Volume 250, number 2, 187–190 FEB 07244 July 1989

Elucidation of multiple forms of nephrocalcin by ^{31}P-NMR spectrometer

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Received 29 March 1989

Four forms of nephrocalcin have been routinely isolated from mammalian kidney tissues and urine using DEAE-cellulose column chromatography with a linear NaCl gradient. We have demonstrated that these four forms of nephrocalcin, isolated from bovine kidneys, contain different amounts of phosphate residues, and that alkaline phosphatase digestion converts these to only one form of nephrocalcin. The changes in the nephrocalcin before and after removal of phosphate residues were measured by ^{31}P-NMR spectrometer. Loss of phosphate residues decreased the dissociation constant of nephrocalcin 10-fold toward calcium oxalate monohydrate crystals, suggesting the phosphate residues appear to be important in the inhibitory effects of calcium oxalate monohydrate crystal growth.

Nephrolithiasis; Nephrocalcin; Calcium oxalate crystal; Crystal growth inhibitor; Glycoprotein; NMR, ^{31}P-

1. INTRODUCTION

Nephrocalcin, an acidic urinary glycoprotein inhibitor of calcium oxalate monohydrate crystal growth (kidney stone inhibitor), has been isolated from normal human urine [1], the urine of kidney stone formers [2], kidney stones [3], rat urine and kidney tissues [4], and human kidney tissue culture media [5]. This inhibitor usually elutes in four different ionic strength regions during DEAE-cellulose column chromatography using a linear NaCl gradient, and the amino acid compositions of the material in all four regions are identical. By amino acid analysis [1], all four forms contain phosphoserine and variable numbers of γ-carboxyglutamic acid (Gla) residues. Nephrocalcins isolated from the urine of kidney stone formers [2] and from kidney stones did not contain Gla [3].

This paper is dedicated to the memory of the late Professor E.T. Kaiser

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The different amounts of the phosphoserine and Gla residues were not sufficient to explain why these inhibitors eluted at widely different ionic strength during DEAE-cellulose column chromatography. We report here that the phosphate contents of each of the four elution peaks differ; also we report ^{31}P-NMR results before and after incubation with alkaline phosphatase and glycosidases to remove phosphate and carbohydrate residues, respectively. These results indicate that variations in the degree of phosphorylation account for heterogeneity of elution from DEAE cellulose column.

2. EXPERIMENTS

2.1. *Materials*

Nephrocalcin was purified from bovine kidneys in a manner similar to that described for purification from rat kidneys [4]. Alkaline phosphatase was in an insoluble form (25 units, Lot No.55F-9550), purchased from Sigma. Mixed glycosidases (Lot No.13A) were obtained from Miles Laboratory. Sephacryl S-200 was purchased from Pharmacia and packed in a 2 × 110 cm glass column. DEAE-cellulose was obtained from Pierce Chemicals Co. and cycled with 0.5 M HCl and 0.5 M NaOH, then equilibrated in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.3, before use.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2.2. *Analytical methods*

Phosphate was determined by the Fiske-Subbarow method after ashing [6]; 0.01 M KH_2PO_4 was used for preparing a calibration curve. Calcium oxalate crystal growth inhibition activity was measured by incorporation rate of [^{14}C]oxalate into preformed calcium oxalate seed crystals [1]. The dissociation constant for inhibitor-crystal interaction was determined by a spectrophotometric assay [7]. The proton-decoupled ^{31}P-NMR spectrum was recorded on a Brucker 200 MHz FT-NMR spectrophotometer at 80.02 MHz in 99% D_2O as the solvent at an ambient temperature. Typically, an 8 μs pulse followed by 0.41 s acquisition with 1 s pulse delay was employed. The chemical shift was calibrated by using 85% H_3PO_4 as the external standard.

Nephrocalcin, 13 mg, was dissolved in 4 ml of 0.1 M sodium acetate, pH 5.0, and 2 mg of mixed glycosidases were added, followed by incubation overnight at 37°C. The solution was subjected to a Sephacryl S-200 column, and proteins were eluted using 0.02 M Tris-HCl, 0.2 M NaCl, pH 7.3, as a solvent. The elution of protein was monitored at 280 or 230 nm absorbance. For alkaline phosphatase digestion, 13 mg of nephrocalcin was dissolved in 4 ml of 0.2 M Tris-HCl, pH 8.0, and 12.5 units of insoluble alkaline phosphatase were added twice at 6 h intervals during a 12 h incubation at 37°C. The solution was centrifuged at 12000 × *g*, then chromatographed on a Sephacryl S-200 column.

