

Characterization of Immunochemically Nonreactive Urinary Albumin

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Background: Conventional immunoassays underestimate the urinary albumin concentration because intact albumin in urine exists in two forms, immunoreactive and immunochemically nonreactive.

Methods: Urinary albumin concentration measured by HPLC (which measures total albumin, i.e., the sum of immunoreactive albumin + immunochemically nonreactive albumin) or RIA was compared with densitometric analysis of albumin bands in diabetic urine samples separated by either native polyacrylamide gel electrophoresis (PAGE) or reducing sodium dodecyl sulfate (SDS)-PAGE. Immunochemically nonreactive albumin was also isolated from diabetic urine (relative amount detected, 70–80% of the expected) and was tested for contamination by common urinary proteins by native PAGE, ELISA, and capillary electrophoresis.

Results: Urinary albumin concentrations measured by native PAGE and HPLC were better correlated ($r^2 = 0.83$) than concentrations measured by native PAGE and RIA ($r^2 = 0.62$) because under native conditions both native PAGE and HPLC detect total albumin and not only the immunoreactive albumin alone that is measured by RIA. Urinary albumin concentrations measured by reducing SDS-PAGE and RIA were better correlated ($r^2 = 0.84$) than concentrations measured by reducing SDS-PAGE and HPLC ($r^2 = 0.65$) because under reducing conditions immunochemically nonreactive albumin is unstable and fragments into many smaller peptides. The partially purified preparation was found to contain <1% contamination by common urinary proteins and is stable to freezing and frequent freeze/thaw cycles.

Conclusions: The results are consistent with the interpretation that immunochemically nonreactive albumin

has a limited number of polypeptide chain scissions and is held together by noncovalent intrachain bonding and disulfide bonds. Detection of this molecule is likely to be of clinical importance in diagnosing kidney disease as well as cardiovascular disease.

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Measurement of albumin (M_r 66 000) in urine is complicated because urinary albumin may exist in multiple forms. This has been highlighted by the discovery that diabetic rats excrete increased quantities of intact albumin during the development of albuminuria that is undetectable by conventional immunoassays (immunochemically nonreactive albumin) (1). Immunochemically nonreactive albumin has also been found in urine from diabetic patients analyzed by HPLC, which is able to measure both immunoreactive albumin and immunochemically nonreactive albumin (total albumin) (2). In addition, we have found in patients with type 1 and type 2 diabetes that measurement of total albumin by HPLC can predict the onset of persistent albuminuria 3.9 and 2.4 years earlier, respectively, than can measurement of immunoreactive albumin alone by conventional RIA (3). These results not only identify that progression from normo- to microalbuminuria in diabetic patients is associated with an increase in urinary immunochemically nonreactive albumin, but raises the possibility that measurement of total albumin may allow earlier detection of progression to kidney disease (3). Similar results have also been found in peripheral vascular disease (4).

The aim of the present study was to further characterize the immunochemically nonreactive albumin species present in human diabetic urine. We compared quantitative analysis of total urinary albumin measured by HPLC or immunoreactive albumin measured by RIA with densitometric analysis of the albumin band in urine samples separated by native polyacrylamide gel electrophoresis (PAGE)³ and reducing sodium dodecyl sulfate (SDS)-

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³ Nonstandard abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HSA, human serum albumin; and LC/MS/MS, liquid chromatography–tandem mass spectrometry.

PAGE. The purified immunochemically nonreactive albumin preparation was also analyzed for contaminants by HPLC, ELISA, and capillary electrophoresis.

