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MULTIPLE FORMS OF THE PROLINE-RICH POLYPEPTIDE (PRP) BOUND TO RAT PROSTATIC BINDING PROTEIN

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SUMMARY : The proline-rich polypeptide, that is bound to rat prostatic binding protein displays a marked heterogeneity on isoelectric focusing, with major bands at pH 7.6 and pH 6.9. The same complex pattern is obtained for PRP prepared from prostates of individual rats from several strains. Using carboxymethylcellulose chromatography 6 different forms of PRP can be separated. Five of them have the same size (MW : 4000) and respectively glycine and lysine as N- and C-terminal amino acid. Their amino acid composition suggests that these forms differ by internal substitution respectively of aspartic acid and glycine and of proline and histidine. The sixth form (MW : 3500) lacks several amino acids at its N-terminal.

The major protein, synthetised and secreted by the rat ventral prostate is prostatic binding protein (PBP), an androgen-dependent oligomeric protein (1-3), that has been studied by other authors as α-protein (4), prostatein (5) or estramustine-binding protein (6). This protein was originally characterized as a steroid-binding protein (1), but, as shown recently (7), it also binds a proline-rich polypeptide (PRP), which is markedly androgen-dependent. During its characterization by means of isoelectric focusing PRP was found to be heterogeneous in this respect. This heterogeneity is studied more in detail in the present communication. Our data indicate that the various forms of PRP differ significantly in their amino acid composition, probably due to internal substitutions of a few amino acids.

ABBREVIATIONS : PBP, prostatic binding protein; PRP, proline-rich polypeptide; SDS, sodium dodecyl sulfate.

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METHODS

Purification of PRP. The method for the purification of PRP on a preparative scale has been described recently (7). In short, the PBP.PRP complex is purified first from prostatic cytosol using DEAE-cellulose chromatography. Thereafter, PRP is separated from the subunits of PBP by chromatography on DEAE-Sepharose, performed in the presence of 8 M urea and then purified further on a Sephadex G-50 column.
For genetic screening a simplified purification procedure was developed, which could be executed individually on each prostate. This method is based on the same principle as the preparative method but combines both ion exchange steps on a single column without intermittent elution of the PBP. PRP complex. Therefore, each ventral prostate was minced in 4 ml of 50 mM NaCl in Tris buffer (25 mM Tris-HCl, pH 7.4 at 4°C) and shaken gently for 5 min. After centrifugation (10 min at 10000 rpm and 4°C) the supernatant was applied to a small column (1.5 x 3.0 cm) of DEAE-Sepharose equilibrated with the same buffer. This column was rinsed successively with 20 ml 0.1 M NaCl and 10 ml 0.1 M NaCl, 0.5 M urea in Tris buffer. Under these conditions the PBP.PRP complex is retained on the column. Thereafter, PRP is eluted selectively with 6 ml of 25 mM NaCl 6 M urea, due to dissociation of the PBP.PRP complex, PBP being retained on the column. Before isoelectric focusing urea is removed by gel filtration on a small PD-10 column. The yield of this method is 0.53 $\pm$ 0.17 (SD) mg per prostate (about 76%) and the obtained PRP was electrophoretically pure except for a weak contaminating band (MW 25,000) present in some samples.

Separation of different forms of PRP. The different isoelectric forms of PRP (see below) could be partially separated by chromatography on carboxymethylcellulose (Whatman, CM-52). Therefore, purified PRP (about 15 mg) was equilibrated with 50 mM acetate buffer (pH 5.0) on a Sephadex G-25 column, and applied to a carboxymethylcellulose column (2.5 x 13 cm), equilibrated with the same buffer. After rinsing the column with 10 ml buffer, the various forms of PRP were eluted with a linear gradient (2 x 150 ml; 0 to 0.5 M) of NaCl in this buffer. The whole procedure was performed at 4°C using a flow rate of 20 ml/hour.
Isoelectric focusing. PAG plates (pH range 3.5 to 9.0) from LKB were used at 4°C on an LKB Multiphor. The focusing was performed in the transverse direction. After a 1 hour prerun at a constant current of 10 mA (for a 12 cm large gel), the samples were applied on the gels at 0.5 cm from the cathodic strip and focused for 3 hours at a constant current of 5 mA. The final voltage was 900 V. After focusing the gel was immersed for 60 min in 100 ml of 20% (w/v) trichloroacetic acid, resulting in the precipitation of proteins. The ampholytes were washed out at room temperature under continuous but gentle shaking. The TCA solution (100 ml) was changed 4 times over a 15 hours period. Thereafter, the gel was stained twice for 20 min with a mixture of 1 volume of 0.2 g % Coomassee Brillant Blue R in methanol added to 4 volumes of an aqueous solution of 15 g % (w/v) trichloroacetic acid and 4.5 g % (w/v) of sulfosalicylic acid. The gel was destained first for 20 min with 1 volume of methanol added to 7 volumes of the TCA-sulfosalicylic acid solution and destained further in 10 % acetic acid,10% glycerol. After this last step, which resulted in a marked enhancement of colour, the gel could be dried as indicated by the manufacturer. The pH-profile was determined at 4°C on a parallel zone of the gel. This zone was cut in 1 cm strips, which were eluted with distilled water.