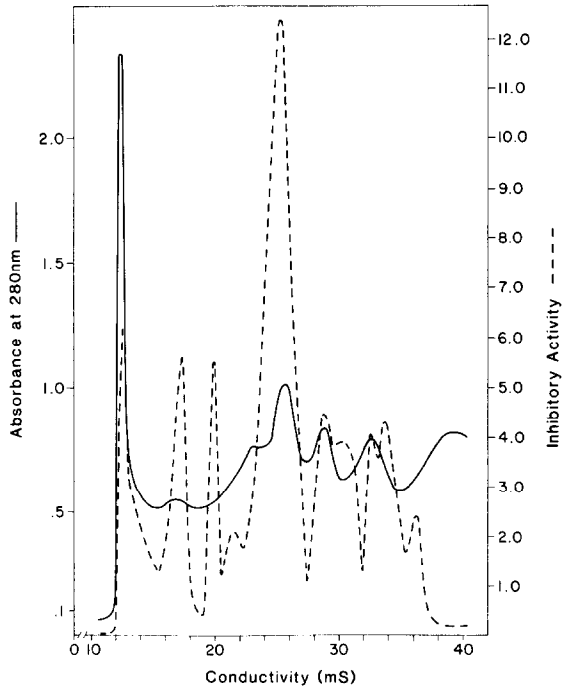

Fig.1. Elution profile of nephrocalcin from DEAE-cellulose column. Bovine kidney homogenate was prepared by a similar manner as described previously [4], and four fractions of nephrocalcin were separated by DEAE-cellulose column chromatography (2 × 15 cm) using a linear NaCl gradient from 0.1 M to 0.5 M in 0.05 M Tris-HCl, pH 7.3.

Table 1

Phosphate contents and dissociation constants of nephrocalcin fractions

Sample	Phosphate content (μg/mg protein)	K_d (M)
Fraction A	48.5	8.45×10^{-8}
Fraction B	470.6	1.10×10^{-7}
Fraction C	1338.5	2.18×10^{-7}
Fraction D	1931.0	
Fraction C after alkaline phosphatase digestion	614.5	2.30×10^{-6}
Fraction C after glycosidase digestion	168.0	

3. RESULTS

The elution pattern of bovine kidney tissue inhibitors from DEAE-cellulose chromatography as previously reported [1–5] is shown in fig.1. The inhibitor fractions were eluted at A (12–15 mS), B (16–22 mS), C (23–28 mS) and D (29–40 mS) at increasing NaCl concentrations (fig.1). Phosphate content increased from A to D fraction (table 1). The C fraction was incubated with alkaline phosphatase and rechromatographed on a DEAE-cellulose column with a linear NaCl gradient (because of high concentration of protein required for NMR studies, we used the most abundant fraction). The protein was eluted at 10–14 mS (fig.2).

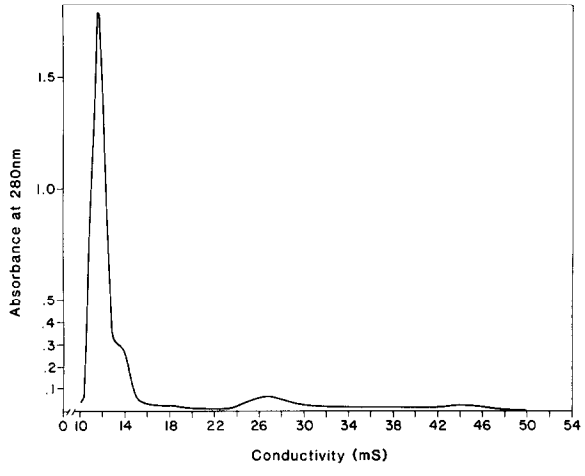

Fig.2. Elution pattern of dephosphorylated nephrocalcin fraction C. Fraction was dephosphorylated by alkaline phosphatase and chromatographed under the identical conditions described in fig.1.

