Characterization and cellular distribution of acidic peptide and oligosaccharide metal-binding compounds from kidneys

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Two low-molecular-mass Ni-binding fractions first isolated from human kidneys [Templeton & Sarkar (1985) *Biochem. J.* **230**, 35–42] are further characterized. Both components are acidic and are readily separated from each other by gel chromatography on Bio-Gel P-2. After equilibration with ^{63}Ni the largest complex constitutes about 30% of the radioactive ^{63}Ni and is an approx. 3.5 kDa peptide and the smallest species comprise short oligosaccharides containing 70% of the radioactivity. Both of these components are found in human, bovine and porcine kidneys as well as in a porcine proximal tubule-like cell line LLC-PK1. There is a small variation in amino acid composition between species. The oligosaccharides are reducing sugars and contain sulphate, glucosamine, glucuronic acid and iduronic acid with two to four overall negative charges. The monosaccharide composition was determined by h.p.l.c. with pulsed amperometric detection of the acid hydrolysates and by gas chromatography. In the LLC-PK1 cell line the acidic peptide is both intracellular and extracellular, whereas the oligosaccharides are only intracellular. The concentration of extracellular peptide, as measured by ^{63}Ni binding, is found to increase after exposure of the cells to low micromolar concentrations of Ni, whereas the oligosaccharide concentrations, also measured by ^{63}Ni binding, remain constant. The oligosaccharide component is decreased by 40% in the presence of NH_4Cl , suggesting that it is derived from degradation of internalized heparan sulphate.

INTRODUCTION

Kidneys are the target organ for the excretion of a number of toxic and essential metals [1] but little is known about the actual compounds involved in the trace metal homeostasis in kidney. For example, although the majority of serum Ni is bound to albumin [2], Ni is rapidly excreted in the urine after intraperitoneal administration in rat [3,4] through an unknown mechanism. It has been postulated that albumin-bound Ni exchanges with low-molecular-mass ligands to accomplish this excretion [2,5], but this remains to be demonstrated. Characterization of such compounds would be useful for designing protocols for the treatment of metal toxicity [6].

A second and more subtle form of metal ion metabolism must exist but has been scarcely investigated, which is intracellular or perhaps more distinctly intraorganelle metal ion transport. For instance, the carcinogenicity and toxicity of Ni is thought to correlate with the amount of intracellular Ni [7], and Ni taken up in Chinese hamster ovary cells is known to be distributed preferentially in the nucleus through an unknown mechanism [8]. Unravelling the biochemical components responsible for such accumulations could be important to an understanding of metal-ion-related carcinogenesis.

Previous results from our laboratory have consistently found a low-molecular-mass Ni-binding pool from a variety of sources including trophoblast cells [9], serum [10] and kidney cytosol [11]. In this work we report on the further characterization of two components first demonstrated to bind injected ^{63}Ni in rats [11] and subsequently isolated from human kidneys [12]. One is an approx. 3.5 kDa acidic peptide and the second is a fraction comprised of small acidic oligosaccharides. That acidic peptides can be involved in metal ion homeostasis *in vivo* is not surprising but the presence of metal-binding oligosaccharides is novel.

