Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 535-543

# ISOLATION OF A NEW LOW MOLECULAR WEIGHT $\beta_2$-GLOBULIN FROM URINE OF A WORKER WITH CHRONIC CADMIUM POISONING

Alfred M. Bernard[1-3], Robert R. Lauwerys[1], Vincenzo Starace[2], and Pierre L. Masson[2]

[1]Unit of Industrial and Medical Toxicology
University of Louvain
Clos Chapelle-aux-Champs 30.54
1200 BRUXELLES

[2]Unit of Experimental Medicine
Institute of Cellular and Molecular Pathology
1200 BRUXELLES

[3]Aspirant du Fonds National Belge de la Recherche Scientifique

Received January 21,1980

SUMMARY A low molecular weight $\beta_2$-globulin which does not correspond to any known protein has been isolated from urine of a cadmium-exposed worker with proteinuria. The isolation procedure included chromatography on Sephadex G-75 and DEAE-cellulose, followed by preparative agarose gel electrophoresis. The purified protein was homogeneous in ultracentrifugation and in electrophoresis in agarose gel. Electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate confirmed the homogeneity of the preparation and suggested a molecular weight of about 21,000. No carbohydrate was detected by the anthrone reaction. The ultraviolet spectrum showed a minimum at 250 nm and a maximum at 279 nm.

Urine from patients with renal tubular dysfunction contains various proteins with molecular weights lower than 40,000. The best known are $\beta_2$-microglobulin ($M_r$ = 11,800) (1), $\alpha_2$-microglobulin or retinol binding protein (RBP, $M_r$ = 21,400) (2) and the post-$\gamma$-protein ($M_r$ about 10,000) (3).

The present paper describes the isolation of another low molecular weight protein from the urine of a worker with proteinuria resulting from cadmium exposure. This protein, hereafter called $\beta_{2x}$-globulin, to distinguish it from $\beta_2$-microglobulin,

Abbreviations : Mr, molecular weight; RBP : retinol binding protein; SDS : sodium dodecyl sulfate.

0006-291X/80/060535-09$01.00/0

was first detected as a contaminant of preparations of RBP from which it could be separated only by preparative electrophoresis. The yield was only about 5 % so that only a few physicochemical characteristics were determined.

## EXPERIMENTAL PROCEDURES

Materials Total (24 h) urine was stored at 4°C after addition of one volume of 0.4 M phosphate buffer pH 7.4, containing 1 % sodium azide, to 10 volumes of urine. Superfine Sephadex G-75 and G-25 from Pharmacia (Uppsala, Sweden) and DEAE-cellulose (Whatman DE-52, Maidstone, England) were prepared according to the instructions of the suppliers.
Antisera against $\beta_2$-microblobulin, κ and λ light chains were from Dako Immunoglobulin (Copenhagen, Denmark). Antisera against RBP and $\beta_2$-glycoprotein III were from Behringwerke AG (Marburg/Lahn, West Germany). Those against albumin, IgG, IgA and IgM were prepared in our laboratories. Antisera against $\beta_{2x}$-globulin were prepared in two rabbits by injecting intradermally 0.5 mg of $\beta_{2x}$-globulin in 0.5 ml saline emulsified with 0.5 ml of complete Freund's adjuvant. The intradermal injections were repeated three times at two-week intervals and the animals were bled one week after the last injection.

