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Unique Human Glycoprotein, α_1-Microglycoprotein: Isolation from the Urine of a Cancer Patient and Its Characterization†

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ABSTRACT: A human glycoprotein was isolated from the urine of a patient with plasma cell leukemia. It appears pure and homogeneous when examined by immunoelectrophoresis, sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis, gel filtration in 6 M guanidine hydrochloride (Gdn·HCl), and NH_2-terminal amino acid sequence analysis. It has a brown color due to a tightly (most likely covalently) bound chromophore group(s) and migrates to the α_1 region in immunoelectrophoresis. A molecular weight (mol wt) of 27 000 was obtained for the glycoprotein by gel filtration in 6 M Gdn·HCl. Its approximate mol wt determined by $NaDodSO_4$-polyacrylamide gel electrophoresis is 29 000 on 5% and 7.5% and 10% gels. Amino acid and hexosamine analyses showed that it is a glycoprotein and indicated that it contains four half-cystine residues per molecule. Based on the above observations we designated it "α_1-microglycoprotein" (α_1-MGP). Isoelectric focusing of α_1-MGP showed a significant charge heterogeneity, although only a single NH_2-terminal amino acid sequence was obtained for α_1-MGP, i.e., Gly-Pro-Val-Pro-( )-Pro-Pro-Asx-Asx-Ile-Glx-Val-Glx-Glx-Asx-Phe-Phe-Ile-(Ser or Ala)-Arg. The α_1-MGP was found in significant concentrations in the urine of many patients with neoplastic diseases.

We have previously reported the results of our study of several immunoglobulin-related proteins in the urine of a patient TSCH with plasma cell leukemia (Seon et al., 1977a; Seon & Pressman, 1977). The amounts of these proteins in the patient paralleled the clinical manifestations during the course of illness (Gailani et al., 1977a).

In the present study we have isolated a unique glycoprotein, α_1-microglycoprotein,[1] from the urine of the same patient TSCH by a relatively simple procedure and have characterized its chemical, physicochemical, and immunological properties. Recently several groups reported similar proteins, although there are some discrepancies in the properties of these proteins (Tejler & Grubb, 1976; Frangione et al., 1976; Svensson & Ravnskov, 1976; Ekström & Berggård, 1977). None of these reported proteins were obtained from cancer patients and the procedures used for their isolation were significantly different from that used here.

Experimental Section

Materials

Urine. The urine specimens were collected from a single patient TSCH with relapsed plasma cell leukemia during 24-h periods. During the collection, NaN_3 was always present at concentrations higher than 0.1%.

Antisera. Rabbit or goat antisera specific to each of the immunoglobulin component chains, i.e., κ, λ, γ, α, μ, δ and ϵ chains, were prepared and characterized in our laboratory (Seon & Pressman, 1974; Seon et al., 1977a). The antiserum specific to β_2m[2] (Seon & Pressman, 1977) and antisera to normal human serum and normal human urine were also prepared and characterized in our laboratory. Antisera specific to carcinoembryonic antigen and urinary kallikrein were kindly provided by Dr. S. Harvey of Roswell Park Memorial Institute and Dr. O. Ole-MoiYoi of Harvard Medical School, respectively. The antisera specific to the following human serum components were purchased from Behring Diagnostics (through American Hoechst Corp., N.J.): α_1-acid glycoprotein, albumin, α_1-antichymotrypsin, α_1-antitrypsin, antithrombin III, C-reactive protein, C1q component, C1s component, C1s inhibitor, C3/C3c, C3 activator, ceruloplasmin,

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[1] The glycoprotein we purified was designated as α_1-microglycoprotein since it migrates to the α_1 region in immunoelectrophoresis and is a small glycoprotein with molecular weight of 27 000.

[2] Abbreviations used: α_1-MGP, α_1-microglycoprotein; β_2m, β_2-microglobulin; Dnp, dinitrophenyl; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; mol wt, molecular weight.

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factor XIII subunit A, factor XIII subunit S, ferritin, fibrinogen, Gc-globulin, α_1B-glycoprotein, α_1T-glycoprotein, α_2AP-glycoprotein, α_2HS-glycoprotein, Zn-α_2-glycoprotein, β_2-glycoprotein I, β_2-glycoprotein III, haptoglobin, hemopexin, inter-α-trypsin inhibitor, α_1-lipoprotein, β-lipoprotein, lysozyme, α_2-macroglobulin, plasminogen, prealbumin, prothrombin, retinol binding protein, and transferrin.