EXPERIMENTAL

Materials

Sodium acetate was puris P.A. grade from Fluka Chemical AG, Ronkonkoma, NY, U.S.A. Chelate-N, Chelex-100, C₁₈ Sepaks and Bio-Gel P-2 (200–400 mesh) were from Bio-Rad (Mississauga, Ont., Canada). SP-Sephadex and Sephadex G-10 (ultrafine) were from Pharmacia (Etobicoke, Ont., Canada). Buffers were of analytical grade and the water used had low conductivity from an ion-exchange system. HCl (ultrapure from Baker Chemicals, Phillipsburg, NJ, U.S.A.), KH_2PO_4 and trifluoroacetic acid (Fisher Scientific, Nepean, Ont., Canada) were used to adjust the pH of buffer solutions. *N*-Ethylmorpholine (Aldrich-Terochem, Toronto, Ont., Canada) was redistilled before use. Radioactive $^{63}\text{NiCl}_2$ and aqueous counting scintillant were purchased from Amersham Corporation (Oakville, Ont., Canada). $^{54}\text{MnCl}_2$, $^{109}\text{CdCl}_2$ and $^{65}\text{ZnCl}_2$ were obtained from New England Nuclear Corporation (Lachine, PQ, Canada). These last two isotopes were converted into acetates before use by dissolving the stock solution in acetate buffers. $^{67}\text{CuCl}_2$ was supplied by Los Alamos National Laboratory (Los Alamos, NM, U.S.A.) and NaB^3H_4 was from New England Nuclear. Foetal calf serum (FCS) was obtained from BDH, Toronto, Ont., Canada, and α -modified Eagle's medium (α -MEM) and phosphate-buffered saline (PBS; 2.69 mM-KCl/1.47 mM- KH_2PO_4 /137 mM-NaCl/8.07 mM- $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.1) were purchased from Gibco, Burlington, Ont., Canada.

Kidney preparations

Bovine kidneys were obtained from freshly butchered cattle, collected on ice and used immediately or stored frozen. Human kidneys were obtained at autopsy, transported on ice and processed immediately. Four kidneys were examined in this

Abbreviations used: FCS, foetal calf serum; α -MEM, α -modified Eagle's medium; PBS, phosphate-buffered saline; f.a.b.-m.s., fast atom bombardment mass spectra; h.p.l.c.-p.a.d., high performance liquid chromatography with pulsed amperometric detection.

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study: from a 71-year-old woman (heart failure), a 77-year-old male (sclerotic kidney, heart failure and colon cancer but no chemotherapy), 69-year-old male (myocardial infarction with sclerotic kidney) and a 71-year-old male (arterial disease). Individual kidneys were homogenized in ice-cold 0.9% NaCl with 50 μ M-NaF and 1 mM-phenylmethanesulphonyl fluoride. The soluble fraction was obtained as the supernatant after centrifugation at 20000 *g* for 1 h at 4 °C. This supernatant was immediately passed through a 10000 Da molecular-mass cut-off (YM-10) ultrafiltration membrane (Amicon, Oakville, Ont., Canada) using about 345 kPa N_2 at 4 °C and the filtrate was concentrated on a nominally 500 Da molecular-mass ultrafiltration membrane (YC05) using about 172 kPa N_2 at 4 °C. The retentate was either used directly for chromatography or freeze-dried and stored at -20 °C. Approximately 5 ml samples were pre-equilibrated overnight with approx. 10^6 – 10^7 c.p.m. of $^{63}\text{NiCl}_2$ and then loaded and eluted in water on a Bio-Gel P-2 (200–400 mesh) permeation column (2.5 cm $\times$ 90 cm). The void volume was determined with Dextran Blue and the column volume with NaCl using AgNO_3 to test for Cl^- ions. The fractions (with or without pre-equilibration with radioactive tracer) corresponding to the radioactive peaks (Fig. 1) were pooled and freeze-dried for h.p.l.c. or f.p.l.c. in plastic tubes. Gel permeation on a Sephadex G-10 column (4.5 cm $\times$ 90 cm) was also performed in a similar manner. Fractions were collected and analysed for radioactivity. Carbohydrate was measured either using the phenol/sulphuric acid test [13] or the Dimethylmethylene Blue (Serva-Terchem, Toronto, Ont., Canada) test [14].

Dispose treatment of kidney

Bovine kidney was finely minced and washed with PBS at 4 °C to remove contaminating erythrocytes. This was then exposed to disperse for 2 h at 37 °C in PBS. Free cells were isolated by filtering through a 70 μ m mesh nylon membrane and this was confirmed by microscopic examination. The cells were washed three times in homogenization buffer to remove any extraneous extracellular material and ruptured by homogenization at 4 °C. The cytosolic fraction was isolated as the supernatant from a 1 h 100000 *g* ultracentrifugation and processed as described.

H.p.l.c.

