Isolation of active subforms of α_2 -macroglobulin

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Anion-exchange chromatography is shown to permit resolution and separation of subforms of the serum glycoprotein α_2 -macroglobulin. The subforms differ dramatically in their stability as judged by differential scanning calorimetry, undergoing thermally induced unfolding at temperatures of 61 and 69°C respectively. In addition, the proteinase-binding stoichiometry of the subforms differs by a factor of 2, with the more- and less-stable forms binding 2 and 1 mol of proteinase per mol of tetramer respectively. The calorimetric stability of the two forms is differentially affected on treatment with neuraminidase, suggesting that the nature of glycosylation may in part account for the observed differences in physical and functional properties.

The glycoprotein α_2 -macroglobulin (α_2 M) is present in human serum at a concentration of 2–4 mg·ml⁻¹ *in vivo* (Starkey & Barrett, 1977; Laurell & Jeppsson, 1975). α_2 M is capable of binding and sequestering endoproteinases of all four major classes (Barrett & Starkey, 1973; Werb *et al.*, 1974). Attendant on proteinase binding at the 'bait' region of the macromolecule (Starkey & Barrett, 1977) a pronounced structural alteration of α_2 M occurs that has been characterized as a condensation of the macroglobulin–proteinase complex (Starkey & Barrett, 1977; Barrett *et al.*, 1979). The bound proteinase does not lose activity, but is shielded from access to macromolecular substrates and inhibitors (Barrett & Starkey, 1973; Sottrup-Jensen *et al.*, 1981). The binding mechanism involves proteolytic cleavage of α_2 M in the bait region; thus only active trypsin is capable of effecting the structural change of α_2 M (Sayers & Barrett, 1980).

Alterations in protein conformation can be detected by changes in parameters characterizing the thermally induced unfolding of a macromolecule employing differential scanning calorimetry (Biltoen & Friere, 1978; Privalov, 1979). This method was previously used to provide comple-

mentary evidence for the proteinase-induced structural alteration of α_2 M (Chlebowski & Williams, 1983). In the case of those studies, evidence was also obtained suggesting that the α_2 M consisted of more than a single form, as deduced from the appearance of multiple transitions in the calorimetric scan. Multiple forms of α_2 M have been previously suggested on the basis of investigations of the proteinase-binding capacity of sera (Topping & Craven, 1979; Topping & Seilman, 1979) and more recently in electron microscopy of the native protein (Schramm & Schramm, 1982). The present paper reports the physical isolation of subforms of α_2 M and a partial characterization of differences in their physical and functional properties.

Materials and methods

α_2 M

α_2 M was prepared from outdated human plasma by using a modification of a previously reported procedure (Swenson & Howard, 1979). This procedure employs a series of poly(ethylene glycol) and (NH₄)₂SO₄ precipitation steps and was followed as described to obtain crude α_2 M. Anion-exchange chromatography employing a biphasic linear NaCl gradient was employed in the final purification step. Samples of the crude protein were loaded on a column (4 cm × 18 cm) of DEAE-cellulose equilibrated with 0.025 M-Tris buffer, pH 8.0. Successive 500 ml gradient elutions were then performed, linearly increasing the NaCl concentration from 0

Abbreviations used: α_2 M, α_2 -macroglobulin; STI, soya-bean trypsin inhibitor; Bz-Arg-OEt, *N*^α-benzoyl-L-arginine ethyl ester; α_2 M-1 and α_2 M-2 refer to the resolved α_2 -macroglobulin species enumerated in the order of their elution from an anion-exchange column; f.p.l.c., fast protein liquid chromatography.

to 0.1M-NaCl and from 0.10M-NaCl to 0.15M-NaCl respectively. When protein samples were purified by f.p.l.c. methods, a preliminary DEAE-cellulose column was run to remove contaminants, a linear 0–0.15M-NaCl gradient in 0.025M-Tris, pH8.0, being employed. Column dimensions and total elution buffer were as described above.

Purification of α_2 M on the Pharmacia f.p.l.c. system was performed on the Mono-Q ion-exchange column using a biphasic linear salt gradient. After centrifugation to remove particulate matter, 0.5–1.0 ml of sample was loaded on the column, which had been equilibrated with 0.025M-Tris buffer, pH8.0. The column was washed with buffer at a flow rate of 1 ml/min for 5 min. Successive linear gradients of 0–0.2M-NaCl and 0.20–0.60M-NaCl were run with elution volumes of 7 ml and 25 ml respectively. Protein elution was monitored at 280 nm.

The concentration of samples of α_2 M was determined on the basis of an absorption coefficient ($A_{280}^{1\%}$) of 9.1 (Dunn & Spiro, 1967). Calculations of the molar concentration of α_2 M assumed an M_r of 725 000 (Jones *et al.*, 1972).