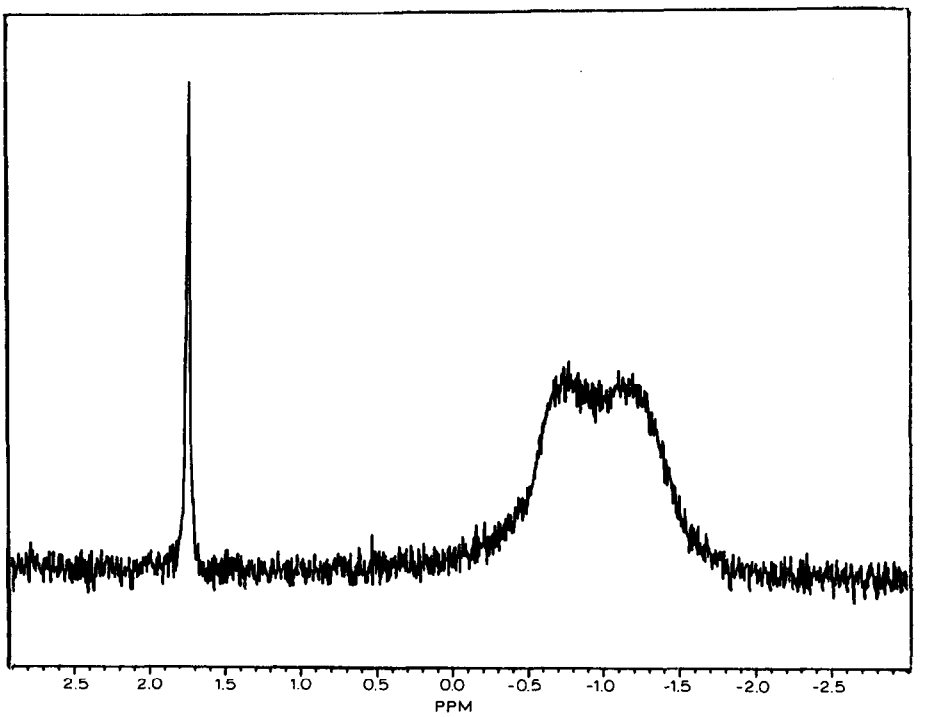

Fig.3. NMR spectrum of nephrocalcin fraction C. The experimental details are described in the text.

After deglycosylation by glycosidases, the protein was similarly eluted at 13–15 mS (not shown).

The removal of phosphate by alkaline phosphatase decreased the dissociation constant 10-fold (table 1). The removal of carbohydrate caused precipitation of the protein. The ^{31}P-NMR spectrum of the original fraction C is shown in fig.3. The chemical shifts were +1.75 ppm, −0.76 ppm and −1.16 ppm. After alkaline phosphatase digestion, the chemical shift at +1.75 ppm disappeared, but +0.45 ppm was observed (not shown). After deglycosylation, −0.86 ppm and −1.26 ppm were observed (not shown).

4. DISCUSSION

Multiple forms of nephrocalcin eluted from a DEAE-cellulose column with a linear NaCl gradient suggesting a difference in negative charge. Amino acid analyses of four fractions were identical [2], and different amounts of Gla in each fraction were not enough to account for this elution pattern. The results presented in this report indicate that number of phosphate residues per nephrocalcin molecule can vary; the more phosphorylated forms eluted from DEAE-cellulose column at a higher salt concentration.

Substantial amount of phosphate residues remained after alkaline phosphatase digestion, indicating that a phosphate group is present on other amino acid residues in addition to serine. Deglycosylation decreased phosphate content more than alkaline phosphatase digestion. These results suggest that a carbohydrate moiety of nephrocalcin could also be phosphorylated. It was observed that the removal of carbohydrate caused precipitation of the protein portion of nephrocalcin unless the suspension was made alkaline by *N*-ethylmorpholine. The exact function and type of the phosphorylated sugars in nephrocalcin are presently unknown; however, it appears that at least a conformational difference of nephrocalcin may be associated with phosphorylated sugars.

It is known that phosphoserine is essential for biological calcifications: for example, proline-rich protein in saliva [8,9], phosphophoryn in dentin

matrix [10,11], and acidic phosphoprotein in mollusk shells [12] contain phosphoserine. We determined that phosphoserine residues are present in urinary calcium oxalate crystal growth inhibitor, nephrocalcin. Dephosphorylation of serine caused a 10-fold decrease in the dissociation constant toward calcium oxalate monohydrate crystals. We conclude that the phosphorylation of serine residues may be important in the binding of nephrocalcin to calcium oxalate monohydrate crystals.

Acknowledgements: This study was supported by NIH grant 5 PO1 AM-33949. We thank Siok Le Dun for technical assistance.

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