H.p.l.c. was performed on a Waters 510 chromatograph, equipped with an automated gradient controller and 481 spectrometric detector, using a Waters I-60 gel-permeation column (4.5 cm $\times$ 30 cm) in either 20 mM- NH_4HCO_3 , pH 7.6, or 10 mM-*N*-ethylmorpholine/HCl + 50 mM-NaCl, pH 7.4, buffers or a C-4 reversed-phase column (2.5 $\times$ 30 cm) in 0.015% trifluoroacetate, pH 3.0. A flow rate of 1 ml/min and u.v. spectral monitoring at 210–215 nm was used. Typically 1 min fractions were collected and counted for radioactivity using a liquid-scintillation β -counter (Beckman LS 8100) for ^{63}Ni or a γ -counter (Beckman Gamma 8000) for ^{67}Cu , ^{65}Zn , ^{54}Mn and ^{109}Cd .

F.p.l.c.

A Pharmacia f.p.l.c. system using a fixed 214 nm wavelength detector and a Pharmacia Mono Q HR 5/5 strong anion-exchange column (0.5 cm $\times$ 5 cm) was used. The flow rate was 1.0 ml/min. The starting buffer, A, was 5 mM- KH_2PO_4 , pH 4.8, and the final buffer, B, was 5 mM- KH_2PO_4 , pH 4.8, plus 150 mM- Na_2SO_4 . A typical gradient was A for 0–12 min, to 85% + 15% B over 4 min, hold at 85% A + 15% B for 20 min, increase to 100% B over 20 min and then hold at 100% B for 20 min. As much as 20 mg of peak 2 can be separated in one run under these conditions. However, it should be noted that extra salt in the injection sample seriously deteriorates the separation. Also the

length of equilibration in the starting buffer and the amount of sample injected alter the retention times. Thus it is necessary to optimize the gradient for every sample.

Desalting

Pooled fractions (4–20 ml) from the f.p.l.c. Mono Q column were subjected to ultrafiltration such that about 5 ml of retentate was kept for freeze-drying in 50 ml plastic centrifuge tubes and more than 100 ml of filtrate was discarded. The dry retentate was redissolved in 1–2 ml of water and 2 vol. of 100% ethanol was added. After 1 h at 4 °C the mixture was centrifuged at low speed and the supernatant kept. The precipitate containing the oligosaccharides was washed with a little ethanol and the liquids were added to the supernatant. This procedure was repeated. If ^{63}Ni was added before ultrafiltration to active fractions, about 80% of the radioactivity was recovered in the ethanol/water-insoluble precipitate.

N.m.r. and m.s.

^1H n.m.r. spectra were recorded at 500 MHz with a Bruker spectrometer at the n.m.r. spectroscopy laboratory of the Carbohydrate Research Centre, University of Toronto. Spectra were obtained at 19 ± 2 °C in $^2\text{H}_2\text{O}$ (99.98%, MSD, Pointe Claire, PQ, Canada) containing a trace of acetone (2.225 p.p.m. relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulphonate, ^1H) as the internal standard. Samples for n.m.r. analyses in $^2\text{H}_2\text{O}$ were passed through Chelex-100 or Chelate-N ion-exchange resins in H_2O , to remove paramagnetic impurities, freeze-dried and then further freeze-dried three times with $^2\text{H}_2\text{O}$.

Fast atom bombardment mass spectra (f.a.b.-m.s.) were recorded with a VG analytical ZAB-SE spectrometer at the m.s. spectroscopy laboratory of the Carbohydrate Research Centre, University of Toronto [15]. Samples were run in the negative ion mode with triethanolamine as the matrix [16].

Monosaccharide analysis

Hydrolysis was performed in sealed Pyrex glass tubes using 1 M-HCl (aq.) at 100 °C for 4 h or 4 M-methanolic HCl at 100 °C for 24 h. H.p.l.c. with pulsed amperometric detection (h.p.l.c.-p.a.d.) of the hydrolysed monosaccharides was carried out using a Dionex BioLC system with sodium acetate (0–150 mM) gradients in 50 mM-NaOH as described in detail in ref. [17]. Samples used mannitol (hydrolysis) or inositol (methanolysis) as internal standards. Methanolysis samples were neutralized, dried, acetylated with acetic anhydride and treated with Tri-Sil (Pierce, Mississauga, Ont., Canada) before injection into the gas chromatograph (Varian GLC, model 3400) (details in ref. [17]). The hydrolysis samples were dissolved in deionized water and directly injected into the Dionex h.p.l.c. system.