Methods After a short centrifugation, the urine was concentrated x 100 by pressure dialysis through a PM 10 membrane (Amicon) at 4°C. The concentrate was chromatographed 10 ml at a time, on a column (100 x 2.6 cm) of Sephadex G-75 in 0.05 M Tris-HCl buffer pH 7.4, containing 0.2 M NaCl and 0.02 % sodium azide. 4 ml fractions were collected at a flow rate of 16 ml/h. The fractions were analyzed by electrophoresis (4), immunoelectrophoresis (5) or electroimmunoassay (6), using Coomassie blue for protein staining. The fractions containing the $\beta_{2x}$-globulin were pooled and equilibrated with a 0.01 M Tris-HCl buffer pH 7.9, containing 0.02 M NaCl, by filtration through Sephadex G-25. This material was then applied to a column of DEAE-cellulose and the proteins were eluted with 400 ml of a linear NaCl gradient (0.02 M to 0.3 M) in the starting Tris-HCl buffer. Fractions containing $\beta_{2x}$-globulin were pooled and concentrated by ultrafiltration through 8/32 inch Visking dialysis tubing which had been boiled in distilled water for 20 min before use to avoid loss of low molecular weight proteins (7). Final purification was obtained by preparative zone electrophoresis in agarose gel in 0.075 M Na-barbital/barbital buffer, pH 8.6 (4).
Sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis was done in 0.1 % SDS after reduction with β-mercaptoethanol, as described by Weber and Osborn (8). Carbohydrate was determined by the anthrone method (9). The ultraviolet spectrum was determined in Sørensen phosphate buffer (pH 7.4, 0.067 M) with a Zeiss PMQ II spectrophotometer. $E^{1\%, 1\,cm}_{280\,nm}$ was measured after drying to constant weight over $P_2O_5$ at 50°C. Amino acid analysis was performed with a LKB 3201 analyzer after overnight hydrolysis of the sample in 6 M HCl at 105°C with 0.1 % phenol to protect tyrosine. Tryptophan was not determined.
The apparent sedimentation coefficient at 20°C was measured in a isokinetic gradient of sucrose using a Spinco Model L 265 B ultra-

centrifuge and a SW 41 rotor, as described by Johns and Stanworth (10). The measurement was made in 0.05 M Tris-HCl buffer, pH 7.4. The molecular weight was determined by high speed equilibrium ultracentrifugation with a Beckman model E ultracentrifuge and a An-D rotor, as described by Chervenka (11).

## RESULTS AND DISCUSSION

Purification On Sephadex G-75 the concentrated urine from the cadmium exposed worker was resolved into six major peaks (Fig. 1.). Electroimmunoassay with specific anti-RBP and anti-$\beta_2$-microglobulin showed that the sixth peak (fractions 52 to 62) contained mainly $\beta_2$-microglobulin and the fifth, RBP. The fifth peak was characterized by a parallel absorbance at 280 and 340 nm (Fig. 1.) and in agarose gel electrophoresis at pH 8.6, gave three major bands in the $\alpha_2$-region and a weak band in the $\beta$ region (Fig. 2.). The three $\alpha_2$-components were identified in immunoelectrophoresis as the various forms of RBP. The $\beta_2$-band failed to react in immunoelectrophoresis with antisera against $\beta_2$-microglobulin and RBP.

