) and ^{131}I -labelled S (▲). Values are expressed as percentages of the respective protein-bound radioactivities in a plasma sample obtained 5 min after injection of the dose (0.2 mg of each protein).

(5–7 h) at 1.10. A t value of 3.53 was calculated for these two groups of values by using Student's t -test ($P < 0.01$). The specific radioactivity of the protein samples obtained by pooling the overlapping portions of the two α_1 -antitrypsins was at any time intermediate between the F- and S-form specific radioactivities.

Results pertinent to the question of metabolic interconversion of the F- and S-forms in the circulation are summarized in Table 3. The two forms, each containing approx. 10% cross-contamination by the other, were labelled differentially (F, ^{131}I ; S, ^{125}I) and injected together. Combined electrophoretic and γ -spectrometric analyses of plasma samples collected over a 5 h period showed fairly constant amounts of radioactivity to be associated with the appropriate α_1 -antitrypsin bands. Analysis of the variances of the values in Table 3 revealed no statistically significant differences as a function of time.

Table 1. Half-lives of the terminal plasma slopes of simultaneously injected α_1 -antitrypsins F and S in rabbits. The two proteins were labelled with different isotopes of iodine. Preparations are numbered 1–4, suffixes A and B refer to the two methods of purification outlined in the Experimental section. r^2 denotes the coefficients of determination. Half-lives are in hours.

Animal		Preparation	F-form		S-form		a/b
No.	Weight (kg)		Half-life (a)	r^2	Half-life (b)	r^2	
1	2.5	1-B	62.4	0.93	46.8	0.97	1.33
2	2.5	1-B	67.8	0.99	51.3	0.99	1.32
3	2.5	1-B	62.6	0.99	46.2	0.99	1.35
4	3.2	2-A	64.5	0.99	54.3	0.99	1.18
5	2.8	2-A	64.0	0.99	50.6	0.99	1.26
6	4.0	3-B	72.2	0.99	61.2	0.99	1.17
7	2.4	3-B	86.5	0.98	72.3	0.99	1.19
8	3.5	4-A	64.4	0.99	56.4	0.99	1.14
9	3.5	4-A	68.8	0.98	58.9	0.99	1.16
Mean $\pm$ S.D. . . .			68.1 $\pm$ 7.6		55.3 $\pm$ 8.1		1.23 $\pm$ 0.08

Table 2. ^{14}C specific radioactivities of rabbit α_1 -antitrypsins F and S at different intervals after the injection of [^{14}C]leucine into rabbits

S/F is the ratio of the specific radioactivities of both forms. The F- and S-forms were prepared as described under 'Methods', except for animal no. 3, where the proteins were isolated as for iodination by Method A (see also under 'Methods').

Animal no.	Time of sample (h)	α_1 -Antitrypsin ^{14}C specific radioactivity (d.p.m./mg)		S/F
		F-form	S-form	
1	1	253	326	1.28
	3	446	566	1.26
2	1	246	295	1.20
	5	405	443	1.09
3	1	197	276	1.40
	5	443	503	1.13
4	1	207	298	1.43
	7	351	389	1.10

Table 3. Distribution of the radioactivity between the two α_1 -antitrypsins in plasma samples obtained at different times after the simultaneous injection of ^{131}I -labelled F-form and ^{125}I -labelled S-form in rabbits

Plasma samples were chromatographed on Sepharose–concanavalin A before being electrophoresed in analytical polyacrylamide gels. Values are the means $\pm$ s.e.m. for assays done in triplicate. Percentages are those of the total ^{125}I or ^{131}I activity present in the sample. For further details, see under ‘Methods’.

Animal no.	Percentage of ^{131}I migrating with the S-form after:				Percentage of ^{125}I migrating with the F-form after:			
	5 min	1 h	3 h	5 h	5 min	1 h	3 h	5 h
1	11.1 $\pm$ 0.3	11.5 $\pm$ 1.7	11.0 $\pm$ 0.7	9.0 $\pm$ 0.9	11.3 $\pm$ 1.0	11.1 $\pm$ 0.3	11.5 $\pm$ 0.2	12.7 $\pm$ 0.4
2	—	9.6 $\pm$ 2.9	9.1 $\pm$ 1.8	7.7 $\pm$ 0.6	—	12.1 $\pm$ 1.5	11.4 $\pm$ 0.5	13.2 $\pm$ 0.5
3	8.8 $\pm$ 1.9	5.2 $\pm$ 0.7	6.0 $\pm$ 1.2	8.7 $\pm$ 2.3	10.1 $\pm$ 0.9	13.4 $\pm$ 1.0	14.6 $\pm$ 1.4	11.9 $\pm$ 0.5

Fig. 3. ^3H activity of α_1 -antitrypsin (●), albumin (△), antithrombin III (○) and fibrinogen (▲) in plasma at various times after the injection of ^3H leucine into rabbits

Points are the means for three experiments; the vertical bars indicate s.e.m. Radioactivities in albumin are expressed as d.p.m./mg, those in fibrinogen and antithrombin III as d.p.m. in these proteins from 1 ml of plasma. Values for α_1 -antitrypsin represent d.p.m. in a fraction of the total plasma α_1 -antitrypsin as obtained by immunoprecipitation at antigen excess.