Cell lines

A porcine proximal tubule-like cell line, LLC-PK1, was used [18]. Cells were cultured at 37 °C in 5% CO_2 initially in α -MEM/10% (v/v) FCS until the cells were nearly confluent. The medium was then changed to α -MEM/1% FCS and the cells were grown for 12 h before the medium was changed to α -MEM pre-equilibrated with NiCl_2 , NH_4Cl or neither, as indicated in the text, and the cells were grown for a further 12 h. Subsequently cells were washed three times in PBS and exposed to trypsin in PBS (0.5 mg/ml) until the cells depleted. The trypsin was 'inhibited' by the addition of an excess of α -MEM/10% FCS and the cells were gently spun down and washed three times in homogenization buffer.

Assays

For tissue culture experiments carbohydrate and peptide were

quantified by saturable nickel binding and expressed as saturable nickel binding/mg of protein. Concentrations of carbohydrate and peptide were determined by the amount of ^{63}Ni bound under saturating concentrations of Ni(II) . Cytoplasmic fractions isolated by spinning cell homogenates at 100 000 *g* for 1 h at 4 °C were fractionated on a Bio-Gel P-2 column to separate peptide and carbohydrate (peaks 1 and 2 in Fig. 1). Carbohydrate or polypeptide fractions from this column were incubated with a saturating concentration of ^{63}Ni and rerun on the P-2 column or alternatively on the I-60 column equilibrated in ammonium acetate as described above [11]. The radioactivity associated with carbohydrate or polypeptide was found to be proportional to the amount of sample under the conditions used. When peptide was assayed in the medium (extracellular), the concentration of Ni in the medium was adjusted to 5 μM before fractionation on the Bio-Gel P-2 column. Although the stoichiometry of Ni/peptide or Ni/carbohydrate under these conditions is unknown, Scatchard analyses with human and bovine peptides had indicated that one high-affinity nickel site exists per peptide [19], although similar data for the carbohydrates are lacking.

Metal binding to oligosaccharides was tested using the following procedure. To a plastic centrifuge tube (1.5 ml) were added sequentially aqueous samples of the radioactive tracer (typically 10^5 – 10^6 c.p.m.), the sample and the buffer, *N*-ethylmorpholine/HCl, pH 7.0–7.5 or NH_4HCO_3 , pH 7.6. This solution was equilibrated overnight in the cold-room, centrifuged in a desk-top centrifuge, and portions were taken for either h.p.l.c. (I-60) or gel-permeation chromatography (Bio-Gel P-2 or G-10).

^3H labelling

Partially desalted (one cycle) fractions corresponding to peaks 2a and 2b (Fig. 2) were individually dissolved in water/methanol (1:5, 6 ml) and 50 μl of 1 M-NaOH was added and the solution cooled to 4 °C. To this solution was added 40 μl of NaB^3H_4 (in dimethyl sulphoxide; approx. 4×10^{10} c.p.m.) and left to stir for 3 h. Then NaBH_4 (10 mg) was added to ensure complete reduction. Subsequently the solution was acidified with acetic acid and evaporated at high vacuum. The residue was dissolved in water (2 ml), vortexed and Amberlite IRA-743 resin was added [20]. After being stirred for 2 h, the resin was removed by filtration and rinsed with water (2×10 ml). The combined filtrates were freeze-dried. The filtrates were further purified by Bio-Gel P-2 permeation chromatography, eluted with water as above, and fractions were counted for ^3H , pooled and freeze-dried. Electrophoresis of small portions was carried out on paper using ^3H -labelled heparin fragments (Research Development Corporation, Toronto, Ont., Canada) as standards. Papers were developed in 100 mM- NH_4HCO_3 (pH 7.8) for 2 h at 50 mA or in 1.5 M-formic acid (pH 1.9) for 3 h at 50 mA. The papers were cut into 1 cm strips, placed in plastic counting vials and scintillation fluid (15 ml) was added; they were then vortexed and counted for ^3H .