Various techniques. Polyacrylamide gel electrophoresis in the presence of SDS was performed on 15% acrylamide gels (0.4 % bisacrylamide) as described previously (7). For the immunological detection after isoelectric focusing an immunoblot technique was used on strips of antiserum-impregnated cellulose acetate membranes (8). Total protein was measured by the Lowry method (9), PRP by a radial immunodiffusion technique (7). The amino acid composition was determined on a Biotronik

LC-2000 amino acid analyzer as described previously (2). The N-terminal amino acids were identified by use of 4-N,N'-dimethylaminoazobenzene-4'-isothiocyanate (10), the C-terminal amino acids after carboxypeptidase B treatment in 0.2 M N-methylmorpholine buffer (pH 8.2) at an enzyme-substrate ratio of 1/20 (w/w) (11).

RESULTS AND DISCUSSION

Isoelectric focusing. Several difficulties were encountered, when isoelectric focusing of PRP was performed on thin layer polyacrylamide gels. The first problem was related to the detection of PRP after focusing. Indeed, because of its small size PRP is completely eluted from the gel during staining and destaining by the usual techniques. This problem was overcome by the use of the staining method described in this paper, which may be of a more general value for staining of polypeptides of similar size. Another important point was the position of application of the samples. Indeed, when the sample strips were applied in the anodic region of the gel, almost no PRP was detectable.

The isoelectric focusing pattern of PRP was surprisingly complex (Fig. 1). Indeed, multiple protein bands were scattered over the gel,

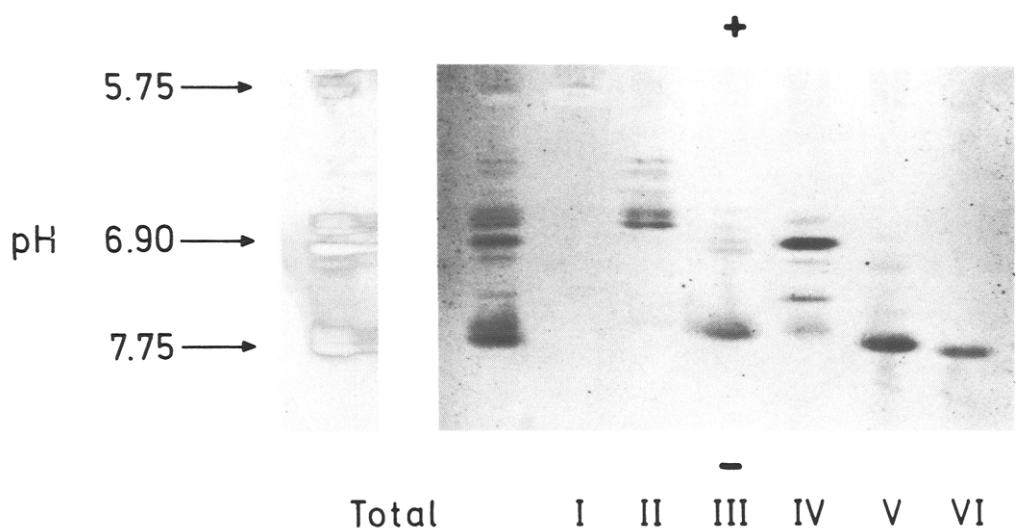

Fig. 1. Isoelectric focusing pattern of purified PRP on polyacrylamide gels (LKB PAG plates : pH 3.5 to 9.0). The roman numerals refer to peak fractions of the carboxymethylcellulose column (see Fig. 2), the arrows indicate the corresponding pHs. Note the negative staining of peak fraction I. The isoelectric focusing pattern obtained by immunodetection on cellulose acetate membranes impregnated with anti-PRP antiserum is shown on the left.

with isoelectric points situated between pH 5.7 to 7.8. The most prominent bands were localized at pH 6.9 and 7.6, respectively. This heterogeneity was not due to impurities of the PRP-preparation, since a very similar pattern (Fig. 1) was observed when an immunoblot technique was used for the detection of PRP. The large range of isoelectric points can be explained by the low molecular weight of PRP, which results in a marked effect of a single additional charge.