Proteins, Synthetic Peptides, and Other Chemicals. Human monoclonal IgG, Bence-Jones protein, and β_2m were prepared as described previously (Seon & Pressman, 1974; Seon et al., 1977a). Lysozyme and trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone were purchased from Worthington Biochemical Corp. Sperm whale apomyoglobin was from Beckman Instruments, Inc. The following proteins and chemicals were purchased from Sigma Chemical Co.: bovine serum albumin, bovine liver L-glutamic dehydrogenase, ovalbumin, rabbit muscle aldolase, rabbit muscle lactate dehydrogenase, α-chymotrypsinogen A, β-lactoglobulin, lysozyme, ribonuclease A, and *N*-2,4-Dnp-alanine. The BDH molecular weight marker mixtures (mol wt range 14 300–71 500) were purchased from BDH Chemicals, Ltd., Poole, England, and ϵ-2,4-Dnp-lysine was from Pierce Chemical Co. Iodoacetamide from Eastman Kodak Co. was recrystallized from distilled-deionized H_2O until the reagent became free of color (yellow or brown).

Methods

Fractionation of the Urinary Proteins. Three 24-h urine specimens were concentrated 20-fold by use of an Amicon ultrafilter with an UM-10 membrane. The resulting concentrated urine was subjected to ammonium sulfate precipitation at 65% saturation, gel filtration on Sephadex G-200 and G-100 columns, and DEAE-cellulose chromatography. During the fractionation, the protein-containing fractions were monitored by their light absorbance at 280 nm and examined by NaDodSO$_4$-polyacrylamide gel electrophoresis and immunoelectrophoresis. The NaDodSO$_4$-polyacrylamide gel electrophoresis and immunoelectrophoresis were carried out as described previously (Seon & Pressman, 1976a; Seon et al., 1972).

The purified protein was tested against 48 antisera described above by a micro double-gel diffusion method (Crowle, 1973; Seon & Pressman, 1977).

Reduction and Alkylation. The protein was exhaustively reduced in 0.1 M dithiothreitol in 0.8 M Tris-HCl (pH 8.7) containing 7 M Gdn·HCl and 1 mM EDTA for 24 h at 37 °C. The alkylation of the resulting SH groups was carried out in 0.24 M recrystallized iodoacetamide for 2 h at room temperature. The amount of iodoacetamide was in 20% excess on a molar basis over SH groups of dithiothreitol. The reaction was stopped by chilling in ice-water followed by the dropwise addition of acetic acid until the pH was lowered to 5. Throughout the above experiment air was replaced with nitrogen and light was excluded. Extensive dialysis was carried out first against 0.01 M formic acid and subsequently against 0.5 M propionic acid in the cold room with exclusion of light.

Amino Acid Analysis. The reduced-alkylated protein was hydrolyzed in evacuated, sealed Pyrex tubes for 24 and 72 h at 110 °C in constant boiling HCl. Replicate hydrolyses were carried out. The quantitative analysis of amino acids was carried out on a Beckman Model 119H amino acid analyzer by use of a single-column methodology. Two different elution systems were used. The first elution system consisted of pH 3.20, 4.25, and 6.40 buffers. In the second elution system, pH 3.20 buffer was replaced with pH 3.49 buffer. The peaks of leucine and glucosamine overlapped in the first elution system but separated in the second one. The peaks of *S*-carboxymethylcysteine and aspartic acid overlapped in the second elution system but separated in the first one.

Amino Acid Sequence Study. The determination of NH_2-terminal amino acid sequence of the protein was carried out with a Beckman sequencer, Model 890C, as described previously (Seon et al., 1977b) but using program 122974 instead of program 072172C. The α_1-MGP was examined twice in the sequencer, once using the reduced-carboxamidomethylated protein and next using the untreated protein. The anilinothiazolinone derivatives from the first and second sequencing were hydrolyzed in 6 N HCl containing 0.1% $SnCl_2$ for 4 h at 150 °C (Mendez & Lai, 1975) and in 57% HI for 20 h at 126 °C, respectively (Smithies et al., 1971). The anilinothiazolinone derivative of methionine yields free methionine in the HCl hydrolysis but no identifiable amino acid in the HI hydrolysis. The resulting free amino acids were determined on an amino acid analyzer. The repetitive yield of Edman degradation was 91%.

Molecular Weight Determination. The mol wt of α_1-MGP was determined by NaDodSO$_4$-polyacrylamide gel electrophoresis (Shapiro et al., 1967; Weber & Osborn, 1969) and by gel filtration in the presence of 6 M Gdn·HCl on a Sephadex G-150 column (Fish et al., 1969; Seon & Pressman, 1975). NaDodSO$_4$-polyacrylamide gel electrophoresis of α_1-MGP was carried out in 5%, 7.5%, and 10% gels and a standard curve with mol wt markers was constructed for each gel concentration. The mol wt markers used in NaDodSO$_4$-polyacrylamide gel electrophoresis were L-glutamic dehydrogenase (liver), H and L chains of two monoclonal human IgG proteins, aldolase (muscle), trypsin, β-lactoglobulin, apomyoglobin, lysozyme, B and C chains of chymotrypsin, cytochrome *c* (horse heart), and BDH mol wt marker mixtures (14 300–71 500). Trypsin and chymotrypsin were treated with 1 mM phenylmethanesulfonyl fluoride prior to the incubation with NaDodSO$_4$ and dithiothreitol. The mol wt markers used in the gel filtration were ribonuclease A, lysozyme, monomer, and covalent dimer of Bence-Jones protein, chymotrypsinogen A (pretreated with 1 mM phenylmethanesulfonyl fluoride), and bovine serum albumin.