Portions were treated with 0.1 ml of freshly prepared 3.9 M- NaNO_2 in 0.28 M-acetic acid for 10 min at room temperature [21]. Then acetic acid (1 ml) was added and the mixture freeze-dried. The residue was passed through the Bio-Gel P-2 column and fractions were counted for ^3H .

Similarly, portions dissolved in 0.2 M-acetate buffer (100 ml, pH 5.0) were treated with about 1000 units of β -glucuronidase (EC 3.2.1.31; bovine lung type B.10; lot 55F 7325) for 24 h at 37 °C. The samples were placed in boiling water for 10 min and then either frozen or added directly to a Sephadex G-10 column which was eluted as above and fractions were counted for ^3H . Essentially identical protocols were used for metal-bound fractions except that counting was for ^{63}Ni .

RESULTS

Fig. 1 shows a typical profile of the fractionation of low-molecular-mass ^{63}Ni -binding components of the cytosolic fraction from kidneys after pre-equilibration of the sample with ^{63}Ni . Two Ni-binding peaks were observed. The first peak (peak 1), eluted just after the void volume, corresponds to an acidic peptide and accounts for about 30 % of the ^{63}Ni radioactivity. The profile of the second peak, which is a reproducible double peak, corresponds to heterogeneous small oligosaccharides (peak 2) and retains about 70 % of the radioactivity. Peptide and carbohydrate were eluted at identical volumes irrespective of pre-equilibration with ^{63}Ni . Purified oligosaccharides (peak 2) were also shown to bind ^{67}Cu , ^{54}Mn , ^{109}Cd and ^{65}Zn .

The peptide (peak 1) was further purified by passage through a C_{18} Sepak column, then through an h.p.l.c. I-60 column and finally through a C-4 reversed-phase column. Final purification was obtained by repassing the fraction through the I-60 column. The purified peptide is eluted as one sharp peak on either I-60 or C-4 columns and other columns tested to date (results not shown). Attempts to sequence human and bovine peptide have been unsuccessful because of blockage of the *N*-terminal amino acid. This procedure has been repeated for human, bovine, porcine and rat kidneys and amino acid compositions of the peptides from each of these species are shown in Table 1. There is a high degree of similarity between the amino acid compositions of the peptides of each of these species. For instance, the peptide is rich in serine, glycine, alanine and acidic residues (Glx, Asx) and is devoid of both aromatic and sulphur-containing amino acids. It is assumed that most of the Glx and Asx are in the acidic forms as the peptide migrates as an anion electrophoretically at low pH [11]. The bovine peptide was also isolated in the presence or absence of a variety of proteinase inhibitors and with heating to 95 °C before homogenization of the kidney. In each case similar quantities of peptide with virtually identical amino acids were obtained (results not shown).

The oligosaccharides (peak 2) were further purified by taking advantage of their negative charge using a Mono Q (Pharmacia)

Fig. 1. Fractionation of low-molecular-mass ^{63}Ni -binding components of the cytosolic fraction from a human kidney on a Bio-Gel P-2 column

Profiles follow recovered radioactivity from ^{63}Ni plus cytosol ($\blacklozenge$) or ^{63}Ni alone ($\square$).

Table 1. Amino acid compositions of peptides from human, bovine, rat and porcine kidneys

Amino acid compositions were determined on a Waters PicoTag system. Numbers are rounded to the nearest whole number based on Thr = 1.