Materials and Methods

CHARACTERIZATION OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN

PAGE. Urines collected from diabetic patients ($n = 32$) over 24 h (stored at -20°C) were analyzed by native PAGE and reducing SDS-PAGE. The total albumin content of the samples was determined by HPLC (see below). Approximately $0.3\ \mu\text{g}$ of protein was separated by electrophoresis in 12.5% gels at 200 V for 2 h (Bio-Rad Laboratories) and then silver-stained using Proteosilver staining reagents (Sigma-Aldrich). Variation between gels was corrected through the use of a standard urine included in each gel. Albumin band thickness was quantified by computer-aided densitometry (MCID software, Ver. 7.0; Imaging Research Inc.). The background density of the gel was subtracted from each band, and results were expressed as relative absorbance. The relative absorbance determined for the albumin band in each urine was quantified by use of an albumin calibrator run on each gel, and this was compared with the urinary albumin concentrations measured by HPLC analysis, which detects total albumin (i.e., the sum of immunoreactive albumin + immunochemically nonreactive albumin) and RIA, which detects immunoreactive albumin only. Densitometry quantified in this manner was found to be linear for human serum albumin (HSA) calibrator solutions up to 100 mg/L. In addition, bands excised from the native gels were sequenced by liquid chromatography–tandem mass spectrometry (LC/MS/MS) by the Australian Proteome Analysis Facility (North Ryde, Sydney, Australia).

HPLC. Another group of fresh (unfrozen) diabetic urines were also analyzed by HPLC and then reanalyzed after freezing at -20 and -80°C for 24 h, 1 week, and 2 months to determine whether freezing had an effect on urine composition. In addition, urines containing large amounts of immunochemically nonreactive albumin were analyzed after multiple (six) freeze/thaw cycles.

ALBUMIN ASSAYS

Immunoassays. Immunoreactive albumin was measured by either a double-antibody RIA (in-house) or by immunoturbidimetry on a Dade-Behring Turbitimer with reagents supplied by Dade-Behring Marburg GmbH as described previously (2, 3).

HPLC analysis. Total urinary albumin (immunoreactive albumin plus immunochemically nonreactive albumin) was determined by analyzing urine samples using an Agilent 1100 HPLC system (Agilent Technologies) (2, 3). Aliquots of urine ($25\ \mu\text{L}$) were injected on a Zorbax GF-250 analytical column [$9.4\ \text{mm}$ (i.d.) \times $250\ \text{mm}$]. The mobile phase was phosphate-buffered saline, pH 7.0, at a

flow rate of 1–2 mL/min. The urinary albumin peak was identified to within $\pm 2\%$ of the elution time of the monomeric albumin calibrator. The intraassay CVs were 5.6% and 6.0% at concentrations of 44.7 mg/L and 141 mg/L, respectively, the interassay CV was 2.4% at 95.9 mg/L, and the detection limit was 2 mg/L.

ISOLATION OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN FROM HUMAN URINE

Urine was collected for 24 h from diabetic patients and analyzed for albumin content by both immunoturbidimetry and HPLC. Urine containing $\sim 50\%$ immunoreactive (e.g., 64 mg/L) and 50% immunochemically nonreactive (e.g., 47 mg/L) albumin was chosen for further purification. Urine was concentrated ~ 5 - to 10-fold by use of a 50 kDa cutoff membrane (Millipore Corporation). Concentrated urine was purified by an immunosubtraction procedure using cyanogen bromide-activated Sepharose (Sigma-Aldrich) according to the supplier's instructions. The ligand bound to the gel was rabbit anti-human albumin antibody (Dako Cytomation). Unbound albumin (or immunochemically nonreactive albumin) was eluted off the column and was found to have a concentration of <6 mg/L by immunoturbidimetry and 69 mg/L by HPLC.

Affinity-purified immunochemically nonreactive albumin was further purified to remove any remaining contaminants by chromatography on an Agilent 1100 HPLC system (see method described above). Typically, the detected amounts of immunochemically nonreactive albumin from urine were 70–80% of the expected values. HPLC-purified immunochemically nonreactive albumin was further concentrated by use of a 50 kDa cutoff membrane. Purified immunochemically nonreactive albumin was analyzed by native PAGE and reducing (with dithiothreitol) SDS-PAGE. Common urinary proteins, including HSA, transferrin (M_r 76 500), α_1 -acid glycoprotein (M_r 44 100), α_1 -antitrypsin (M_r 54 000), and α_2 -HS-glycoprotein (M_r 49 000), were used as size markers.