Isoelectric Focusing in Polyacrylamide Gels. The experiment was kindly done by Dr. G. L. Mayers of our Institute. The fully reduced-alkylated α_1-MGP and untreated α_1-MGP were subjected to isoelectric focusing in tubes according to the procedure by Wrigley (1968) as modified by Hoffman et al. (1971) with and without urea in the gel. Samples were placed in the gel mixture prior to polymerization of the gels. The ampholine pH gradient was 3.5–10 (LKB 1809-101). The samples were subjected to electrophoresis at a constant current of 0.5–0.6 mA/tube until the voltage reached 400 V, at which point a constant voltage of 400 V was maintained for about 4 h. The presence of protein was detected by staining with Coomassie Brilliant Blue R-250.

Preparation of Antiserum to α_1-Microglycoprotein. Rabbit antiserum was prepared against the purified α_1-MGP as described previously for the preparation of anti-β_2m serum (Seon & Pressman, 1977). The anti-α_1-MGP serum was absorbed with proteins of urine specimens of six healthy individuals which were coupled to CNBr-activated Sepharose 4B (Porath & Kristiansen, 1975).

Results

Isolation of α_1-Microglycoprotein. The urine of patient TSCH was subjected to ammonium sulfate precipitation at 65% saturation. The resulting precipitate was dissolved in water and dialyzed against water containing 0.05% NaN_3 and

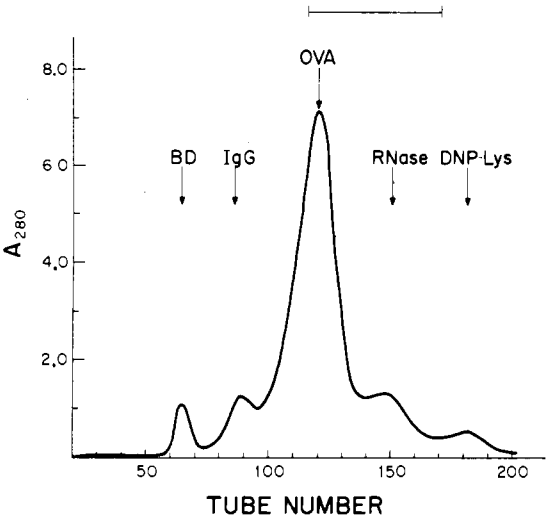

FIGURE 1: Fractionation of urinary proteins of patient TSCH on a Sephadex G-200 column. Three 24-h urine specimens were combined, concentrated 20-fold, and subjected to ammonium sulfate precipitation at 65% saturation. The precipitate was solubilized and approximately a quarter of the material was subjected to gel filtration on a Sephadex G-200 column (2.9 × 80 cm) in 0.1 M Tris-HCl containing 0.2 M NaCl, 2 mM EDTA-Na_2, and 0.02% NaN_3, pH 8.0. The column had been previously calibrated with blue dextran 2000, IgG, ovalbumin, ribonuclease A, and ϵ-Dnp-lysine. The fractions shown by a horizontal line were pooled for the further fractionation.

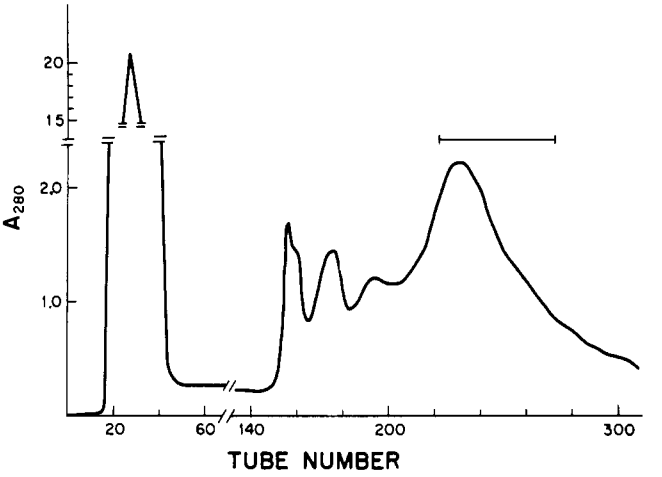

FIGURE 2: DEAE-cellulose chromatography. The fractions pooled as indicated by a horizontal line in Figure 1 were combined with the similarly pooled fractions of two other gel filtrations and concentrated to 42 mL and dialyzed against the starting buffer of the chromatography. The column (1.8 × 43 cm) was prepared with Whatman DE-52 and the elution, after tube no. 17, was carried out by a concave gradient from 0.015 to 0.30 M phosphate at pH 8.0. The gradient was produced by an autograd four-chamber system with 200 mL of 0.015 M potassium phosphate (pH 8.0) in each of the chambers numbered 1, 2, and 3 and with 190 mL of 0.30 M potassium phosphate (pH 8.0) in chamber no. 4. The fractions indicated by a horizontal line were pooled.