Amino acid	Human	Bovine	Rat	Porcine
Asx	3	3	4	4
Thr	1	1	1	1
Ser	6	4	6	6
Glx	5	5	6	4
Pro	2	1	1	1
Gly	8	10	6	7
Ala	4	4	8	6
Val	2	1	2	2
Ile	1	1	—	1
Leu	2	2	1	2
His	1	1	2	—
Lys	1	1	2	1
Arg	—	—	1	1

f.p.l.c. column and Na_2SO_4 gradients in a dilute phosphate buffer. As shown in Fig. 2, two fractions were found to retain radioactivity. The first (peak 2a) corresponds to two charges and the second (peak 2b) to three or possibly four overall negative charges. The ability of these fractions to retain metal-binding activity is surprising since this chromatography is performed at pH 4.5 and high salt. Furthermore the resin contains quaternary ammonium sites at many times the mole fraction of Ni or sugar. This result is suggestive of the unique characteristic of these compounds, which is to bind metals at low pH and high salt concentrations.

Fractions collected from the anion-exchange column which were not pre-equilibrated with ^{63}Ni could be freeze-dried and metal then added. When used in the h.p.l.c. metal-binding assay,

peaks corresponding to 2a and 2b were active with ^{63}Ni and ^{67}Cu , demonstrating that metal binding does not markedly alter the anion-exchange profile. All human, bovine and porcine kidneys exhibited these peaks.

Salt was partly removed from these fractions by using the 500 Da molecular-mass cut-off ultrafiltration system and then ethanol precipitation (2:1, v/v). This procedure was repeated twice. The final precipitate was passed through a cation-exchange resin to remove paramagnetic impurities. This procedure was absolutely necessary as evidenced by extreme paramagnetic broadening in the n.m.r. spectra if omitted. Finally each fraction was filtered through a C_{18} Sepak column. It should be noted that this fraction is also eluted in the void volume of all reverse-phase columns tested, presumably because of its charge.

One characteristic of these compounds is that they have no appreciable optical absorption. We consider that the absorbance observed at 214 nm in Fig. 2 is probably caused by impurities. In fact, reinjection of metal-binding fractions after ultrafiltration and ethanol/water precipitation shows recovered radioactivity but almost no absorbance (not shown). We conclude that the material from bovine and human kidneys is very similar because of its similar retention times on Mono Q (Fig. 2).

Since only very small quantities of material were obtained, only low-resolution ^1H n.m.r. spectra could be obtained at 500 MHz. The fraction corresponding to peak 2a was always contaminated with amino acids. These were judged to be contaminants since neither the amino acid analysis nor the n.m.r. spectra were reproducible. The fraction corresponding to peak 2b was almost devoid of amino acids and subsequent discussion will focus on this peak. All ^1H n.m.r. spectra exhibited a broad envelope of resonances from approximately 3.4 to 4.4 p.p.m. Significant features are partly resolved multiplets at about 3.0 and 3.15 p.p.m., some multiplets between 4.2 and 4.7 p.p.m. and narrow probably anomeric resonances at 5.0, 5.2 and 5.4 p.p.m. These features are consistent with sulphated oligosaccharides such as heparin and heparan sulphate containing both α -iduronic acid and α,β -glucosamine *N*-sulphate [22,23]. It proved impossible to obtain satisfactory negative (or positive) ion f.a.b.-

Fig. 2. Anion-exchange chromatography of oligosaccharides (peak 2, Fig. 1) from bovine (left) and human (right) kidney on a Mono Q anion-exchange column using a Pharmacia f.p.l.c. system

The profile corresponds to the absorbance at 214 nm. The solid linear lines refer to the salt composition (0% B = 0.005 M- KH_2PO_4 and 100% B = 0.005 M- KH_2PO_4 + 0.150 M- Na_2SO_4). The vertical bars correspond to recovered ^{63}Ni radioactivity.

Fig. 3. G.I.c. monosaccharide analysis of methanolysed (4 M-HCl, 100 °C, 24 h) oligosaccharides from bovine kidney (desalted peak 2b from Fig. 2)

Peaks for iduronic acid, glucuronic acid and *N*-acetylglucosamine as well as the internal standard inositol are marked on the chromatogram. Samples were neutralized, dried, acetylated and silylated before injection. Peaks marked with '?' are unidentified components.

m.s. because of the residual salt, under a variety of conditions. These n.m.r. and m.s. procedures have been successfully applied to heparin-derived disaccharides purified by f.p.l.c. (B. Sarkar & D. M. Whitfield, unpublished observations).

