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NEUTRAL MICROPROTEIN, A NOVEL HUMAN PLASMA AND URINARY PROTEIN ASSOCIATED WITH A YELLOW-BROWN CHROMOPHORE. ISOLATION FROM PROTEIN HC PREPARATIONS AND PARTIAL CHARACTERIZATION

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An apparently novel human plasma and urinary protein of low molecular weight was isolated from several highly purified preparations of protein HC by gel chromatography and high voltage electrophoresis with a yield of about 8 mg/g. The protein has a molecular weight of about 20,000, neutral electrophoretic mobility at pH 6.5 and a high content of half-cystine. It is associated with a yellow-brown chromophore like protein HC and could be demonstrated in all investigated preparations of isolated human, rabbit and guinea-pig protein HC and α_1-microglobulin.

Protein HC (and the closely related α_1-microglobulin) is a recently described low molecular weight human glycoprotein originally isolated from normal urine (1,2). It carries an unidentified yellow-brown chromophore, displays a considerable charge heterogeneity despite a unique invariable amino acid sequence of its single polypeptide chain and is present in human plasma to a large extent as an IgA-complex (3-5). Protein HC and α_1-microglobulin are produced in hepatocytes (6,7) and possess immunoregulatory properties (8) but their normal physiological roles are not finally established. Conflicting results have been presented concerning the presence of the two proteins on the surface of lymphoid cells (9-11), their carbohydrate prosthetic groups (4,12,13) and their half-cystine content (1,14-16).

The present work describes the isolation of an apparently novel protein in small amounts from highly purified preparations of human urinary protein HC. This new protein was found in all investigated preparations of isolated human, rabbit and guinea-pig protein HC and α_1-microglobulin and its ubiquitous presence in such protein preparations can possibly explain some of the conflicting results. The protein was strongly associated with a yellow-brown chromophore like protein HC and α_1-microglobulin and may be a new member of the protein HC-α_1-microglobulin protein family.

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MATERIALS AND METHODS

Human protein HC isolated as earlier described by ion-exchange- and gel chromatography followed by immunosorption from the urine of a single individual with tubular proteinuria (1) was used as starting material for the isolation. Human plasma protein HC was produced by precipitation, immunosorption and gel chromatography as earlier reported (5). Isolated urinary α_1-microglobulin from human, rabbit and guinea-pig was a generous gift from Bo Åkerström, Department of Physiological Chemistry, University of Lund, 220 07 Lund 7. Sweden. Sephacryl S-200 Superfine, Sephadex G-25 and molecular weight calibration proteins were obtained from Pharmacia, Uppsala, Sweden, dithiothreitol, iodoacetic acid, guanidinium chloride, tris(hydroxymethyl)aminomethane and ninhydrin from Sigma, St. Louis, USA, ^{14}C-iodoacetic acid from the Radiochemical Centre, Amersham, England, and cellulose thin layer plastic sheets and other reagents not specified from Merck, Darmstadt, West Germany.

Amino acid analysis. Hydrolysis was performed at 110°C for 20 h with 200 µl of 5.7 M HCl containing 0.05 % (v/v) 2-mercaptoethanol in evacuated and sealed tubes. The analyses were carried out with a Beckman 121-MB analyzer.

Reduction and alkylation. Native protein HC (20 mg/ml) in 2.0 M Tris-HCl buffer, pH 8.6, containing 2 mM EDTA and 6 M guanidinium chloride was reduced with 35 mM dithiothreitol at 37°C for 100 min. Radioalkylation was accomplished by addition of ^{14}C-iodoacetic acid to the mixture and incubation at room temperature in the absence of light for 15 min. Unlabelled iodoacetic acid was then added to a final concentration of 80 mM and excess reagents removed by gel chromatography on a column of Sephadex G-25.

Electrophoresis at pH 6.5. Native protein material in 10 % (v/v) formic acid was applied (0.2 mg/cm) on cellulose plates (20 x 20 cm). Electrophoresis at pH 6.5 (acetic acid-pyridine-water 1:25:225 v/v) was run at 20 V/cm for 1 h. Visible yellow-brown cellulose sections containing proteins were scraped off the plate and the proteins eluted three times with 0.4 ml of 10 % (v/v) formic acid.