subsequently against 0.1 M Tris-HCl buffer, 0.2 M in NaCl, 2 mM in EDTA-Na_2, and 0.02% in NaN_3, pH 8.0. Approximately a quarter of the solution was subjected to gel filtration on a Sephadex G-200 column in the same Tris-HCl buffer (Figure 1). The column was previously calibrated with blue dextran 2000, IgG, ovalbumin, ribonuclease A, and ϵ-Dnp-lysine. Since we were interested in proteins with relatively small sizes which may be present in large quantities in the urine of patients with neoplastic diseases but only in small quantities in plasma (Rudman et al., 1969; Seon et al., 1977a), we pooled the fractions indicated by a horizontal line in Figure 1 which include the proteins with approximate sizes between 6000 and 50 000 daltons. The gel filtration was carried out twice more with the remaining solution of ammonium sulfate precipitate and the fractions similar to the pooled fractions of Figure 1 were collected. The pooled materials of the three gel filtrations were combined and subjected to DEAE-cellulose chromatography using a concave gradient from 0.015 M to 0.30 M phosphate at pH 8.0 (Figure 2). The fractions in Figure 2 were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis and immunoelectrophoresis using antisera to β_2m and immunoglobulin κ and γ chains. The first big peak contained a κ-type Bence-Jones protein. The last peak in Figure 2 showed a 29 000[3] mol wt component (α_1-MGP) on $NaDodSO_4$-polyacrylamide gel electrophoresis and the presence of this material was accompanied by a brown color. The α_1-MGP was different from any of the previously reported proteins in the urine of the same patient (Seon & Pressman, 1977; Seon et al., 1977a). The fractions pooled as indicated by a horizontal line in Figure 2 were again subjected to DEAE-cellulose chromatography using a linear gradient from 0.015 to 0.30 M phosphate at pH 8.0 and the major fraction was subjected to gel filtration on a Sephadex G-100 (Superfine) column (Figure 3). The fractions were examined by $NaDodSO_4$-polyacrylamide gel electrophoresis and immunoelectrophoresis using antisera to normal serum, normal urine, and antisera specific to β_2m and immunoglobulin κ and γ chains. The second large peak contained the brown color and the α_1-MGP. The fractions shown by a horizontal line in Figure 3 contained the pure α_1-MGP and were pooled. The α_1-MGP showed a single protein band on $NaDodSO_4$-polyacrylamide gel electrophoresis. In immunoelectrophoretic analysis α_1-MGP did not react with any of the antisera to normal serum, β_2m, κ chain, and γ chain but did react very weakly with one of two antisera to normal urine. The content of α_1-MGP in the urine of patient TSCH was determined by radial immunodiffusion (Mancini et al., 1965; Fahey & McKelvey, 1965) and was 140 mg/L or 130 mg/24-h urine volume.

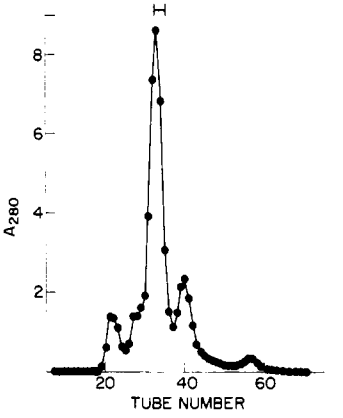

FIGURE 3: A Sephadex G-100 gel filtration. A pool of fractions from DEAE-cellulose chromatography (see Figure 2 and the text) was subjected to gel filtration on a Sephadex G-100 (Superfine) column (1.5 × 85 cm) in the same buffer as that in Figure 1. The eluate was collected in 2.6-mL fractions in each tube. The fractions bracketed by a horizontal line contained the pure α_1-MGP. The front part of the large peak was slightly contaminated with serum albumin.

More detailed studies of the chemical, physicochemical, and immunological properties of α_1-MGP are described below.

[3] The apparent mol wt of α_1-MGP determined in $NaDodSO_4$-polyacrylamide gel electrophoresis was 29 000 but a more accurate value of mol wt determined by gel filtration in 6 M guanidine hydrochloride was 27 000.

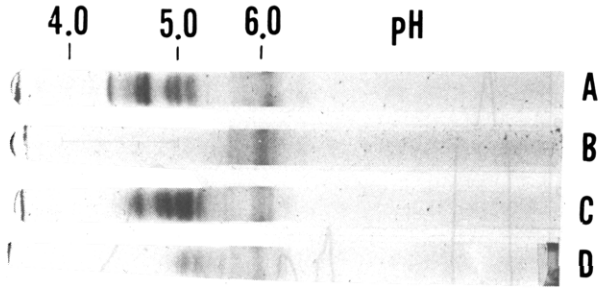

FIGURE 4: Isoelectric focusing of α_1-microglycoprotein. The fully reduced–carboxamidomethylated (gels B and D) and the untreated (gels A and C) proteins were analyzed in a pH gradient of 3.5–10 in polyacrylamide gels. The experiment was done both in the presence (gels C and D) and absence (gels A and B) of urea. The concentration of urea was approximately 5 M.

