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DIFFERENTIAL ISOELECTRIC FOCUSING PROPERTIES OF CRUDE AND PURIFIED HUMAN α_2-MACROGLOBULIN AND α_2-MACROGLOBULIN—PROTEINASE COMPLEXES

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SUMMARY

The isoelectric focusing (IEF) properties of human α_2-macroglobulin (α_2M) and α_2M—proteinase complexes from crude and partially purified preparations were studied by column IEF. The average isoelectric point (pI) of the major form was 6.5 for α_2M from crude plasma but was 5.3 for purified samples. Following preincubation with either trypsin, chymotrypsin or pancreatic elastase the crude α_2M—proteinase complexes displayed p*I* values ranging from 5.3 to 6.1 and the purified α_2M—proteinase complexes ranged from pH 6.0 to 6.1. A comparison of recoveries for focused crude or purified α_2M and trypsin-preincubated α_2M indicated enhanced recovery for the trypsin-preincubated samples suggesting that the binding of the proteinase enhances the stability of α_2M. α_2M thus displays column IEF properties which appear to be dependent upon the state of purity of the molecule. These findings are of particular significance to investigators concerned with using expressions of altered α_2M electrophoretic patterns for clinical diagnostic purposes in such diseases as multiple sclerosis, diabetes and cystic fibrosis.

INTRODUCTION

The human serum glycoprotein α_2-macroglobulin (α_2M) is a relatively non-specific proteinase inhibitor which has been found to inhibit to varying degrees the activated forms of most of the known endopeptidases (for reviews see refs. 1 and 2). The broad specificity displayed by α_2M toward proteinases appears

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central to its proposed regulatory role in a number of important physiological processes including blood coagulation, fibrinolysis, and inflammatory processes [3]. In addition, the inhibitory effect of α_2M on several in vitro, proteinase-mediated immunological processes has suggested a possible regulatory role for α_2M in various immune responses [4].

Several investigators have demonstrated by isoelectric focusing (IEF) that α_2M displays multiple forms but the reported number of forms and their respective isoelectric points differs significantly. The isoelectric point (p*I*) of the principal form of α_2M ranges from 4.2 [5] to 5.4 [6,7]. Frénoy and Bourillon [8] resolved four forms of α_2M with p*I* values ranging from 4.97 to 5.50. Rosén et al. [5] reported seven forms with p*I* values ranging from 4.1 to 4.9. Significantly different separations of α_2M from α_2M—proteinase complexes by IEF have also been obtained depending upon the system employed [9].

Since knowledge of the electrophoretic forms of α_2M is particularly important for studying possible abnormalities of α_2M in diseases such as cystic fibrosis [10], diabetes [11] and multiple sclerosis [12], the present study has utilized column IEF to compare the number, p*I* values and relative amounts of the forms of α_2M from crude and purified samples. IEF was employed for our studies on α_2M because this high-resolution technique has proven to be very useful for analytical and diagnostic purposes as well as for preparatively separating proteins with different pI values [13]. Liquid media were used to minimize the amount of molecular sieving which can occur for very large molecules like α_2M [1,2] in solid media such as polyacrylamide [7,9] and, to a lesser extent, agarose [5]. Our findings suggest that the IEF profiles of α_2M differ in crude and purified states and that the binding of proteinases enhances the stability of α_2M.

MATERIALS AND METHODS

General

Sources of chemicals and other materials were as follows: α_1-antitrypsin (human), N-benzoyl-L-tyrosine ethyl ester, α-chymotrypsin (type II), elastase (porcine, type I, 2× crystallized suspension), N-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanalide, trypsin (bovine, type III), trypsin inhibitor (soybean, type I-S), trypsin inhibitor (turkey eggwhite, type II-T) from Sigma (St. Louis, MO, U.S.A.); α_2-macroglobulin (human, rabbit antiserum) and tosyl-L-arginine methyl ester hydrochloride from Calbiochem-Behring (La Jolla, CA, U.S.A.); *p*-toluene sulfonyl-L-arginine from ICN K and K Labs. (Plainview, NY, U.S.A.).

Ampholytes were from LKB Instruments (Rockville, MD, U.S.A.) and from Bio-Rad Labs. (Richmond, CA, U.S.A.). Blue Sepharose Cl-6B was from Pharmacia (Piscataway, NJ, U.S.A.). Agar double-immunodiffusion plates were from Hyland Diagnostics (Deerfield, IL, U.S.A.).