COMPETITIVE ELISAs

The immunochemically nonreactive albumin preparation was also tested by ELISA for the presence of other common urinary proteins: IgG (M_r 160 000), transferrin, α_1 -acid glycoprotein, and α_1 -antitrypsin. The presence of Na^+/K^+ -ATPase β -subunit was also tested by ELISA. IgG, transferrin, α_1 -acid glycoprotein, and adenosine 5'-triphosphatase were purchased from Sigma-Aldrich. α_1 -Antitrypsin was purchased from ICN Biomedicals Inc. Rabbit anti-human IgG (polyclonal to whole molecule), mouse anti-human α_1 -acid glycoprotein (monoclonal), and rabbit anti-human α_1 -antitrypsin (polyclonal) were purchased from Sigma-Aldrich. Rabbit anti-human transferrin (polyclonal) was from Dako Cytomation. Mouse anti-human Na^+/K^+ -ATPase β -subunit (monoclonal) was from ABR Affinity Bioreagents. The detection antibody used was alkaline phosphatase-conjugated goat anti-rabbit IgG (Dako) or goat anti-mouse IgG (Sigma-

Aldrich) diluted 1:2000 in assay buffer. The enzyme substrate used was *p*-nitrophenyl phosphate (Sigma-Aldrich). The plate was read at 405 nm.

Results

COMPARISON OF URINARY ALBUMIN ANALYSIS BY HPLC OR RIA AND DENSITOMETRIC ANALYSIS OF ALBUMIN BAND IN ELECTROPHORETIC PROFILES

Urines collected from diabetic patients over 24 h and stored at -20°C were analyzed by native PAGE. A representative gel is shown in Fig. 1. For three diabetic urines containing relatively large quantities of immunochemically nonreactive albumin, the bands that migrated the same distance on the gel as the albumin calibrator were excised from the gel. These bands were sequenced by LC/MS/MS and were demonstrated to contain only albumin (two of these are shown in Fig. 1). Native PAGE analysis of other plasma protein markers, including α_1 -antitrypsin, transferrin, α_1 -acid glycoprotein, and α_2 -HS-glycoprotein, showed that these proteins migrated differently from urinary albumin (Fig. 2). IgG was too large to enter the native gels.

The results of the native PAGE analysis (Fig. 1) are striking because the major protein found in all of the diabetic urines analyzed was albumin, and the RIA clearly underestimates the amount of albumin in urine. For example, for the urine sample in the fourth lane from the right in Fig. 1, the RIA would suggest that there is only minimal albumin (3.78 mg/L), whereas from the band intensity and from the HPLC-estimated value (41.3 mg/L), there is clearly a far greater amount of albumin present. To further demonstrate this, we determined the correlation between urinary albumin concentration measured by native PAGE and either HPLC or RIA (Fig. 3A) and found good correlation ($r^2 = 0.83$) between native PAGE and HPLC analysis in spite of the varying ratio of HPLC/RIA concentrations (Fig. 1) but a poor correlation

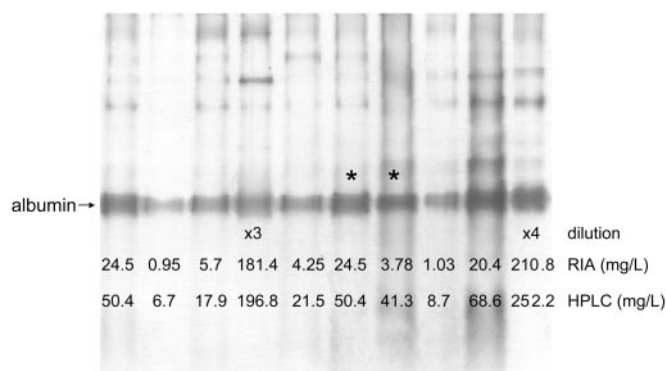


Fig. 1. Native PAGE analysis of diabetic urines containing various amounts of immunoreactive albumin and immunochemically nonreactive albumin.

The amounts of albumin determined by HPLC and RIA are shown, as is the position of the albumin monomer. *, bands sequenced by LC/MS/MS by the Australian Proteome Analysis Facility. These bands were demonstrated to contain only albumin.