Analysis of α_1-Microglycoprotein by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The purified α_1-MGP showed a single protein band in $NaDodSO_4$–polyacrylamide gel electrophoresis either before or after the full reduction. The α_1-MGP is a glycoprotein (see below) and the mol wt of glycoproteins estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis tend to be higher than actual ones and to vary with the acrylamide concentration (Segrest et al., 1971). We, therefore, examined the mol wt of α_1-MGP in 5%, 7.5%, and 10% acrylamide gels. A standard curve was prepared for each of the acrylamide gels using the marker proteins described in the Experimental Section. The average values of duplicate analyses were 29 100, 28 900, and 29 300 in 5%, 7.5%, and 10% gels, respectively. There was, therefore, no significant difference in the mol wt of α_1-MGP observed at the three acrylamide gel concentrations. The experiment reported below, however, indicates that the mol wt of α_1-MGP determined by $NaDodSO_4$–polyacrylamide gel electrophoresis is indeed higher than the real one. The fact that there was no significant difference in mol wt between the reduced α_1-MGP and the unreduced one indicates that α_1-MGP is composed of a single polypeptide chain.

Molecular Weight Determination by Gel Filtration in 6 M Gdn·HCl. The mol wt of α_1-MGP was estimated by gel filtration as described previously (Seon & Pressman, 1975) according to the procedure of Fish et al. (1969). Since we used the present gel filtration for preparative purposes as well as analytical purposes, α_1-MGP was incubated at 37 °C for 18 h in the presence of 6 M Gdn·HCl but without a reducing agent. Accordingly, all the marker proteins used to prepare a standard curve were treated in the same manner. A single symmetrical peak was obtained for α_1-MGP. The results of gel filtration in 6 M Gdn·HCl were analyzed by two different procedures, one plotting $K_d^{1/3}$ as a function of mol wt to the 0.555 power and the other using K_d and log mol wt instead of $K_d^{1/3}$ and $(\text{mol wt})^{0.555}$. Essentially the same mol wt was obtained, i.e., 27 300 by the former procedure and 27 000 by the latter.

The brown color of α_1-MGP remained after incubation in 6 M Gdn·HCl and also remained after full reduction with 0.1 M dithiothreitol in 7 M Gdn·HCl.

Immunological Analyses. The purified α_1-MGP was used to immunize rabbits and the resulting antiserum showed a single precipitin arc against the α_1-MGP in immunoelectrophoresis. The electrophoretic mobility of α_1-MGP was compared in immunoelectrophoresis with those of whole human serum, albumin, α_2-macroglobulin, transferrin, β_2m, and fibrinogen, which showed that α_1-MGP migrates to the α_1 region. Since we did not know the nature and origin of the α_1-MGP, we tested for α_1-MGP by immunoelectrophoresis and/or micro double-gel immunodiffusion against a variety of antisera, i.e., antisera to normal serum and normal urine, antisera specific to β_2m, carcinoembryonic antigen, urinary kallikrein, and immunoglobulin κ, λ, γ, α, μ, δ, and ϵ chains, and commercial (Behring Diagnostics) antisera specific to 36 different serum proteins (see Experimental Section). One of two antisera to normal urine reacted very weakly with α_1-MGP. The only monospecific antiserum that reacted was anti-inter-α-trypsin inhibitor serum (Behring Diagnostics, lot 1916 BM) but the reaction was weak. Inter-α-trypsin inhibitor is a serum glycoprotein with mol wt of 160 000 (Daniels, 1975) and tends to degrade to yield 4.4 S and smaller fragments (Heide et al., 1965), although the degradation products are not well characterized. To examine the possibility that our α_1-MGP is contaminated with the degradation fragments of inter-α-trypsin inhibitor, α_1-MGP was subjected to gel filtration on a Sephadex G-100 (Superfine) column and each fraction of the resultant single symmetric peak was tested against anti-inter-α-trypsin inhibitor serum in the micro double-gel diffusion test. The antigenic activity was evenly distributed throughout the peak. It, therefore, appears that the antigenic activity of our α_1-MGP is not due to contamination by inter-α-trypsin inhibitor or its degradation products. This conclusion is supported by the facts that α_1-MGP showed a single protein band in $NaDodSO_4$–polyacrylamide gel electrophoresis and that a single NH_2-terminal amino acid sequence was found for α_1-MGP (see below). It is likely that the anti-inter-α-trypsin inhibitor serum has an activity to α_1-MGP as well as to inter-α-trypsin inhibitor.[4] Alternatively α_1-MGP may antigenically cross-react with inter-α-trypsin inhibitor, or α_1-MGP may be a degradation product of inter-α-trypsin inhibitor.