Discussion

Two possibilities seemed feasible at the outset of these studies to explain the presence of two α_1 -antitrypsins in the plasma of rabbits: synthesis of two metabolically unrelated proteins (more precisely, groups of proteins), or post-translational modification of a single product of protein synthesis either before or after its release from the hepatocyte. Differences between the amino-acid compositions of both forms, affecting phenylalanine, aspartic acid and arginine, did not favour the idea of a common gene product modified before secretion; however, the slightly lower apparent molecular weight of the S-form (Koj *et al.*, 1978), taken in conjunction with the inferior reactivity of the S-form with serine proteinases (A. Koj & E. Regöczy, unpublished work), was suggestive of the S-form deriving from

the F-form, perhaps as the result of limited extracellular proteolysis. A post-secretional transformation of this kind should manifest itself in a gradual shift of the injected labelled α_1 -antitrypsin F to the electrophoretic position of the S-form. Assuming the consecutive reactions, $\text{F} \rightarrow \text{S} \rightarrow \text{catabolism}$, with constant k_{12} for the former and k_{23} for the latter, the ratio S/F in the plasma pool at any time may be calculated by the formula:

$$\text{S/F} = [k_{12}/(k_{23} - k_{12})][1 - e^{-(k_{23} - k_{12})\Delta t}]$$

Application of this calculation to the data from the rabbits in Table 3 makes it clear that if the above metabolic scheme prevailed, the fraction of the injected F-form that electrophoresed as S-form should have risen from 8–9% to 14.7% in 5 h. Similarly, the less likely alternative, namely, $\text{S} \rightarrow \text{F} \rightarrow \text{catabolism}$, should have resulted in a rise from 12.6 to 17.2% in the fraction of S-form migrating as F-form over the same period of time. The lack of changes in these orders of magnitude in our experiments suggests that interconversion is not a major pathway in α_1 -antitrypsin metabolism.

From previous analyses (Koj *et al.*, 1978), the F-form is known to contain 49.9 residues of leucine as compared with 48.4 residues in the S-form. Clearly, these values provide no explanation for the unequal ($\text{S} > \text{F}$) specific radioactivities of both forms after the injection of ^{14}C leucine. The data in Table 2 could therefore imply that the precursor leucine (or leucyl-tRNA) pool from which the two rabbit α_1 -antitrypsins are synthesized, is heterogeneous. This could be the case, for example, if the two forms were synthesized by different cells or in different compartments within the same cell. Limitations of the concept of a uniform intracellular amino-acid pool have been emphasized by Christensen (1964) and, indeed, Ilan & Singer (1975) found that, in newt liver, the specific radioactivity of ^3H leucine in nascent peptides from free ribosomes was twice that of peptides from membrane-bound ribosomes. Alternatively, the unequal ^{14}C specific radioactivities of both rabbit α_1 -antitrypsins could be an expression of

different turnover rates. This explanation is favoured by the results of the studies with the iodine-labelled F- and S-proteins.

After the intravenous injection of an iodine-labelled plasma protein, the logarithms of the plasma radioactivities decrease in a characteristic manner as a function of time (Matthews, 1957; McFarlane, 1964). The early curvilinear section of the plasma-radioactivity curve (between 0 h and approx. 50 h in Fig. 2) is due to combined losses of the dose through equilibration with the extravascular pool and catabolism, whereas the straight section (the so-called 'terminal plasma slope') represents catabolic loss alone. Two or more exponential terms can be fitted to the whole plasma curve for the calculation of capillary transfer and catabolic rates by compartmental analysis (Jacquez, 1972). Capillary transfer rates and pool sizes were not computed in the present study for the following reason. Analyses of the hydrolysates of iodinated α_1 -antitrypsins F and S by paper chromatography indicate that a portion of the substituted radioactivity is present as sulphenyl iodide; unlike iodotyrosine, this labelled residue is unstable and its deiodination *in vivo* is complete in 24–30 h after the injection of the protein (E. Regoeczi & M. Bolyos, unpublished work). Loss of radioactivity through this mechanism from the plasma during the equilibration period may be expected to result in overestimation of the capillary transfer rate and extravascular pool (McFarlane, 1964) of α_1 -antitrypsin. In contrast, the rate constant of the terminal plasma slope is not affected by this process. Since the terminal plasma slope and the total-body-radioactivity slope of iodine-labelled proteins parallel each other (Matthews, 1957), the former slope was used to compare the catabolic rates of the F- and S-forms. From the mean half-lives in Table 1, the catabolic rate of the F-form is calculated to be approx. 24% of the total body pool per day, the corresponding value for the S-form being 30%.

In final analysis, rabbit α_1 -antitrypsin emerges from our present and previous studies as a rather complex system of proteins that shows a striking similarity to plasminogen in several mammalian species (Sodetz *et al.*, 1972; Wallén & Wiman, 1972; Siefring & Castellino, 1974). The similarity encompasses the existence of two major electrophoretic forms of plasminogen (by analogy to α_1 -antitrypsins F and S) with several isoenzymes within each form (by analogy to the subcomponents

of the F- and S-forms), their immunological identity, the close, but not identical, amino-acid compositions and molecular weights, differences in the rates of incorporation of [^3H]leucine, different catabolic rates, and the lack of interconvertibility.

The question as to the control of this complex α_1 -antitrypsin system is intriguing. Allelic genes, a common source of microheterogeneity in plasma proteins, is unlikely. In contrast, the existence of two autosomal genes, or two sets of autosomal allelic genes, for α_1 -antitrypsin in the rabbit seems a distinct possibility.

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