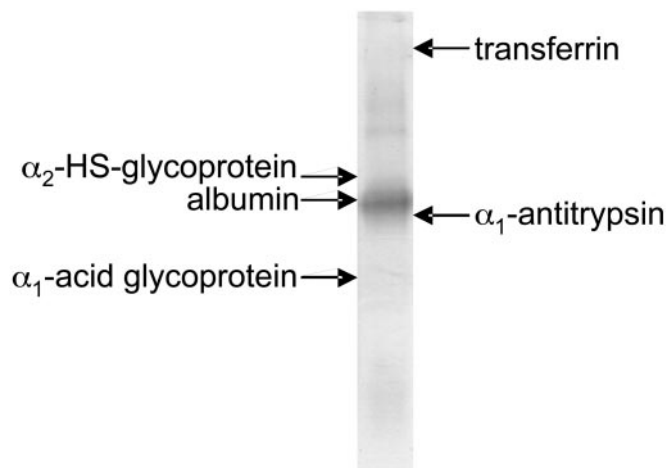


Fig. 2. Native PAGE analysis of HPLC-purified immunochemically nonreactive albumin.

The positions of albumin, α_1 -antitrypsin, transferrin, α_1 -acid glycoprotein, and α_2 -HS-glycoprotein are indicated.

between native PAGE and RIA [$r^2 = 0.62$ ($P = 0.0778$ vs the correlation coefficient for native PAGE and HPLC)]. Similar analyses were performed for the same diabetic urines analyzed by reducing SDS-PAGE (Fig. 3B). The results of this analysis were the opposite of those for native PAGE, with a good correlation ($r^2 = 0.84$) between reducing SDS-PAGE and RIA and a poor correlation between reducing SDS-PAGE and HPLC [$r^2 = 0.65$ ($P = 0.0895$ vs the correlation coefficient for reducing SDS-PAGE and HPLC)].

These results can be further explained by calculating the relative amount of albumin that was detected (quantity calculated by comparing densitometry values with HSA calibrators run on each gel) by native PAGE and reducing SDS-PAGE compared with that determined by either HPLC or RIA. Under native conditions, the mean (SE) amounts of albumin detected by native PAGE and HPLC were similar [89 (8.1)% of the expected value] compared with that detected by native PAGE and RIA [30 (6.8)% of the expected value]. Under reducing conditions, however, the amounts of albumin detected by reducing SDS-PAGE and RIA were equivalent [98 (5.1)% of the expected value] compared with the amounts detected by reducing SDS-PAGE and HPLC [28 (2.4)%] because, under native conditions, both native PAGE and HPLC detect total albumin (immunoreactive albumin + immunochemically nonreactive albumin). Only immuno-reactive albumin is detected by the RIA; therefore, the amount detected is lower. On the other hand, under reducing conditions, the immunochemically nonreactive albumin fragments into many smaller bands (Fig. 4), and therefore, the amounts detected by reducing SDS-PAGE and RIA become equivalent because both are detecting the immunoreactive albumin species.

Sera from patients with a high urinary content of immunochemically nonreactive albumin were also ana-