Isoelectric Focusing in Polyacrylamide Gels. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration in the absence and in the presence of 6 M Gdn·HCl showed the α_1-MGP to be homogeneous in size. Homogeneity with respect to charge was examined by isoelectric focusing. As shown in Figure 4, there is a significant charge heterogeneity in α_1-MGP both in the absence (gels A and B) and presence (gels C and D) of urea. To examine the role of S–S bonds in the heterogeneity, the fully reduced–carboxamidomethylated α_1-MGP (*S*-CM-α_1-MGP) was analyzed (gels B and D) along with the unreduced α_1-MGP (gels A and C) (Figure 4). Although the *S*-CM-α_1-MGP showed a much restricted heterogeneity in the absence of urea, this is probably due to the poor solubility of *S*-CM-α_1-MGP in aqueous buffer as is suggested by a high background stain. The *S*-CM-α_1-MGP showed more heterogeneity in the presence of urea, although the heterogeneity pattern is different from that of unreduced α_1-MGP in the presence of urea; i.e., the three strong protein bands at pH 4.45, 4.7, and 4.85 in the nonreduced protein (gel C) are not observed in the reduced–carboxamidomethylated protein (gel D), while more protein bands are observed between pH 5 and 6 in the latter gel.

Amino Acid and Hexosamine Analyses. The amino acid and hexosamine compositions of α_1-MGP are shown in Table I. In replicate analyses the residue numbers of amino acids were consistent when expressed relative to the residue number of arginine. When the residue number of amino acids was calculated on the basis of 10.00 residues of arginine, total number of amino acid residues recovered (190.2 excluding tryptophan) corresponds approximately to the expected number based on the mol wt of 27 000 and its carbohydrate content of ap-

[4] We found that the same antiserum reacted with β_2m also.

TABLE I: Amino Acid and Hexosamine Compositions of α_1-Microglycoprotein.[a]

constituent	residue no.[b]
Asp	15.7 ± 0.2
Thr[c]	17.4 ± 0.3
Ser[c]	11.3 ± 0.1
Glu	23.9 ± 0.2
Pro	12.8 ± 0.3
Gly	15.9 ± 0.2
Ala	10.2 ± 0.1
½-cystine[d]	3.7 ± 0.1
Val[e]	12.2
Met	4.8 ± 0.3
Ile[e]	11.8
Leu	11.7 ± 0.1
Tyr	8.0 ± 0.1
Phe	6.1 ± 0.3
His	3.8 ± 0.1
Lys	10.8 ± 0.3
Arg	10.0 ± 0.0
Trp[f]	+
glucosamine[g]	++

[a] Unless stated otherwise, all values are averages of quadruplicate hydrolysates for 24 h. [b] Calculated on the basis of 10.00 residues of arginine per molecule. The values are means ± standard deviation (except for valine and leucine). [c] Corrected for 3% and 10% destruction during acid hydrolysis for threonine and serine, respectively (Downs & Pigman, 1969). [d] Determined as *S*-carboxymethylcysteine. [e] From a 72-h hydrolysate. [f] Tryptophan was detected by the hydrolysis of the protein in 6 N HCl containing 4% thioglycolic acid (Matsubara & Sasaki, 1969). Since the presence of carbohydrate inhibits the yields of tryptophan, quantitative recovery of tryptophan was not achieved for α_1-MGP, a glycoprotein. [g] Under the hydrolysis conditions that we used, some of the glucosamine is destroyed (Neuberger & Marshall, 1966).

TABLE II: Amino Acids Obtained after HI Hydrolysis of the Thiazolinone Derivatives Which Were Derived from the Unreduced α_1-Microglycoprotein.[a]

sequence position	major amino acid obtained	nmol[b]	amino acid[c] in the sequence
1	Gly	103.3	Gly
2	Pro	42.6	Pro
3	Val	36.4	Val
4	Pro	46.9	Pro
5	none		
6	Pro	45.2	Pro
7	Pro	62.4	Pro
8	Asp	21.5	Asx
9	Asp	31.1	Asx
10	Ile + *a*Ile	16.1	Ile
11	Glu	17.8	Glx
12	Val	15.2	Val
13	Glu	21.7	Glx
14	Glu	26.3	Glx
15	Asp	10.8	Asx
16	Phe	6.3	Phe
17	Phe	6.6	Phe
18	Ile + *a*Ile	8.5	Ile
19	Ala	6.9	Ser or Ala
20	Arg	+	Arg

[a] Approximately 140 nmol of the unreduced α_1-MGP was subjected to the sequence study. [b] No corrections were made for the varying degrees of incomplete recoveries from the individual thiazolinone derivatives by HI hydrolysis (see Smithies et al., 1971). [c] See Smithies et al. (1971). The Asx and Glx represent aspartic acid or asparagine and glutamic acid or glutamine, respectively.

proximately 20% (see the Discussion). Quantitative determinations of tryptophan and glucosamine were not achieved since both were partially destroyed under the acid hydrolysis conditions used. The polarity of the protein part of α_1-MGP was calculated according to the procedure of Capaldi & Vanderkooi (1972) and was 48.8% excluding tryptophan.