Blood was obtained from healthy human donors and plasma prepared according to the method of Harpel [14]. Plasma was dialyzed 18 h at 0—4°C against 0.05 *M* Tris—HCl buffer, pH 8.0, containing 0.02% sodium azide and stored at 2°C or at −20°C.

Assays of proteinase-binding activity of α_2M

All assay procedures were based upon the hydrolysis of a low-molecular weight substrate by α_2M-bound proteinase in the presence of an excess of an inhibitor specific for the unbound proteinase and each assay varied by less than 10%.

The assay for α_2M-bound trypsin is based upon the method of Ganrot [15]. In each duplicate tube, 10—40 μl of plasma were brought to a final volume of 50 μl with 1 m*M* hydrochloric acid, preincubated at 22°C for 1 min with 10 μl of 10 mg/ml trypsin, and preincubated 1 min longer with 40 μl of 10 mg/ml soybean trypsin inhibitor. The reaction is initiated by mixing a 10-μl sample aliquot with 1 ml of freshly prepared 1 m*M* tosyl-L-arginine methyl ester in 0.1 *M* Tris—HCl, pH 8.0, containing 10 m*M* calcium chloride. The increase in absorbance at 247 nm is recorded at 30-sec intervals for 3—4 min at 22°C. A unit of activity is defined as a change in absorbance of 0.01 per min at 247 nm which corresponds to the hydrolysis of 5 nmoles of substrate. The assay was linear with respect to amount of plasma (10—50 μl) and time (0.5—10.0 min).

The assay for α_2M-bound chymotrypsin was developed from the method of Walsh and Wilcox [16] using benzoyl tyrosine ethyl ester as substrate. In each duplicate tube, 10—40 μl of plasma is brought to a final volume of 50 μl with 0.1 *M* Tris—HCl buffer, pH 7.8, containing 0.1 *M* calcium chloride, preincubated at 22°C for 1 min with 10 μl of 10 mg/ml chymotrypsin in 1 m*M* hydrochloric acid, and preincubated 1 min longer with 40 μl of 10 mg/ml turkey eggwhite trypsin inhibitor. The reaction is initiated by mixing a 20-μl aliquot of sample with 0.50 ml of 1 m*M* substrate in 50% (w/w) aqueous methanol brought to 1 ml final volume with the same Tris buffer. The increase in absorbance at 253 nm is recorded at 30-sec intervals for 3—4 min at 22°C. A unit of activity is defined as a change in absorbance of 0.01 per min at 253 nm. The assay was linear with respect to amount of plasma (10—80 μl) and time (0.5—10.0 min).

The colorimetric assay of α_2M-bound elastase activity was developed from the method of McGillivray et al. [17] using N-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanalide as substrate. In each duplicate tube, a 5-μl aliquot of plasma in 1120 μl of 0.2 *M* Tris—HCl, pH 8.0, is preincubated for 5 min at 37°C in a shaking water bath with 15 μl of elastase suspension, diluted 1:10 in the same Tris buffer. This solution is mixed with 60 μl of 10 mg/ml α_1-antitrypsin and preincubated 5 min longer at 37°C. The reaction is initiated by mixing the entire sample aliquot with 150 μl of 10 m*M* substrate in the Tris buffer, terminated after 20 min with concentrated glacial acetic acid, and the absorbance at 410 nm determined. A unit of activity is defined as an absorbance change of 0.01 per min at 410 nm. The assay was linear with respect to amount of plasma (1—10 μl) and time (5—30 min).

All absorbances were read on a Beckman Model 24 spectrophotometer. Assay of α_2M activity after IEF was measured as for plasma samples, except that aliquot volumes were adjusted to be in the linear range of the assay.

Purification of α_2M

α_2M was partially purified by a procedure based upon that of Virca et al.

[18]. Human plasma (5 ml), stored at 2°C for 2—5 days after collection, was chromatographed at 22°C on a bed (1.0 × 100 cm) of Cibicron Blue Sepharose using 0.05 *M* Tris—HCl, pH 8.0, containing 0.02% sodium azide, at a flow-rate of 5—7 ml/h. Fractions were stored at 2°C as collected. α_2M was detected by double immunodiffusion against α_2M antisera to be in fractions from the void volume. All fractions containing α_2M were combined and concentrated from about 18 ml to 0.5 ml in a collodion bag apparatus using a collodion bag (Schleicher and Schuell, Keene, NH, U.S.A.) with 25,000 MW cut off. Concentrated α_2M samples were stored at 2°C. The purified α_2M samples were determined by SDS-polyacrylamide gel electrophoresis, using the method of Laemmli [19], to contain only minor contamination (less than 5%).