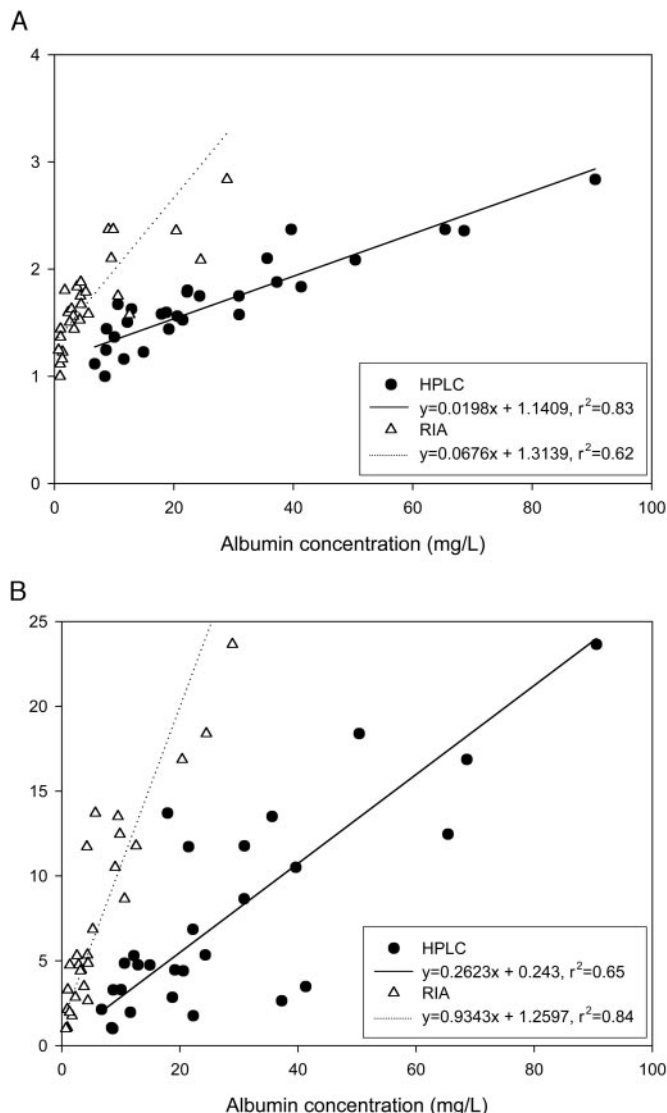


Fig. 3. Relative absorbance of the urinary albumin monomer as determined by computer-aided densitometry after separation by native PAGE (A) or SDS-PAGE (B) compared with urinary albumin concentrations (<100 mg/L) measured by HPLC or RIA.

●, HPLC (mg/L); △, RIA (mg/L). Passing-Bablok analysis lines and correlation coefficients are also shown for relative absorbance vs HPLC (solid line) and relative absorbance vs RIA (dashed line).

lyzed by reducing SDS-PAGE (results not shown). These samples did not contain any of the distinctive bands of immunochemically nonreactive albumin when analyzed under these conditions, indicating that this species of albumin is formed during renal passage and is not present in the blood.

In a separate experiment, fresh (unfrozen) urines collected from diabetic patients were also analyzed by native PAGE and HPLC and then reanalyzed after freezing at -20 and -80 °C for 24 h, 1 week, and 2 months. None of the material present in the fresh urine was lost at either -20 or -80 °C, nor was extra material produced, as detected by HPLC or native PAGE after freezing (results

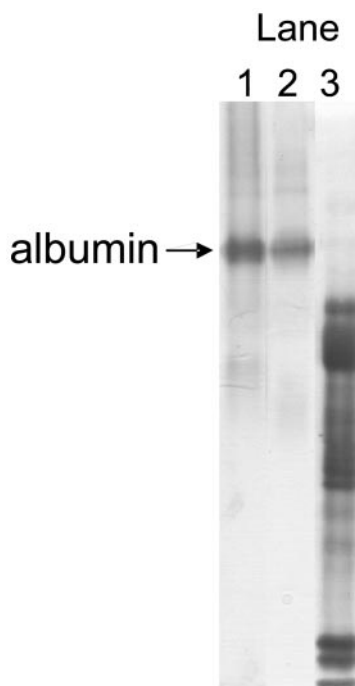


Fig. 4. Electrophoretic analysis of diabetic urine and HPLC-purified immunochemically nonreactive albumin.

Lane 1, undiluted diabetic urine analyzed by native PAGE; lane 2, HPLC-purified immunochemically nonreactive albumin analyzed by native PAGE; lane 3, HPLC-purified immunochemically nonreactive albumin analyzed by reducing SDS-PAGE.

not shown). In addition, there was no loss or increase in immunochemically nonreactive albumin in diabetic urine after six freeze/thaw cycles (mean HPLC value of 20.6 mg/L for fresh samples; mean value of 21.8 mg/L in samples after six freeze/thaw cycles).

ISOLATION OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN

The HPLC profiles obtained at various stages in the isolation of immunochemically nonreactive albumin from diabetic urine are shown in Fig. 5. The elution profile of HSA is shown in Fig. 5A. Both the monomer (retention time, 9.469 min) and dimer can be seen. In a typical urine profile from a diabetic patient (Fig. 5B), the albumin monomer eluted at 9.474 min. The same urine after concentration through a 50 kDa cutoff membrane is shown in Fig. 5C, and the elution profile of concentrated urine after immunosubtraction using anti-albumin antibodies to remove all immunoreactive albumin is shown in Fig. 5D. At this stage, the preparation was found to contain <6 mg/L albumin by immunoturbidimetry but 69 mg/L albumin by HPLC (retention time, 9.496 min). The preparation was further purified by HPLC fractionation (Fig. 5E). After HPLC fractionation, the preparation eluted as a single peak with a retention time similar to that of HSA and also migrated as a single band under native conditions (Fig. 4, lane 2). This preparation was further concentrated to 0.8 g/L by HPLC but was still found to