NH_2-Terminal Amino Acid Sequence. The fully reduced carboxamidomethylated α_1-MGP and unreduced α_1-MGP were both subjected to NH_2-terminal amino acid sequencing. In both the sequence studies, a single amino acid sequence was obtained.

The results obtained with the unreduced α_1-MGP are presented in Table II. The thiazolinone derivatives from the Beckman sequencer were hydrolyzed in 57% HI as described in Methods. A result consistent with that of Table II was obtained from the sequence study with the fully reduced and carboxamidomethylated α_1-MGP in which the thiazolinone derivatives were hydrolyzed in 6 N HCl containing 0.1% $SnCl_2$ (Mendez & Lai, 1975). At each position, except for position no. 5, a single major amino acid was found. In both the sequence studies no major amino acid was found at position no. 5. This suggests that the chromophore group or carbohydrate may be attached to the no. 5 residue. The sequence is unusual because of the high content of proline at the first seven positions.

Discussion

Patients with advanced noeplastic diseases frequently excrete large quantities of proteins into the urine (Rudman et al., 1969; Seon et al., 1977a). In the present study we isolated a unique glycoprotein, α_1-MGP, from the urine of a patient with relapsed plasma cell leukemia. In immunoelectrophoretic analyses α_1-MGP did not react with antisera to normal serum, β_2m, carcinoembryonic antigen, urinary kallikrein, and immunoglobulin κ, λ, γ, α, μ, δ, and ϵ chains. An approximate mol wt of 29 000 was obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis using 5%, 7.5%, and 10% gels. A more accurate mol wt of 27 000 was obtained for this glycoprotein by gel filtration in 6 M Gdn·HCl. The NH_2-terminal amino acid sequence study of α_1-MGP showed a unique sequence (Table II). To examine the origin of α_1-MGP, we purchased Behring antisera specific to 36 different serum proteins and tested α_1-MGP against these antisera in a micro double-gel diffusion test. Only anti-inter-α-trypsin inhibitor serum (Behring Diagnostics, lot 1916 BM) reacted, but the reaction was weak. Several lines of evidences indicate that the reaction is not due to the contamination of α_1-MGP with inter-α-trypsin inhibitor or its degradation products (see the Results). The specificity of the antiserum remains to be proved. To examine the possibility that α_1-MGP is a degradation product of inter-α-trypsin inhibitor which still retains the inhibitory activity against trypsin, we tested the effect of α_1-MGP on tryptic digestions of *N*-benzoyl-DL-arginine-*p*-nitroanilide and azocasein. No effect of α_1-MGP was observed on either digestion. To determine if the concentration of α_1-MGP is elevated in the urine of cancer patients as compared with that of healthy individuals, we tested the urine specimens of 121 cancer patients and 28 healthy individuals against rabbit anti-α_1-MGP serum which had been absorbed with proteins of urine specimens of healthy individuals (see Methods). The absorbed antiserum was used along with anti-β_2m antiserum. Approximately 50% of the cancer specimens reacted with the absorbed anti-α_1-MGP and one-fourth (16 specimens) of the reacting specimens reacted also with anti-β_2m serum. None of the specimens of healthy individuals reacted with the antiserum. The low mol wt proteins (<50 000 mol wt) readily transverse the glomerular filter of

kidney and are largely taken up and catabolized by renal tubular cells. In tubular diseases the low mol wt proteins enter the tubular lumen in normal fashion, but are not taken up by tubular cells. Thus, these proteins are excreted in large quantities into the urine (Strober & Waldmann, 1974). The mol wt of β_2m is 11 800 (Berggård & Bearn, 1968) and it is typical of such proteins (Peterson et al., 1969). We have previously shown that the quantities of β_2m in the urine of a patient with acquired Fanconi syndrome changed in parallel with the extent of renal tubular dysfunction of the patient (Gailani et al., 1977b). The fact that three-fourths of the cancer specimens reacting with the absorbed anti-α_1-MGP serum did not react with anti-β_2m serum suggests that the elevated presence of α_1-MGP in the urine of these patients is not due to renal tubular dysfunction. The 121 cancer patients studied in the present study include patients with a variety of neoplastic diseases such as multiple myeloma, various types of leukemia, malignant lymphoma, melanoma, breast carcinoma, and lung carcinoma, etc. We have analyzed the details of the types of cancer and the stage of the diseases of these patients and will report the results elsewhere. We are now establishing a quantitative radioimmunoassay of α_1-MGP in the urine and serum of cancer patients, patients with benign diseases, and healthy individuals.