Dodecyl sulfate-polyacrylamide gel electrophoresis. The system of Laemmli (17) was used with 15 % (w/v) acrylamide and 0.4 % (w/v) bisacrylamide in the separation gel.

RESULTS

Performic acid oxidation of native urinary protein HC followed by cellulose thin layer electrophoresis at pH 6.5 revealed the presence in the protein preparation of small amounts of two components, in addition to protein HC, which seemed to be released by the oxidation (Fig. 1). The acidic component has been shown to correspond to a single cysteine amino acid bound to a cysteine residue at position 34 in the polypeptide chain of protein HC (4,16) while the relation between the second component migrating in the neutral electrophoretic position and protein HC never has been investigated. When the native, unoxidized, protein HC preparation (1 mg) was subjected to electrophoresis at pH 6.5 two colored bands could be observed by visual inspection directly after the electrophoresis. One band remained at the point of application and was associated with a dark yellow-brown pigment while the other was situated in the neutral electrophoretic zone and had a more light yellow-brown color (Fig. 1). No further component could be visualized by ninhydrin

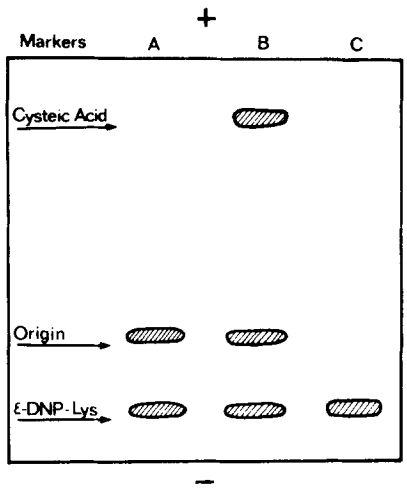

Fig. 1 Diagram of the electrophoresis at pH 6.5 on cellulose plates. (A) Native urinary protein HC, (B) oxidized protein HC, and (C) peak b from Fig. 2.

staining. The material of the application and the neutral section of the cellulose thin layer was eluted with 10 % (v/v) formic acid, oxidized and hydrolised. Amino acid analysis revealed that the material at the application was protein HC while that of the neutral position had a clearly different amino acid composition with a high content of cysteic acid (Table I).

To isolate higher amounts of the half-cystine-rich component the native protein HC preparation was completely reduced, ^{14}C-carboxymethylated and chromatographed on a column of Sephadex G-25. No radioactive peak corresponding to a half-cystine-rich peptide could be observed between the radioactive peak of the void volume and the radioactive peak of the low molecular weight reagents. However, when the reaction mixture was chromatographed on a column of Sephacryl S-200 in 10 % (v/v) formic acid two radioactive peaks (a and b), in addition to the radioactive reagent peak, were obtained (Fig. 2). Although peaks a and b contained approximately equal amounts of radioactivity peak b displayed virtually no absorbance at 280 nm. The elution position of peak b corresponded to a molecular weight of about 20,000. The amino acid composition of the material of the peaks showed that protein HC constituted peak a and the half-cystine-rich component peak b (Table I). Electrophoresis at pH 6.5 of the material of peak b demonstrated in addition that it migrated in the neutral zone and carried a yellow-brown pigment exactly as the half-cystine-rich peptide observed on electrophoresis of the native protein HC preparation (Fig. 1). Autoradiography of the electropherogram displayed a strong band in the neutral zone. The yield of the reduced and carboxymethylated half-