Isoelectric focusing

IEF was performed at 2—4°C in a pH 3.5—10 gradient essentially as previously described [20] using a 40-ml column apparatus. Ampholytes (2%) (pH 3.5—10) were used in a gradient of 0—67% sucrose. The starting amperage was 2.5—3.0 mA at a constant voltage of 600 V. Focusing was conducted for 15 h or until the amperage became constant at 0.5 mA.

Samples of plasma or purified α_2M of comparable trypsin-binding activity (about 2000 units) were focused in all experiments (except where indicated due to limited materials). Samples of proteinase-bound α_2M were prepared for focusing by preincubating an excess of proteinase with plasma samples at 37°C or with purified α_2M samples at 22°C for 30 min. The migration of α_2M in the gradient was monitored by proteinase-binding activity and by use of agar double-immunodiffusion plates in which 5 μl of IEF aliquots were diffused against 5 μl of α_2M antiserum.

Recovery of α_2M following IEF was estimated by measuring the α_2M trypsin-binding activity of pooled focusing fractions, pH 4.5—8.0, dialyzed against 0.05 *M* Tris—HCl, pH 8.0 at 2°C for 12 h. Since it was found that recoveries were lower for small amounts of focused α_2 M activity (< 1000 units), all reported recovery data were based upon IEF of 2000 units of α_2 M trypsin-binding activity.

RESULTS

Fig. 1a depicts the results of IEF of human plasma assayed for α_2M trypsin-binding activity. The profile of activity is representative of profiles obtained for six different plasma samples. Examination of these profiles indicated that a small percentage of the total integrated, recovered activity (6—27%) focused between pH 4.5 and 6.0, and the main isoelectric form in this region had a p*I* value of 5.4—5.7. The greatest percentage of the total integrated, recovered activity for all six samples was from pH 6.0 to 8.0 (73—94%). The major isoelectric form had a p*I* centered around 6.5 and ranging from 6.2 to 6.8.

A comparison of the proteinase-binding activity in Fig. 1a (trypsin) with Fig. 1b (chymotrypsin) and Fig. 1c (elastase) demonstrates that similar focusing profiles were obtained (with regard to the presence of a single main peak above pH 6.0) when α_2M from the same donor was assayed with each of these three proteinases. The total recovery of α_2M trypsin-binding activity was 26—58% of the activity applied to the IEF column (average recovery = 44%).

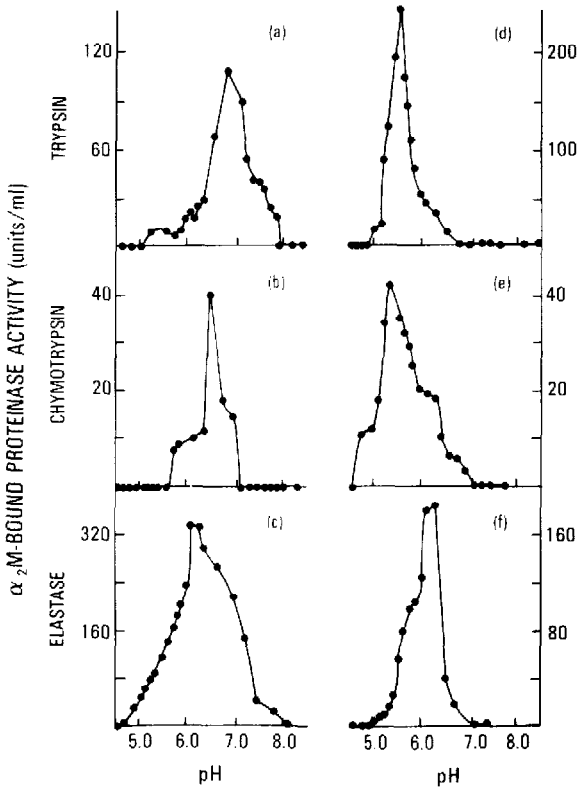

Fig. 1. IEF of α_2M in crude plasma before (a—c) and after (d—f) preincubation with various proteinases. (a, d) Trypsin-binding activity; (b, e) chymotrypsin-binding activity; (c, f) elastase-binding activity. Samples of plasma equivalent to 2000 units of trypsin-binding activity were focused in (a—e) and 1000 units were focused in (f). See Materials and methods section for details.