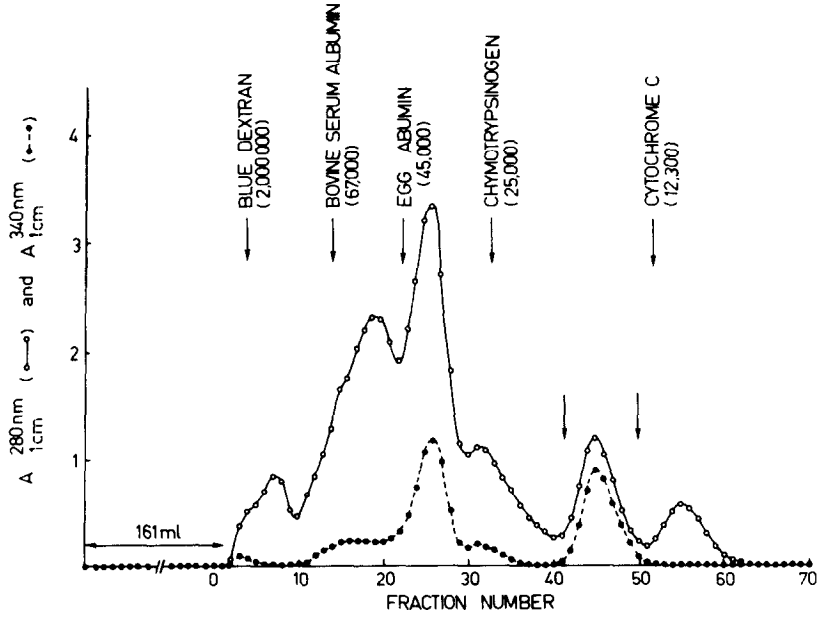


Fig. 1. Chromatogram on Sephadex G-75 of 100-fold concentrated urine from a cadmium-exposed worker. Fractions containing RBP and $\beta_{2x}$-globulin were pooled as indicated by the lower arrows.

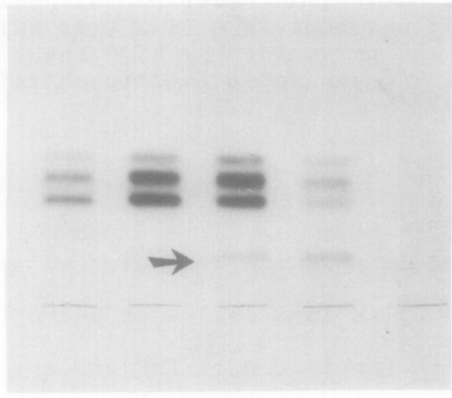


Fig. 2. Electrophoresis in agarose gel at pH 8.6 of the fractions 38 to 48 from Sephadex G-75 containing RBP and $\beta_{2x}$-globulin. The latter is indicated by the arrow.

DEAE-cellulose chromatography of the fifth peak gave three major fractions (Fig. 3.) which, as shown by agarose gel electrophoresis, contained the different forms of RBP (Fig. 4.). This chromatography

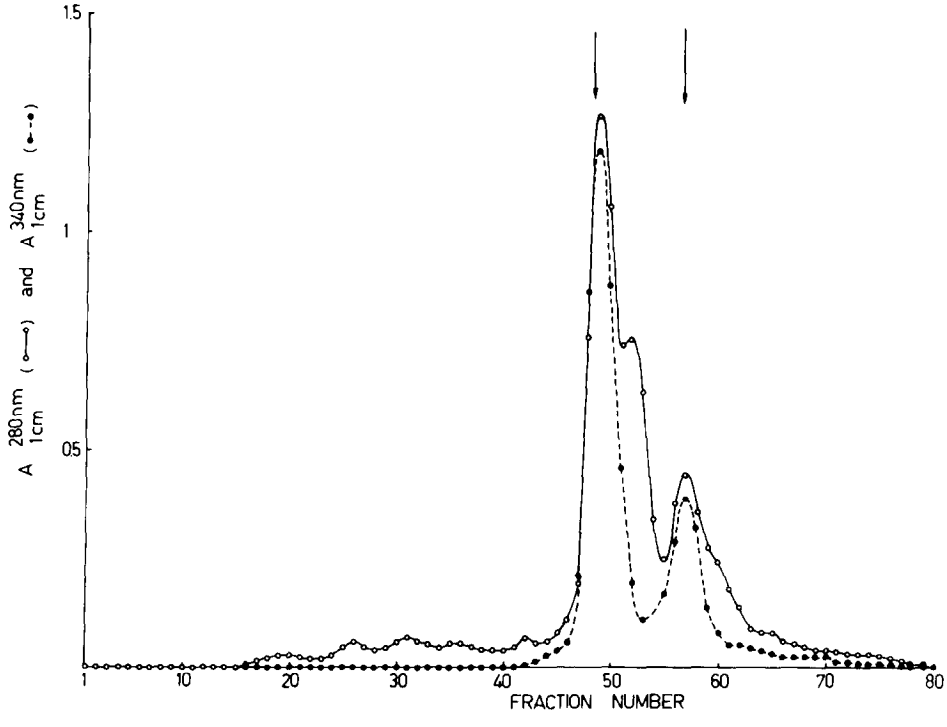


Fig. 3. Chromatogram on DEAE-cellulose of the material from Sephadex G-75 containing RBP and $\beta_{2x}$-globulin. Those fractions between the arrows were pooled and concentrated for preparative electrophoresis.