TABLE I

AMINO ACID COMPOSITION OF VARIOUS PREPARATIONS OF PROTEIN HC AND NEUTRAL MICROPROTEIN

Amino acid	Protein HC[a]	Protein from electrophoretic application zone (Fig. 1)[b]	Protein from first peak of Sephacryl S-200 separation in formic acid (Fig. 2)[b]	Protein from first peak of Sephacryl S-200 separation in ammonium bicarbonate (Fig. 4)[b]	Protein from electrophoretic neutral zone (Fig. 1)[c]	Protein from second peak of Sephacryl S-200 separation in formic acid (Fig. 2)[c]	Protein from second peak of Sephacryl S-200 separation in ammonium bicarbonate (Fig. 4)[c]
Lysine	10	9.1	10.1	10.0	8.2	7.8	7.4
Histidine	4	3.3	3.6	3.8	3.2	3.4	3.3
Arginine	10	8.6	9.9	9.7	8.3	10.6	10.3
Cysteine	3	3.7[d]	2.4[e]	2.8[e]	12.0[d]	11.5[e]	12.8[e]
Aspartic acid	14	14	14	14	13	13	13
Threonine	17	13.9	14.8	15.3	6.1	8.2	7.3
Serine	10	9.2	9.1	10.2	9.9	8.9	9.5
Glutamic acid	21	20.8	20.5	22.0	22.7	25.9	27.1
Proline	12	11.0	11.6	12.0	10.5	16.1	15.3
Glycine	14	14.1	14.4	14.4	22.0	20.8	20.5
Alanine	9	8.3	9.5	9.3	13.0	14.9	14.8
Valine	11	9.5	9.8	10.0	8.2	8.3	7.6
Methionine	5	ND	4.0	3.9	ND	1.5	1.8
Isoleucine	13	11.0	11.8	12.0	1.3	2.7	2.4
Leucine	11	11.1	11.1	11.3	8.5	11.8	11.6
Tyrosine	8	ND	7.9	7.9	ND	2.9	2.6
Phenylalanine	6	4.5	6.2	6.5	ND	2.6	2.7
Tryptophan	4	ND	ND	ND	ND	ND	ND

[a] Data taken from amino acid sequence (references 3 and 4)

[b] Expressed on the basis of 14 residues of aspartic acid (+ asparagine) per molecule

[c] Expressed on the basis of 13 residues of aspartic acid (+ asparagine) per molecule

[d] Determined as cysteic acid

[e] Determined as carboxymethylcysteine

ND = not determined

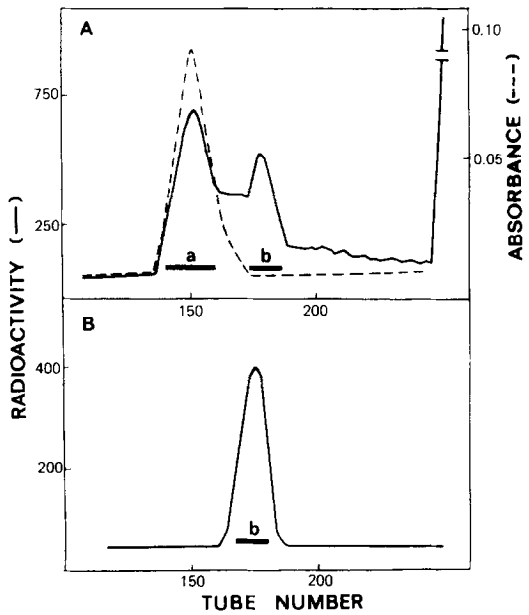

Fig. 2 Fractionation of reduced and ^{14}C-carboxymethylated protein HC (10 mg) on Sephacryl S-200. (A) After reduction and carboxymethylation, the material was chromatographed on a column (2.0 x 200 cm) of Sephacryl S-200, equilibrated and eluted with 10 % (v/v) formic acid. The elution rate was 20 ml/h and 2.0 ml fractions were collected. (--) Absorbance at 280 nm; (——) ^{14}C-radioactivity. (B) Rechromatography of peak b on the same column.

cystine-rich peptide by this chromatographic procedure was approximately 75 µg from 10 mg of the native protein HC preparation.

Dodecyl sulfate-polyacrylamide gel electrophoresis of the reduced half-cystine-rich component demonstrated a single band in a position corresponding to a molecular weight of 20,000 (Fig. 3). The lyophilized protein as well as water solutions of it (1 mg/ml) displayed a strong yellow-brown color similar to the one of protein HC and α_1-microglobulin.