It was possible to label these substances with ^3H by NaB^3H_4 reduction, strongly suggesting that these oligosaccharides are reducing. High-voltage electrophoresis of these compounds at different pHs (7.8 versus 1.9) supports the presence of one or two uronic acids and one or two sulphates in these fractions. These labelled oligosaccharides were sensitive to HNO_2 but insensitive to β -glucuronidase as assayed by changes in elution profiles on Bio-Gel P-2 or Sephadex G-10 columns (not shown). Also ^{63}Ni binding to unlabelled oligosaccharides was abolished by HNO_2 treatment but not by β -glucuronidase treatment.

Further structural information was obtained by analysing for carbohydrates (peak 2b) by g.l.c. after methanolysis, acetylation and silylation [17]. Peaks for *N*-acetylglucosamine (GlcNAc), glucuronic acid (GlcUA) and iduronic acid (IdUA) are clearly observed (Fig. 3). These results were confirmed by monosaccharide analysis of acid hydrolysates (peak 2b) using a Dionex h.p.l.c.-p.a.d. system [17]. Together these results suggest that the acidic oligosaccharides are sulphated α -linked reducing di- or tri-saccharides. Thus probable structures are IdUA or GlcUA α -1,4-GlcNS with at least one more sulphate group. Samples from two bovine kidney preparations and from four

human kidneys were all similar. Porcine kidney exhibited metal-binding peaks such as 2a and 2b whereas rat kidney in one trial showed little or no carbohydrate metal binding.

Both the polypeptide and carbohydrate are isolated from the 'cytoplasmic', or post-100 000 g fraction of the kidney homogenate but could actually correspond to components which originate either intracellularly or extracellularly, as the kidney homogenate contains extracellular components in addition to intracellular ones. In order to differentiate between these possibilities a finely minced bovine kidney was treated with dispase to release free cells. After homogenization of washed cells it was found that both the peptide and carbohydrate could be isolated, thus demonstrating an intracellular localization of these compounds.

Since the peptide and carbohydrate occur intracellularly, a kidney cell line was used to test for the presence of these compounds. Because the peptide was found to be enriched in the renal cortex compared with the medulla (P. F. Predki & B. Sarkar, unpublished work), a porcine proximal tubule-like cell line, LLC-PK1 [18], was used. Soluble low-molecular-mass Ni-binding components were isolated as described above. On the basis of amino acid composition and Dionex h.p.l.c.-p.a.d. carbohydrate analysis respectively, the peptide and carbohydrate fractions isolated were essentially indistinguishable from those isolated from porcine kidney. It is also surprising to find the peptide, but not the carbohydrate, in the media in a quantity of approx. 50% of that isolated intracellularly. The peptide was not, though, isolated from control media unexposed to the cells. As the cells are perfectly viable under the experimental conditions employed (determined by Trypan Blue staining and microscopic examination) it seems unlikely that the peptide has been leaked from dead or injured cells. Thus the peptide isolated from the media is referred to as the secreted form.

Metallothioneins are low-molecular-mass cysteine-rich proteins thought to be involved in metal detoxification and/or zinc regulation [24]. Their synthesis is known to be induced by a number of heavy metals, most notably Cd, but Ni is a much less effective inducer. Furthermore, in liver cells, the induced metallothionein protects against Cd toxicity but not Ni toxicity [25]. We have used the LLC-PK1 cell line to help determine whether the synthesis of the peptide or carbohydrate can be induced by Ni. The cells were exposed to 0–5 μM -Ni for 12 h and levels of peptide and carbohydrate were measured as saturable nickel binding/mg of protein. The intracellular concentration of both peptide and carbohydrate remained constant over this exposure but the extracellular concentration of peptide was observed to double (Fig. 4).

As discussed above, the carbohydrates appear to be degradation products of heparan sulphate. As exposure to NH_4Cl is known to inhibit phagocytosis and lysosomal processing in a number of cell lines [26], the effect of 12 h of exposure to 20 mM- NH_4Cl on the concentration of carbohydrate was determined. Trypan Blue staining indicated that this treatment had no detectable effect on cell viability. The concentrations of intracellular peptide remained constant whereas the amount of carbohydrate was decreased to 61% of control levels.