Recently several papers appeared reporting proteins similar to α_1-MGP, i.e., protein HC (Tejler & Grubb, 1976; Frangione et al., 1976) and α_1-microglobulin (Svensson & Ravnskov, 1976; Ekström & Berggård, 1977). These proteins were isolated from urine of a healthy individual (Tejler & Grubb, 1976), patients with tubular proteinuria (Frangione et al., 1976; Ekström & Berggård, 1977) or a patient with uremia (Svensson & Ravnskov, 1976). We would like to point out the fact that our procedure for the isolation of α_1-MGP is significantly different from those for protein HC and α_1-microglobulin. One of the differences is that the procedures reported by others for the isolation of protein HC and α_1-microglobulin involved the use of an antiserum to each of these glycoproteins. Our procedure for α_1-MGP does not involve the use of anti-α_1-MGP serum. In each case, i.e., protein HC and α_1-microglobulin, the protein was a glycoprotein with a carbohydrate content of approximately 20%, had a brown color, and showed charge heterogeneity. Except for one report (Ekström & Berggård, 1977), the mol wt was determined by Na-DodSO_4-polyacrylamide gel electrophoresis only and was 30 000–31 500. The mol wt determined by gel filtration in 6 M Gdn·HCl and ultracentrifugation was 25 000–27 000. There was a significant difference in amino acid composition for multiple amino acid residues between the glycoprotein of Tejler & Grubb (1976) and those of others. In none of the studies of protein HC and α_1-microglobulin was the glycoprotein tested against as many antisera specific to serum and urinary proteins as we did in the present study. In none of these studies were specimens from cancer patients examined.

The amino acid composition and isoelectric focusing pattern of α_1-MGP are significantly different from those of protein HC of Tejler & Grubb (1976) but the composition is similar to those of protein HC and α_1-microglobulin of other reports. In the latter reports, isoelectric focusing was not studied or not reported. Because of the importance of S–S bonds in maintaining the structure and function of proteins, we would like to discuss the number of S–S bonds in α_1-MGP. Ekström & Berggård (1977) reported that the number of half-cystine residues in their α_1-microglobulin was about 3.4 per molecule when measured as cysteic acid after performic acid oxidation and about 2.3 when measured as carboxymethylcysteine after reduction and alkylation. There were no SH groups. Based on these observations, they postulated that α_1-microglobulin contains two half-cystine residues which are blocked by cysteine, i.e., α_1-microglobulin contains no S–S bonds. Our results for α_1-MGP shown in Table I do not support the above postulation. We determined the half-cystine as carboxymethylcysteine (see Methods) and found that α_1-MGP contained approximately 3.7 half-cystine per molecule. The compositions of α_1-MGP and α_1-microglobulin agree reasonably with respect to other amino acids. The difference in the half-cystine content determined as carboxymethylcysteine is likely due to the different conditions of reduction and carboxamidomethylation. Ekström & Berggård reduced the protein in 0.02 M dithiothreitol at pH 8.0 in the presence of 6 M Gdn·HCl and alkylated in 0.05 M iodoacetamide. We reduced the protein in 0.1 M dithiothreitol at pH 8.7 in the presence of 7 M Gdn·HCl and alkylated in 0.24 M recrystallized iodoacetamide. We previously showed that the alkylation of SH groups at pH 8.0 was incomplete for IgM and that the alkylation improved at higher pH (Seon & Pressman, 1976b). It, therefore, appears to us that the lower half-cystine value (2.3 per molecule) of Ekström & Berggård is due to incomplete reduction–alkylation. In view of the facts that Ekström & Berggård did not find any free SH groups in their protein and that many of the proteins such as α_1-MGP in an extracellular environment contain little SH groups but do contain S–S bonds (Cecil, 1963), the 3.7 half-cystine of α_1-MGP appears to represent 2 S–S bonds per molecule.

Frangione et al. (1976) reported an NH_2-terminal amino acid sequence of their protein HC for the first 25 residues with gaps (not determined) at positions 5 and 16. The present NH_2-terminal sequence of α_1-MGP agrees with the sequence of Frangione et al. except that phenylalanine was found at position 16 of α_1-MGP.

In summary α_1-MGP is very similar to protein HC of Frangione et al. and α_1-microglobulin; however, it is still not known if these proteins are the same protein or not, partly because of a significant charge heterogeneity of these proteins. To this end, the determination of the complete amino acid sequences of these proteins will be necessary.

The origin of the charge heterogeneity of these proteins is not due to sialic acid (Tejler & Grubb, 1976; Ekström & Berggård, 1977) or to NH_2-terminal amino acid sequence heterogeneity.

Recently Pearlstein et al. (1977) found protein HC on the surface of cells such as peripheral blood B and T cells and erythrocytes. Further studies of the origin and function of these glycoproteins will be important.

Acknowledgments

We are much indebted to Dr. George L. Mayers for performing isoelectric focusing. We wish to thank Mrs. H. Tsai, Mr. S. Grimm, and Mr. J. Yeh for their skillful technical assistance. Thanks also go to Dr. V. P. Kreiter and Miss J. Stevens for their help in the preparation of the manuscript.

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