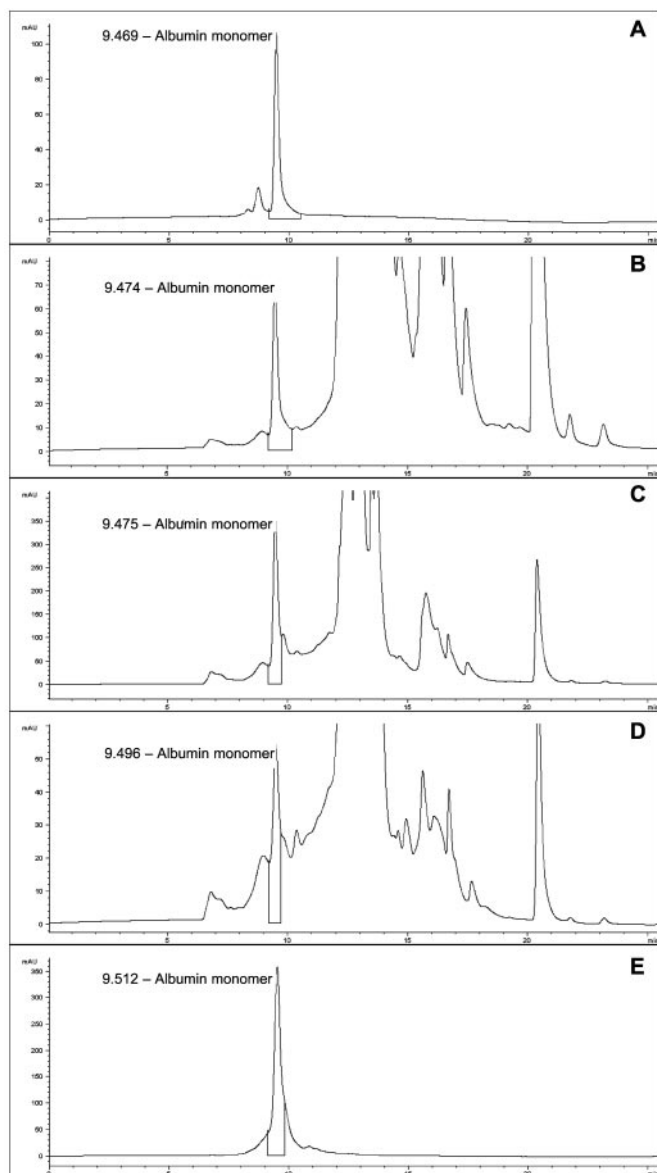


Fig. 5. HPLC profiles obtained at different stages in the isolation of immunochemically nonreactive albumin.

(A), HSA; (B), undiluted diabetic urine; (C), urine after concentration through a 50 kDa molecular cutoff membrane; (D), concentrated urine after immunosubtraction using anti-albumin antibodies; (E), affinity-purified urine after further purification by HPLC fractionation. The *x* axes show the elution times in minutes, and the *y* axes show the absorbance units (*mAU*) at 214 nm. The retention time for albumin monomer is indicated in each panel.

contain no immunoreactive albumin by both immunoturbidimetry and RIA.

ANALYSIS OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN BY ELISA

The immunochemically nonreactive albumin preparation (0.8 g/L) was analyzed by ELISA for the presence of other proteins commonly found in urine, including IgG, transferrin, α_1 -acid glycoprotein, α_1 -antitrypsin, and Na^+/K^+ -ATPase β -subunit. None of the proteins tested was present at a concentration exceeding 1% in the immuno-

chemically nonreactive albumin preparation. The amounts of contamination were most likely less than those indicated because the concentrations of these proteins were below the detection limits of the ELISAs and could not be accurately measured. There was also no immunoreactive albumin present as tested by both RIA and immunoturbidimetry. In addition, <1% Tamm-Horsfall glycoprotein (M_r 80 000–90 000) was detected in the preparation by capillary electrophoresis (5).