Fig. 1d—f (each profile representative of three different plasma samples) depicts the results of IEF of plasma samples preincubated prior to focusing with saturating amounts of trypsin (Fig. 1d), chymotrypsin (Fig. 1e) or elastase (Fig. 1f). For the trypsin- and chymotrypsin-preincubated samples, the α_2M-bound proteinase focused to a lower p*I* value relative to the nonpreincubated sample (Fig. 1a and b). For α_2M-bound trypsin the p*I* value of the principal isoelectric form ranged from 5.5 to 5.7 and for α_2M-bound chymotrypsin from 5.3 to 5.7. Under saturating conditions the α_2M-preincubated elastase displayed no appreciable shift in p*I* to a lower pH (Fig. 1f). However, under subsaturating conditions, the α_2M-bound elastase displayed a profile similar to that seen for the other α_2M-bound proteinases studied (data not shown).

In contrast to the recovery of α_2M obtained for IEF of plasma, the recovery of α_2M for IEF of proteinase-preincubated plasmas was substantially enhanced. The typical total recovery of α_2M-bound trypsin activity was 61—74% (average recovery = 70%).

In Fig. 2 are shown the results of IEF of α_2M and α_2M—proteinase complexes employing purified α_2M samples. A comparison of the α_2M profiles for focused

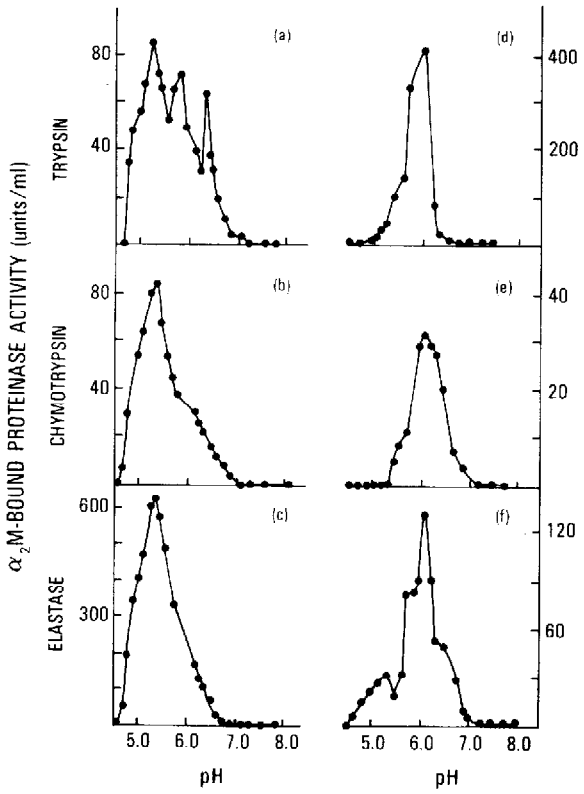

Fig. 2. IEF of purified α_2M before (a—c) and after (d—f) preincubation with various proteinases. (a, d) Trypsin-binding activity; (b, e) chymotrypsin-binding activity; (c, f) elastase-binding activity. Samples of α_2M equivalent to 2000 units of trypsin-binding activity were focused in (a—d) and 800 units were focused in (e) and (f). See Materials and methods section for details.

plasma in Fig. 1 with those for purified α_2M in Fig. 2 indicates that the p*I* values of the α_2M isoelectric forms are quite different. The principal form of α_2M from purified samples had a p*I* value of about 5.3 when assayed with either trypsin, chymotrypsin or elastase (Fig. 2a, b and c, respectively). Better resolution of minor forms was obtained using the trypsin assay and forms with p*I* values at 5.8 and 6.4 were also resolved.

For purified α_2M samples preincubated with either trypsin, chymotrypsin or elastase, the principal isoelectric form for each α_2M—proteinase complex displayed a neutral shift in p*I* value to pH 6.0—6.1 (Fig. 2d, e and f, respectively), when compared to the corresponding nonpreincubated sample.

The recovery of α_2M activity following IEF of purified α_2M samples was similar to that seen for IEF of plasma. The typical recovery of α_2M was 56% for the uncomplexed α_2M and essentially full recovery was obtained for the trypsin-preincubated α_2M.

Throughout these studies double immunodiffusion using α_2M antisera was employed to determine the distribution of α_2M antigen in samples of focused plasma or purified α_2M. In each experiment the presence of proteinase-binding

activity always coincided with the distribution of antigenically cross-reactive material.