DISCUSSION

The evidence presented above demonstrates that human, bovine and porcine kidneys have an acidic Ni-binding peptide and pool of iduronic acid-containing oligosaccharides which also bind Ni.

The amino acid composition of the peptide from this variety of sources was found to display little variation. Furthermore, the peptide has been isolated in the presence or absence of proteinase

Fig. 4. Effect of exposure of LLC PK1 cells to various concentrations of Ni

Cells grown to near confluence were exposed to various concentrations of Ni, equilibrated in α -MEM for 12 h at 37 °C (see the text). Saturable nickel binding per mg of protein as described in the text is expressed defining 0 μ M-Ni-exposed cells as 100%. Values were measured for extracellular peptide ($\square$), intracellular peptide ($\circ$) and intracellular carbohydrate ($\blacktriangle$). Three experiments were performed in duplicate. Values are means $\pm$ S.D.

inhibitors and when the kidney had been boiled before homogenization, suggesting that the peptide is not the product of a larger polypeptide or protein degraded during isolation. In each case the peptide has been found to be N-terminally blocked to sequencing. An internal lysine residue, however, provides an internal trypsin cut site yielding two smaller peptides of which only one is blocked.

The biological function of the peptide remains undetermined. It is possible that the intracellular peptide may be involved in the detoxification of Ni and possibly other metals by tightly binding the metal, thus making it unavailable for binding critical cellular molecules in a manner similar to that proposed for metallothionein [24]. The presence of a secreted, possibly inducible, form of peptide suggests that the secreted form could function to remove Ni from the kidney before it becomes internalized by the kidney cells. It cannot yet be conclusively stated that the synthesis of the secreted form of the peptide was induced, as it remains possible that the peptides may somehow be stabilized by binding Ni added to the media. Also, consistent with the Ni-mobilization hypothesis is the isolation of a heterogeneous pool of acidic Ni-binding peptides in the urine which may represent breakdown products of the original peptide (A. Mas & B. Sarkar, unpublished work). In this respect it should be pointed out that rats pre-exposed to Ni have been shown to accumulate less Ni in the kidney in a later exposure than control rats exposed to the same amount of Ni [27], suggesting that an inducible mechanism for the renal clearance of Ni may exist.

The presence of iduronic acid in the oligosaccharides is significant since we have been able to show using model compounds that iduronic acid-containing oligosaccharides are able to bind heavy metals such as the ones discussed here [28], and bind them better than other uronic acids [29–32].

Using both dispase-treated kidneys and the LLC-PK1 cell line we have been able to show that these oligosaccharides are preferentially located intracellularly. This raises the question of how they are formed. Heparan sulphates containing proteoglycans are known to be components of kidney basement

membranes [33,34]. These have been estimated to turn over rapidly by a mechanism that involves endocytosis and degradation in at least a two-step mechanism involving partial degradation in prelysosomal vesicles followed by complete degradation in the lysosomes [35–37]. Since we were able to demonstrate a decrease in this oligosaccharide fraction in the presence of NH_4Cl which is known to interfere with lysosomal processing [38,39], this observation supports the premise that these oligosaccharides are heparan sulphate-degradation products.

Several authors have demonstrated the presence of low-molecular-mass glycosaminoglycans in both urine [40,41] and blood [41,42]. Unfortunately all of these protocols involved either dialysis or precipitation, so it is not possible to know for sure if components as small as disaccharides exist in blood or urine *in vivo*. However, it has been demonstrated in rats [43] and rabbits [44,45] that administered heparin is accumulated in kidneys and then degraded before excretion in urine. Therefore it is probable that such acidic oligosaccharides are normal constituents of kidney cells. Their ability to bind at low pH and high salt as well as their intracellular location leads us to suggest a possible role for these compounds as scavengers for heavy metals in the low-pH environment of the lysosome, before the metals can have toxic effects on the cell.

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