When 10 mg of the native protein HC preparation was chromatographed on a column of Sephacryl S-200 in 0.05 M ammonium bicarbonate a single peak (a) with absorbance at 280 nm was obtained (Fig. 4). The material of this peak was reduced, ^{14}C-carboxymethylated and rechromatographed on the same column. A single peak of radioactivity was obtained (Fig. 4) and the material of this peak was identified as protein HC by amino acid analysis (Table I). Fourteen of the fractions immediately after those constituting peak a were pooled (Fig. 4, pool b). The material of this pool b was reduced, ^{14}C-carboxymethylated and chromatographed on the same column of Sephacryl S-200. A single radioactive peak (b1) with virtually no absorbance at 280 nm

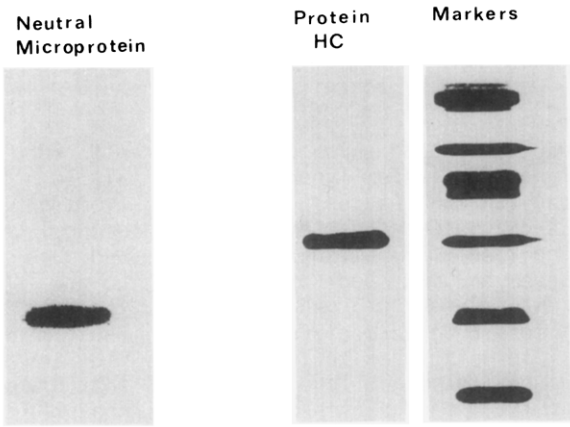

Fig. 3 SDS-polyacrylamide gel electrophoresis of isolated neutral microprotein (20 μg) and protein HC (30 μg).

was obtained (Fig. 4). The material of this peak was identified as the half-cystine-rich protein by amino acid analysis (Table I).

The presence of the half-cystine-rich protein in preparations of isolated human plasma protein HC, isolated human, rabbit and guinea-pig urinary α_1-microglobulin was investigated by subjecting one mg of these preparations to electrophoresis at pH 6.5 on cellulose thin layer plates. All preparations contained a yellow-brown component in the neutral electrophoretic zone in which the half-cystine-rich protein migrated.

DISCUSSION

This work describes the isolation of a 20 kDa protein from highly purified preparations of human urinary protein HC. The protein is composed of one polypeptide chain, has a high content of half-cystine residues, migrates in the neutral zone on electrophoresis at pH 6.5 and is associated with a yellow-brown chromophore. The chromophore(-s) is probably linked to the protein by covalent bonds other than disulfide-bridges since the protein still is colored after reduction and gel chromatography in dissociating media.

The color of the protein which is similar to that of protein HC and α_1-microglobulin and its co-isolation with these proteins suggest that it belongs to the same protein family as protein HC and α_1-microglobulin. It is unlikely that the half-cystine-rich protein is a breakdown product of protein HC or α_1-microglobulin since its amino acid composition is quite different from those of protein HC and α_1-microglobulin, e.g. in its high half-cystine and low isoleucine content. The structures of isolated tryptic cysteine-containing peptides from the protein also differ from those of all conceivable cysteine-con-