Several other findings relative to the IEF studies were noteworthy. No endogenous trypsin, chymotrypsin or elastase activity was detected in samples of plasma or focused plasma. The focusing profiles were very reproducible regardless of the duration of focusing (15—36 h), the amount of α_2M focused (1550—3000 units of trypsin-binding activity), the supplier of ampholytes (LKB or Bio-Rad), or the previous history of plasma samples (fresh or frozen for 1—12 weeks). For comparable studies, similar recoveries of α_2M were obtained for each proteinase employed. However, recoveries were not as good when low levels of α_2M activity (< 1000 units) were focused.

DISCUSSION

Column IEF of human α_2M in crude and purified states demonstrated that both α_2M and α_2M—proteinase complexes displayed markedly different focusing properties depending upon the degree of purity of the α_2M. For purified samples, the p*I* value of the major α_2M form was 5.3 which is in close agreement with previous findings for column IEF [6,8,21] and polyacrylamide gel IEF [7,22]. The form we observed at pH 5.8 appears to correspond to the secondary form at pH 5.5 observed by Frénoy and Bourrillon [8].

In agreement with previous studies employing polyacrylamide gel IEF [9,22], a neutral shift in p*I* was observed for purified α_2M samples preincubated with proteinases prior to IEF. However, we were unable to confirm the observation of Barret et al. [9] using column IEF that purified α_2M and α_2M—proteinase complexes do not differ appreciably in p*I*.

In contrast to the close agreement seen in many IEF studies employing purified α_2M samples, widely differing results have been obtained for IEF of crude α_2M. Rosén et al. [5] employed agarose-crossed immunoelectrofocusing of whole serum to resolve α_2M into seven peaks with p*I* values ranging from 4.1 to 4.9. The main form had an apparent p*I* of 4.2. In contrast, Ohlsson and Skude [23] identified only one α_2M peak with a p*I* value of 5.0 by employing two-dimensional polyacrylamide gel IEF. In the present study, the main α_2M form focused to a p*I* value ranging from 6.2 to 6.8 when monitored by trypsin-binding activity and double immunodiffusion against α_2M antisera. In addition, preincubation of plasma with trypsin or chymotrypsin prior to IEF elicited an acid shift to p*I* values ranging from 5.5 to 5.7 for trypsin and from 5.3 to 5.7 for chymotrypsin. We thus did not observe the consistent basic shift in p*I* value to pH 6.0 for α_2M—proteinase complexes as observed by Ohlsson and Skude [23]. Taken together, these diverse findings suggest that there may be system-related factors [24] as well as unidentified interactions of α_2M with other molecules in plasma which are causing the different observed electrophoretic migrations of crude α_2M.

A number of studies we performed supports the validity of the isoelectric focusing patterns we observed in the crude state. The focusing profiles for both α_2M and α_2M—proteinase complexes were very reproducible regardless of the duration of focusing, the amount of plasma focused, the brand of ampholytes employed, or the previous history of the plasma. In addition, the substantial

recoveries obtained for trypsin-preincubated plasma samples (61—74%) indicate that the focusing profiles for crude α_2M are representative of the majority of the examined proteinase-binding activity. The distinct differences in the focusing profiles for nonpreincubated α_2M (Fig. 1a, b) and α_2M preincubated with trypsin or chymotrypsin (Fig. 1d, e) suggest that the migration of non-preincubated α_2M to p*I* values more neutral than previously reported [5,23] was not due to association of α_2M with proteinases. This is supported by the absence of endogenous trypsin, chymotrypsin or elastase activity in either plasma or focused plasma.

A systematic examination of IEF recoveries for α_2M trypsin-binding activity demonstrated similar recoveries for crude and purified α_2M samples indicating no loss of α_2M stability to IEF due to purification. Consistently enhanced recoveries were observed for both crude and purified trypsin-preincubated samples which suggests that formation of the α_2M-proteinase complex enhances the in vitro stability of α_2M.

The present column IEF study indicates that the p*I* values and focusing properties of α_2M and α_2M-proteinase complexes appear dependent upon the state of purity of the molecule. The present findings are of particular significance to investigators concerned with using expressions of altered α_2M electrophoretic patterns for clinical diagnostic purposes. In addition, our findings indicate the general need for caution in characterizing the isoelectric forms of other macromolecules which may also display variable IEF properties depending upon their state of purity.

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