Immunochemical assay of α_2 M

Ouchterlony immunodiffusion of putative α_2 M against anti- α_2 M serum was performed as previously described (Barrett, 1974).

Materials

Trypsin was obtained as a freeze-dried powder from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. On the basis of the weight ratio of STI required to abolish the esterase activity of trypsin against Bz-Arg-OEt, the trypsin employed in these experiments was approx. 50% active. Calculations of molar concentrations of trypsin present in the experiments described are based on the amount of active trypsin present. Neuraminidase cross-linked to agarose beads, STI and Bz-Arg-OEt were also obtained from Sigma. Anti- α_2 M serum was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). All buffers and other chemicals were of reagent grade.

Determination of the proteolytic activity of α_2 M-trypsin complex

Portions of α_2 M in 0.025M-Tris/0.02M-CaCl₂, pH8.0, were incubated at room temperature for 20 min with portions of a stock trypsin solution prepared in the same buffer. The concentration of α_2 M was typically of the order of 10 μ M with the trypsin concentration adjusted to yield the desired stoichiometry. Stock trypsin solutions were freshly prepared for each series of determinations and titrated with STI to determine the concentration of

active proteinase present. After addition of α_2 M and trypsin as described above, STI was added in excess and the solution incubated for a further 20 min. At this point the reaction volume of α_2 M, trypsin and STI was 100 μ l. A 1 ml portion of Bz-Arg-OEt at a concentration of 0.5 mM was added, the mixture was transferred to a 1 ml cuvette and the esterase activity of the solution monitored at 253 nm as previously described (Schwert & Takenaka, 1955), with an IBM 9420 recording spectrophotometer.

Neuraminidase treatment of α_2 M

Portions of α_2 M containing approx. 2 mg of protein in 0.025M-Tris buffer, pH8.0, were incubated at room temperature with 0.2 ml of a neuraminidase-agarose-bead suspension. The Sigma type VI-A neuraminidase covalently cross-linked to beaded agarose used had an activity of 0.6 unit·ml⁻¹. The suspension was rotated continuously overnight. Agarose beads were removed by centrifugation before calorimetric runs.

Calorimetric experiments

Calorimetric experiments were conducted in an MC-1 differential scanning calorimeter (Microcal, Amherst, MA, U.S.A.). Sample and reference solutions of 1 ml volume were employed with a scan rate of 1°C/min. Transition enthalpies were determined by comparison of the area under the transition curve with standard areas generated by an applied calibration voltage. Areas were measured by cutting and weighing the transition curves in accordance with methods previously described (Privalov & Khechinashvili, 1974). Transition temperatures are reported for the point of maximum amplitude for a given peak.

Results

Isolation of α_2 M subforms

The procedure adopted by this laboratory for the isolation of α_2 M from serum follows that previously reported (Swenson & Howard, 1979) in its initial stages. Although the initial steps in this protocol are common to most investigations of α_2 M, a variety of methods have been employed as the ultimate chromatographic procedure (Swenson & Howard, 1979; Virca *et al.*, 1978; Maxfield *et al.*, 1981; Sottrup-Jensen *et al.*, 1980). Anion-exchange chromatography was adopted as the method of choice; however, instead of a step gradient of NaCl as used previously (Swenson & Howard, 1979) a linear salt gradient was employed. As Fig. 1(a) shows, the peak eluted in the expected position of α_2 M is markedly asymmetric, suggesting an underlying heterogeneity. Fractions through the peak all contain α_2 M as judged by the positive

Fig. 1. Anion-exchange chromatography of α_2 M. Samples were prepared in 0.025M-Tris buffer, pH8.0, as described in the Materials and methods section. (a) Initial chromatography of crude α_2 M employing a single linear gradient of 0–0.25M-NaCl. Partially resolved fractions of α_2 M subforms were pooled as indicated by arrows, dialysed to remove salt, concentrated and rechromatographed as shown in (b) and (c) (corresponding to chromatography of the early- and late-to-be-eluted fractions respectively. A biphasic salt gradient as described in the Materials and methods section was employed; elution of the second portion of the gradient is shown in (b) and (c).

immunoprecipitation reaction observed when portions were exposed to anti- α_2 M serum in an Ouchterlony double-diffusion experiment. Fractions from this column were pooled as indicated and rechromatographed, yielding resolved peaks which were eluted at NaCl concentrations differing by approx. 5mM (Figs. 1b and 1c). Material eluted as the major peak from each column retained the immunochemical response to anti- α_2 M serum.