Discussion

In this study we isolated a species of albumin from urine collected from several diabetic patients that is not detected by conventional antibodies generated against native serum albumin. We named this molecule immunochemically nonreactive albumin. The molecule appears to be the same size as HSA because it elutes at the same position in size-exclusion HPLC and native PAGE. To ensure that the immunochemically nonreactive albumin preparation did not contain other common urinary proteins of similar size to albumin, we tested the preparation for the presence of IgG, transferrin, α_1 -acid glycoprotein, α_1 -antitrypsin, Na^+/K^+ -ATPase β -subunit, and α_2 -HS-glycoprotein. There was <1% contamination by any of these other proteins. Similarly, these possible contaminants did not migrate at the same position as the immunochemically nonreactive albumin in native PAGE. In fact, given that the measured amount the protein was 70–80% of the expected and the much smaller amounts of other proteins found in urine compared with albumin (6), it is highly unlikely that we could have isolated such large quantities of any protein other than albumin. Similarly, we have previously analyzed diabetic urines containing a significant amount of immunochemically nonreactive albumin by two-dimensional electrophoresis (2) and found by sequencing that all of the major bands were HSA, demonstrating that albumin is the major urinary protein (2).

Some investigators have described Tamm-Horsfall glycoprotein as the most abundant protein in mammalian urine (7,8), and the reported effects of diabetes on Tamm-Horsfall glycoprotein excretion vary (9). Using capillary electrophoresis, we have been unable to detect any significant quantities of Tamm-Horsfall glycoprotein in the immunochemically nonreactive albumin preparation, nor have we detected this glycoprotein in any of the fresh or frozen diabetic or control urines, using native PAGE or HPLC. In fact, none of the material present in the fresh urine was lost nor was extra material produced, as detected by both of these techniques, after freezing. We may not have observed the presence of Tamm-Horsfall glycoprotein using the techniques in this study because it has a tendency to become gelatinous and aggregate when the sodium chloride concentration of the sample is close to 100 mmol/L or calcium chloride is 1 mmol/L (10).

Under reducing SDS-PAGE conditions, there was a major loss of immunochemically nonreactive albumin in

both undiluted urine and purified immunochemically nonreactive albumin preparations. These results are consistent with the interpretation that immunochemically nonreactive albumin is a composite of peptide chains resulting from limited proteolytic digestion and are held together by disulfide bonds and noncovalent interpeptide chain bonding. It is probable that immunochemically nonreactive albumin is formed as a result of renal passage, although we cannot eliminate the possibility that it is present in low concentrations in blood and that its removal is facilitated by renal clearance. It is of interest that a similar molecule was proposed by Yagame et al. (11), although they demonstrated the presence of albumin fragments by reducing SDS-PAGE and did not demonstrate that the molecule ever existed in an intact form. They did demonstrate the prevalence of relatively large albumin fragments in diabetic urines compared with control urines.

Conventional antibodies generated against native serum albumin do not recognize modified intact urinary albumin (12–14). In fact, we have tested in excess of 30 different polyclonal and monoclonal albumin antibodies and have none that detect immunochemically nonreactive albumin. These antibodies have been raised against serum albumin and not urinary albumin. It is clear that immunoassays measure an active epitope in the urine; however, this epitope may not be associated exclusively with intact albumin (12–14). The immunochemically nonreactive nature of this albumin molecule may be attributable to the fact that the epitope is altered by a conformational change as a result of incomplete processing by the lysosomal pathway, which is compromised in diabetes but not in healthy individuals (12, 13), or to the attachment of ligands such as glucose or fatty acids, which are increased in diabetes.

We have recently shown in both diabetic rats and in diabetic patients that the progression to microalbuminuria is accompanied by an increase in the concentration of immunochemically nonreactive albumin. In fact, for patients with type 1 or type 2 diabetes, detection of this albumin species by HPLC can predict the onset of persistent albuminuria 3.9 and 2.4 years earlier, respectively, than can detection of immunoreactive albumin by conventional immunochemistry-based tests (3), with similar results found in peripheral vascular disease (4). Isolation and partial characterization of immunochemically nonreactive albumin may therefore be important for early detection of the kidney disease associated with diabetes and possibly of other albuminuric conditions, such as those associated with hypertension and cardiovascular disease (15, 16).

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