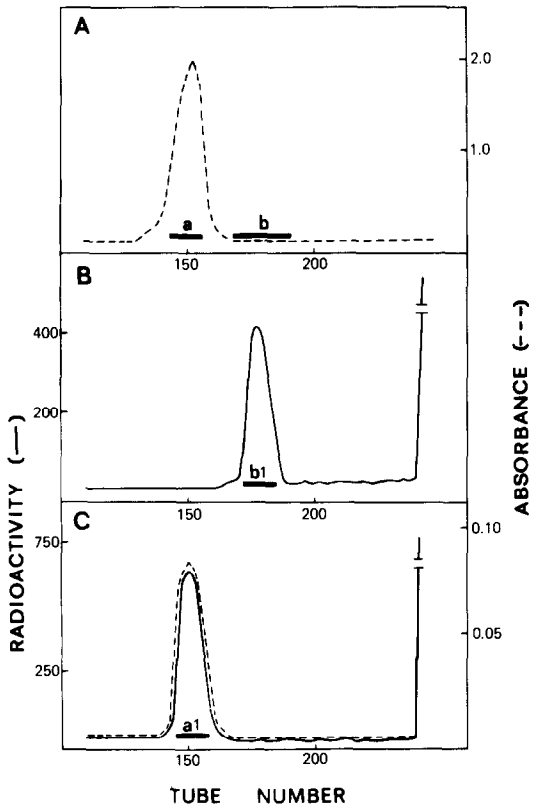

Fig. 4 Chromatography of native protein HC on Sephacryl S-200. (A) The material (10 mg) was chromatographed on a column (2.0 x 200 cm) of Sephacryl S-200, equilibrated and eluted with 0.05 M ammonium bicarbonate. The flow-rate was 20 ml/h and 2.0 ml fractions were collected. (B) Fractionation of the material from pool b in (A) after reduction and ^{14}C-carboxymethylation on the same Sephacryl column equilibrated and eluted with 10 % (v/v) formic acid. (C) Fractionation of the material from pool a in (A) after reduction and ^{14}C-carboxymethylation on the same column in 10 % (v/v) formic acid. (--) Absorbance at 280 nm; (——) ^{14}C-radioactivity.

taining peptides of protein HC and α_1-microglobulin (3,4,12).

Although the half-cystine-rich protein has been found to be present in all investigated preparations of purified protein HC and α_1-microglobulin it is too early to infer that it is functionally carried by or associated with these proteins as it could be dissociated from these proteins by simple gel chromatography in neutral non-dissociating buffers provided that a suitable chromatographic resin was selected.

The conflicting results concerning the cysteine-content of various preparations of proten HC and α_1-microglobulin (1,14-16) and the production of these proteins by lymphoid cells (9-11) can possibly be explained by the observation that all preparations of pro-

tein HC and α_1-microglobulin seem to contain the half-cystine-rich protein as the content of this protein in different preparations of protein HC and α_1-microglobulin may vary and antisera against such protein preparations may contain a varying proportion of antibodies against the half-cystine-rich protein.

Until the biological function of the half-cystine-rich protein described in this work become known, we propose that it be called neutral microprotein.

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REFERENCES

1. Tejler, L., and Grubb, A.O. (1976) Biochim. Biophys. Acta 439, 82-94.
2. Ekström, B., Peterson, P.A., and Berggård, I. (1975) Biochem. Biophys. Res. Commun. 65, 1427-1433.
3. López, C., Grubb, A.O., Soriano, F., and Méndez, E. (1981) Biochem. Biophys. Res. Commun. 103, 919-925.
4. López-Otin, C., Grubb, A.O., and Méndez, E. (1983) Arch. Biochem. Biophys. In press.
5. Grubb, A.O., López, C., Tejler, L., and Méndez, E. (1983) J. Biol. Chem. In press.
6. Tejler, L., Eriksson, S., Grubb, A.O., and Åstedt, B. (1978) Biochim. Biophys. Acta 542, 506-514.
7. Berggård, B., Ekström, B., and Åkerström, B. (1980) Scand. J. Clin. Lab. Invest. 40, Suppl. 154, 63-71.
8. Lögdberg, L., and Åkerström, B. (1981) Scand. J. Immunol. 13, 383-390.
9. Pearlstein, E., Turesson, I., Tejler, L., and Grubb, A.O. (1977) J. Immunol. 119, 824-829.
10. Takagi, K., Kin, K., Itoh, Y., Kawai, T., Kasahara, T., Shimoda, T., and Shikata, T. (1979) J. Clin. Invest. 63, 318-325.
11. Åkerström, B., Nilsson, K., Berggård, B., and Berggård, I. (1979) J. Immunol. 122, 2516-2520.
12. Takagi, T., Takagi, K., and Kawai, T. (1981) Biochem. Biophys. Res. Commun. 98, 997-1001.
13. Ekström, B., Lundblad, A., and Svensson, S. (1981) Eur. J. Biochem. 114, 663-666.
14. Ekström, B., and Berggård, I. (1977) J. Biol. Chem. 252, 8048-8057.
15. Frangione, B., Franklin, E.C., Grubb, A.O., and Tejler, L. (1976) FEBS Lett. 70, 239-240.
16. Méndez, E., Grubb, A.O., López, C., Frangione, B., and Franklin, E.C. (1982) Arch. Biochem. Biophys. 213, 240-250.
17. Laemmli, U.K. (1970) Nature 227, 680-685.