We have previously reported the apparent heterogeneity of preparations of α_2 M on the basis of multiple transitions observed in differential scanning calorimetric assays (Chlebowski & Williams, 1983). Calorimetric scans of material eluted at increasing NaCl concentrations (designated as ' α_2 M-1' and ' α_2 M-2' respectively) are shown in

Fig. 2. Calorimetric scans of α_2 M subforms resolved by DEAE-cellulose chromatography

Samples were prepared in 0.025M-Tris, pH8.0; α_2 M concentration was 2mg/ml. Samples resolved by DEAE-cellulose chromatography and corresponding to α_2 M-1 (a) and α_2 M-2 (b) were evaluated calorimetrically as described in the Materials and methods section. $[C_p]$ is the differential heat capacity between the sample and reference cells.

Fig. 2. In each case a single principal transition is observed, differing markedly in both transition temperature (61 and 69°C respectively) and apparent transition enthalpy (4.8mJ·mg⁻¹ and 12.1mJ·mg⁻¹). The results shown were obtained with equivalent-absorbance amounts of protein, based on the A_{280} and the reported absorption coefficient of the protein. Since both the transition temperature and transition enthalpy of α_2 M-2 are significantly greater than that of α_2 M-1, it would appear that the former species is of greater stability.

The resolution afforded by anion-exchange chromatography on DEAE-cellulose, although satisfactory, proved difficult to reproduce precisely. To improve the isolation method, crudely purified samples of α_2 M were subjected to anion-exchange chromatography employing a Mono-Q column in the Pharmacia f.p.l.c. system. The elution profile, monitored at 280nm, is shown in Fig. 3 for a portion of the gradient. Two major peak fractions were observed, although it is apparent that a further heterogeneity exists within these fractions. As shown in Fig. 4, material eluted in these separate peaks corresponds to α_2 M-1 and α_2 M-2 as judged by calorimetric analysis. The crude material displays two transitions (Fig. 4a), which are separated into homogeneous components after f.p.l.c. chromatography. Material purified in this fashion corresponds to that prepared by cellulose chromatography.

Proteinase binding by α_2 M subforms

Although the resolved components appear to be α_2 M species by immunological criteria (see above), this qualitative test could in principle be compro-

Fig. 3. *F.p.l.c. chromatography and assay of trypsin binding by α_2M subforms*

A sample of α_2M in 0.025 M-Tris, pH 7.5, was chromatographed on a Mono Q anion-exchange column as described in the Materials and methods section. The section of the elution profile, monitored at 280 nm (—), corresponding to the appearance of α_2M , is displayed. Fractions (0.25 ml) were collected and portions were assayed for trypsin-binding capacity as described in the Materials and methods section, the Bz-Arg-OEt esterase activity of trypsin in the presence of excess STI at 253 nm (○) being measured. The activity profile is superimposed on the A_{280} profile recorded directly.

Fig. 4. *Calorimetric scans of crude α_2M and f.p.l.c.-resolved α_2M subforms*

Samples were prepared in 0.025 M-Tris, pH 8.0; the concentration of α_2M was (a) 1.8 mg/ml, (b) 1.0 mg/ml and (c) 2.35 mg/ml. Samples utilized in (b) and (c) correspond to early- and late-to-be-eluted peaks resolved on f.p.l.c. chromatography (cf. Fig. 3); the calorimetric trace of the unresolved mixture before chromatography is shown in (a) (for experimental details, see the Materials and methods section). $[C_p]$ is the differential heat capacity between the sample and reference cells.

mised by minor cross-contamination. In addition, since the commercially available antiserum is raised against what may be a mixture of components, this identification is not unequivocal. To resolve these possible ambiguities, the proteinase-binding capacity of the subforms was examined. Proteinase (e.g. trypsin) bound to α_2M retains activity, but is not accessible to macromolecular substrates or inhibitors. Thus although trypsin bound to α_2M hydrolyses Bz-Arg-OEt, this activity is not abolished by the presence of STI. In contrast, STI binds to free trypsin and abolishes activity. Fig. 3 shows the Bz-Arg-OEt esterase activity of trypsin added to fractions of putative α_2M in the presence of STI. Two resolved peaks of activity were observed, confirming that the separated components in fact represent active α_2M species.

To determine if differences in the proteinase-binding capacity of the two resolved forms of α_2M were present, equivalent amounts (as judged by A_{280}) of these species were titrated with trypsin (Fig. 5). Trypsin binding was assayed as the activity against Bz-Arg-OEt in the presence of STI. The specific activity of bound trypsin against Bz-Arg-OEt, as judged by the initial slope of the titration, appears identical for proteinase bound to either α_2M -1 or α_2M -2. However, the stoichiometry of trypsin binding differs for the two forms; titration of α_2M -1 is complete at a trypsin/protein ratio of 1.1, whereas for α_2M -2 the titration is not complete until a trypsin/protein ratio of 2.2 is achieved. This 2-fold difference in apparent proteinase-binding capacity has been repeatedly observed in separate preparations of α_2M subforms.