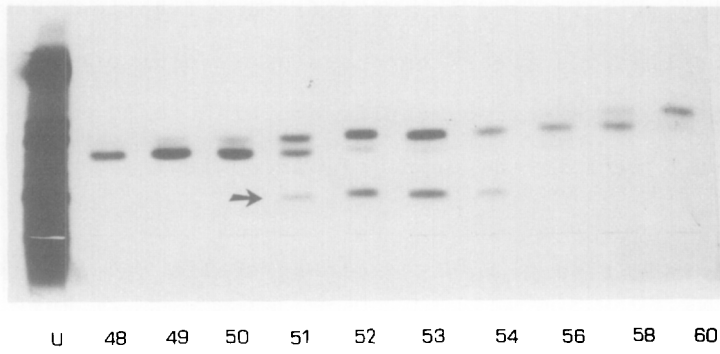


Fig. 4. Electrophoresis in agarose gel at pH 8.6 of the fractions 48 to 60 of the DEAE-cellulose chromatogram. The arrow indicates the $\beta_{2x}$-globulin. U = concentrated urine used as starting material.

resulted in the elimination of small amounts of $\beta_2$-microglobulin, which was eluted earlier (0.11 M NaCl gradient, fraction 31) than $\beta_{2x}$-globulin. The latter, recognized in agarose gel electrophoresis, was eluted together with RBP, in the unresolved second peak (0.17 M NaCl gradient) (Fig. 4).

$\beta_{2x}$-globulin was finally purified by preparative agarose gel electrophoresis at pH 8.6 (Fig. 5.). The yield, which was estimated approximately from the intensity of the electrophoretic band of $\beta_{2x}$-globul

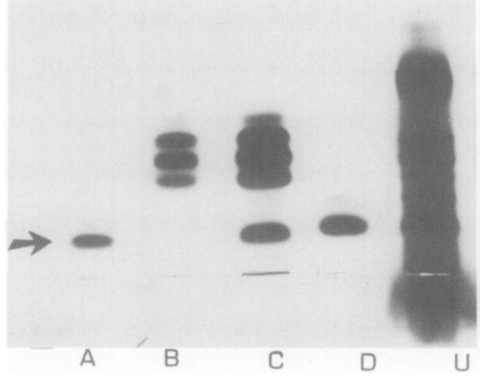


Fig. 5. Electrophoresis in agarose gel at pH 8.6 of isolated $\beta_{2x}$-globulin (A), isolated RBP (B), starting material of the preparative electrophoresis (C) and of isolated $\beta_2$-microglobulin (D). The electrophoretic mobility of $\beta_{2x}$-globulin is slightly less than that of $\beta_2$-microglobulin. The latter was isolated by chromatographing the sixth peak of Sephadex G-75 chromatogram on a DEAE-cellulose column using 400 ml of a linear gradient of NaCl (0.02 to 0.2 M) in 0.01 M Tris-HCl pH 7.9. U = concentrated urine used as starting material.

in the starting material and in the different fractions was about 5 %. $\beta_{2x}$-globulin is a minor component of tubular proteinuria. Whereas the concentration of $\beta_2$-microglobulin amounted to 30 mg/l, $\beta_{2x}$-globulin did not exceed 5 mg/l in the urine of our patient

Properties In addition to anti-$\beta_2$-microglobulin and anti-RBP sera we tested in immunoelectrophoresis antisera directed against the following serum proteins : $\kappa$ and $\lambda$ immunoglobulin light chains, $\beta_2$-glycoprotein III, IgG, IgA, IgM and albumin. No precipitin arc was obtained with purified $\beta_{2x}$-globulin at a concentration of about 1 mg/ml. The two rabbit antisera prepared against $\beta_{2x}$-globulin gave a slight precipitin arc in the $\beta_2$-region with most samples of 100-fold concentrated urines from normal males (n = 7) and females (n = 5). This suggests that $\beta_{2x}$-globulin is a normal constituent of urine. When tested against normal human plasma, no precipitin arc was observed in the $\beta_2$-region. The ultraviolet spectrum of $\beta_{2x}$-globulin was characterized by a minimum at 250 nm and a maximum at 279 nm. $E^{1\ \%,\ 1\ cm}_{280\ nm}$ of $\beta_{2x}$-globulin, of which the concentration was determined by dry weight (see Materials and Methods), was 7.1. No sugar was detected by the anthrone reaction when applied to a solution containing about 1 mg/ml.

A molecular weight of 21,000 was estimated by SDS/polyacrylamide gel electrophoresis in the presence of ß-mercaptoethanol. Preliminary data were also obtained by ultracentrifugation. The apparent sedimentation coefficient at 20°C in 0.05 M Tris-HCl buffer 7.4, containing 0.15 M NaCl, was 2.4 S. The molecular weight was also estimated by high speed equilibrium ultracentrifugation. The calculations were made by assuming a partial specific volume of 0.73, similar to that of $\beta_2$-microglobulin (1) and RBP (2). Two runs were performed and the values found at 32,270 rpm for 45 h and 30,200 rpm for 47 h were 21,900 and 19,700 respectively.

As the $\beta_{2x}$-globulin has nearly the same elution volume on Sephadex G-75 as the RBP (Fig. 1. and 2.), its molecular weight should be rather similar to that of RBP. By calibrating the column on Sephadex G-75 wit reference proteins (Fig. 1.), we calculated a molecular weight of 18,700 for the RBP. However, Peterson and Berggård (2) obtained a value of 21,400 for this protein with the more reliable technique of sedimentation equilibrium ultracentrifugation.

A preliminary amino acid analysis performed on one preparation revealed an unusual absence of cystein, methionine and histidine. Tryptophan was not determined.

Comparison with other low molecular weight urinary proteins Among the urinary proteins with molecular weight below 40,000 (Table 1), only the free immunoglobulin light chains and the 3 S $\gamma_1$-globulin show some similarities regarding molecular weight and electrophoretic mobility

Table 1.

Comparison of $\beta_{2x}$-globulin with other low molecular weight urinary proteins.

| Protein | Molecular Weight | Electrophoretic Mobility | Ref. |
|---|---|---|---|
| $\beta_{2x}$-globulin | 21,000 | β | |
| γ-trace | 10,000 | post-γ | 12 |
| β-trace | 30,000 | β | 12 |
| Minialbumin | 10,000 | α | 13 |
| $\beta_2$-microglobulin | 11,800 | β | 1 |
| Lyzozyme | 14,600 | post-γ | 14 |
| Ribonucleases | 13,000-20,000-32,000 | - | 15 |
| Retinol binding protein | 21,400 | α | 2 |
| Free immunoglobulin light chains | 23,000 | α-γ | 16 |
| 3 S $\gamma_1$-globulin (Carbonic anhydrase B) | 25,000 | γ | 16,17 |

with $\beta_{2x}$-globulin. However, the latter had, whatever the technique used, a molecular wieght lower than that of light chains. In SDS-polyacrylamide gel electrophoresis the $\beta_{2x}$-globulin band was clearly distinc from that of light chains. Also, light chains do not bind to DEAE-cellulose in 0.02 M Tris-HCl buffer, pH 8 (18) or at pH 8.5 (19), whereas the elution of $\beta_{2x}$-globulin at pH 7.9 required 0.17 M NaCl. The amino acid compositions were also different (20) and the $\beta_{2x}$-globulin failed to react with an anti-$\kappa$ or $\lambda$ light chains serum.