Fig. 5. Determination of trypsin-binding stoichiometry for subforms of α_2M

Portions of a stock solution (0.3 mM) of active trypsin were added to equivalent-absorbance amounts of isolated subforms of α_2M . After the addition of excess STI, the remaining trypsin activity against Bz-Arg-OEt was monitored at 253 nm (for details, see the Materials and methods section). Equal concentrations of α_2M -1 (○) and α_2M -2 (●) were compared.

Effect of neuraminidase on α_2M subforms

As isolated, α_2M has been reported to display a pronounced microheterogeneity with respect to carbohydrate content, as deduced from the appearance of forms of distinguishable pI, a heterogeneity reportedly eliminated on treatment of α_2M with neuraminidase (Frenoy & Bourrillon, 1974). The effect of neuraminidase treatment on the stability of the α_2M subforms was evaluated calorimetrically as shown in Fig. 6. The discrete transitions of α_2M -1 and α_2M -2 (Fig. 6a) are merged into a broadened transition after exposure to neuraminidase (Fig. 6b). When a more dilute sample was examined, it is apparent that the enzymic treatment did not, in fact, result in conversion of these species into a single form. The transition corresponding to α_2M -1 is essentially unchanged in transition temperature, whereas that corresponding to α_2M -2 is shifted from 69 to 66°C.

Discussion

Reported procedures for the final purification step in the preparation of α_2M have employed a variety of chromatographic procedures, including gel filtration (Maxfield *et al.*, 1981), step-gradient salt elution from an anion-exchange matrix (Swenson & Howard, 1979) dye affinity chromatography (Virca *et al.*, 1978) and metal-ion affinity chromatography (Sottrup-Jensen *et al.*, 1980). Resolution of subforms of α_2M is not achieved in these procedures. However, given the low resolving

Fig. 6. Effect of neuraminidase treatment on α_2M as monitored by calorimetry

Samples were prepared in 0.025 M-Tris, pH 8.0, at an α_2M concentration of 2 mg/ml. Calorimetric scans of untreated and neuraminidase-treated samples are shown in (a) and (b) respectively. The trace displayed in (c) is that for an approx. 5-fold dilution of sample (b). Experimental details are given in the Materials and methods section. $[C_p]$ is the differential heat capacity between the sample and reference cells.

power of these methods and the demonstrated difficulty in separating these forms described herein, it is reasonable to suggest that the presence of these species has escaped detection. In addition, we have observed variability in the distribution of a given preparation of α_2M from different serum sources. This would contribute to difficulty in the observation of these species.

The observed difference in proteinase-binding capacity of α_2M -1 and α_2M -2 may in part explain deviations from precise integral values obtained for the proteinase-binding capacity of α_2M in previous reports. For example, on the basis of the calorimetric profile shown in Fig. 4, the distribution of α_2M -1 and α_2M -2 in the crude mixture is 55 and 45% of the sample respectively. On the basis of the titrimetric data of Fig. 5, the proteinase-binding capacity of the sample is 1.6 mol of trypsin per mol of α_2M . As mentioned above, the distribution of the subforms appears variable. In addition, systematic elimination of, for example, leading fractions of an unresolved peak of α_2M

activity would enrich a given sample in α_2 M-2. It is therefore conceivable that the apparent observation of a trypsin-binding stoichiometry of 2 mol per mol of α_2 M could mask the presence of a significant concentration of α_2 M-1.

The calorimetric assay of neuraminidase-treated α_2 M indicates that the presence of subforms is not simply due to variation in the pattern of oligosaccharide substitution. Glycosylation does, however, clearly affect the stability of α_2 M-2, reducing the transition temperature by 3°C. Although glycosylation has been suggested as a mechanism for altering macromolecular stability (Sharon & Lis, 1981), this has not been well documented. The persistence of discernible subforms is not incompatible with the prior observation of the elimination of variants on neuraminidase treatment (Frenoy & Bourrillon, 1974). The earlier study employed isoelectric focusing as a detection method, which is not necessarily sensitive to the presence of conformational variants. The scanning calorimetry method is particularly sensitive to alterations of macromolecular conformation that may escape detection by other criteria (Roberts & Chlebowski, 1984a,b). Although the calorimetry results would suggest that these species are conformationally distinct, in accord with the observation of α_2 M forms of distinguishable shape by electron microscopy (Schramm & Schramm, 1982), it is premature to attempt to correlate these results. Further work is required to characterize more comprehensively the physical and functional differences between these forms.

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