The urinary 3 S $\gamma_1$-globulin (14), which has a molecular weight of 25,000, probably corresponds to the plasma 3 S $\gamma_1$-globulin described by Ikenata et al. (17). This protein has an amino acid composition different from that of $\beta_{2x}$-globulin. Furthermore, its ultraviolet absorption curve has a shoulder at 290 nm which was not observed with $\beta_{2x}$-globulin. The extinction coefficient ($E^{1\%}_{280\ nm}$) of 3 S $\gamma_1$-globulin is 14.7 (17), twice that estimated for $\beta_{2x}$-globulin.

The occurrence in urine of ribonucleases with molecular weights of 13,000, 20,000 and 33,000 has been reported recently (15). These enzymes have not yet been characterized sufficiently to be compared with $\beta_{2x}$- globulin

The hypothesis that $\beta_{2x}$-globulin could be a degradation product of fibrinogen or fibrin was not supported by tests with anti-fibrinogen serum. No fragment with a molecular weight around 20,000 has been reported to result from fibrinolysis (16).

The $\beta_{2x}$-globulin seems then to be a so far undescribed low molecular weight protein occurring in normal human urine. Whether this protein is present in plasma or is related to another plasma component remains to be answered.

ACKNOWLEDGEMENTS We are grateful to Dr C. RICHARDS, for correcting the manuscript. The competent technical assistance of E. VAN ROOST who performed the ultracentrifugation work is gratefully acknowledged.

REFERENCES

1. Berggård, I. and Bearn, A.G. (1968) J. Biol. Chem. 243, 4095-4103.
2. Peterson, P.A. and Berggård, I. (1971) J. Biol. Chem. 246, 25-33.
3. Butler, E.A. and Flynn, F.V. (1961), J. Clin. Path. 14, 172-178.
4. Johansson, B.G. (1972) Scand. J. Clin. Lab. Invest. 29, 17-19.
5. Laurell, C.B. (1965) Anal. Biochem. 10, 358-361.
6. Laurell, C.B. (1966) Anal. Biochem. 15, 45-52.
7. Davis, J.S., Flynn, F.V. and Platt, H.S. (1968) Clin. Chim. Acta 21, 357-376.
8. Weber, K and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
9. Scott, T.A. and Melvin, E.H. (1953) Anal. Chemistry 25, 1656-1661.
10. Johns, P. and Stanworth, D.R. (1976) J. Imm. Meth. 10, 231-252.
11. Chervenka, C.H. (1970) Anal. Biochem. 34, 24-29.
12. Link, H. (1967) Acta Neur. Scand. 43, suppl. 28, 7-130.
13. Kench, J.E. and Sutherland, E.M. (1966) South Afr. Med. J. 40, 1109-1116.
14. Manuel, Y., Revillard, J.P. and Betuel, H. (1976) Proteins in normal and pathological urine. S. Karger Basel (Switzerland) New York.
15. Yamanaka, M., Akagi, K., Murai, K., Hirao, N., Fujimi, S. and Omae,T. (1977) Clin. Chim. Acta 78, 191-201.
16. Jamieson, G.A. and Greenwalt, T.J. (1976) In trace components of plasma. Isolation and chemical significance. Progress in chimical biological Research, vol. 5.
17. Ikenata, T., Gitlin, D., and Schmid, K. (1965) J. Biol. Chem. 240, 2868-2876.
18. Deutsch, H.F. (1963) Science 141, 435-436.
19. Deutsch, H.F. (1965) Immunochemistry 2, 207-208.
20. Dayhoff, M.O. (1972) Atlas of proteins sequence and structure, vol. 5.