Electrofocusing pattern of PRP in individual rats from different strains. Since the initial electrofocusing experiments were performed on PRP prepared batchwise from a large number of rat prostates the observed heterogeneity of PRP could be due to genetic polymorphism. To explore this possibility a method was developed (see methods), which allowed the preparation of sufficiently pure PRP from an individual rat ventral prostate. Using this method individual rats from the following strains were screened for PRP-heterogeneity: WIST, BN, BD/IX, GUNN and R/A. In all instances the obtained pattern was identical.

Separation of different forms of PRP on carboxymethylcellulose. An interaction of PRP with ampholines could offer another explanation for

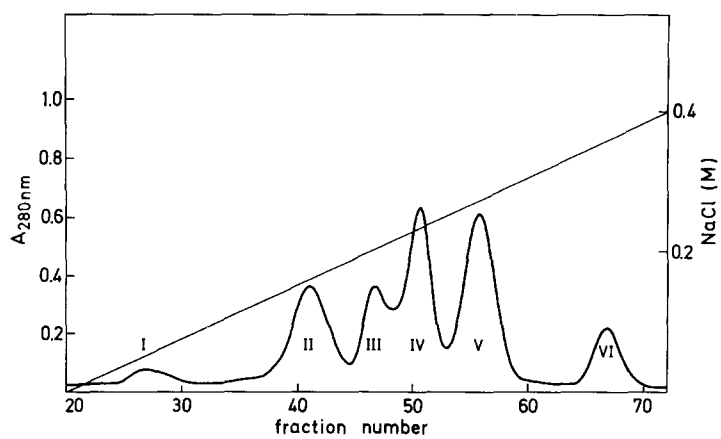

Fig. 2. Ion exchange chromatography of purified PRP on carboxymethylcellulose. PRP (15 mg) was applied to a column (2.5 x 13.2 cm) of carboxymethylcellulose in 50 mM acetate buffer (pH 5.0) at 4°C and eluted with a linear NaCl gradient in the same buffer at a flow rate of 20 ml per hour. Fractions of 4 ml were collected. The extinction at 280 nm (——) and the concentration of NaCl(——) are indicated. No protein was eluted in the previous fractions. The roman numerals (I to VI) refer to the different peaks in the manuscript.

TABLE I

General properties of the different forms of PRP separated by carboxymethylcellulose chromatography.

Peak	Extinction[1] (280 nm)	Immunological[2] Response	pI[3]	N-terminal Sequence	C-terminal Sequence
I	1.4	1.14	5.75	Thr-Pro-	-Thr-Lys
II	2.2	1.01	6.75	Gly-Trp-	-Thr-Lys
III	2.0	0.90	7.50	Gly-Trp-	-Thr-Lys
IV	2.0	1.00	6.90	Gly-Trp-	-Thr-Lys
V	2.3	0.80	7.65	Gly-Trp-	-Thr-Lys
VI	2.8	0.99	7.75	Gly-Trp-	-Thr-Lys

[1] For a solution containing 1 mg/ml of protein in the Lowry assay.
[2] Relative response per mg of Lowry protein, using peak IV as reference.
[3] The isoelectric points refer to the major band of each fraction on thin layer isoelectric focusing.

its complex isoelectric focusing pattern. This possibility can be readily excluded since the major forms of PRP can also be separated by ion exchange chromatography on carboxymethylcellulose at pH 5.0. The elution profile of PRP, which is reproducible for different preparations of PRP, consists of 6 different peaks (Fig. 2). On SDS-polyacrylamide gel electrophoresis they all produce a single protein band (MW 4000), except for peak I, which consists of a faster migrating polypeptide (MW: 3500). On the other hand, on focusing, these peaks correspond to different bands of the isoelectric focusing pattern (Fig. 1) and although they are not completely homogeneous, their separation is sufficient to allow a more detailed characterization of the various forms of PRP (see below).

It is noteworthy that the sequence of elution is not completely parallel to the position of the bands on the focusing gel. Indeed, whereas peak I and II are situated most anodically as expected, peak IV is localized more anodically than peak III.Furthermore, peak III, V and VI (or peaks II and IV), which have nearly the same position on the focusing gel, are very well separated by CMC chromatography. A possible explanation for this apparent discrepancy could be the involvement of a

TABLE II

Amino acid composition (mol/100 mol) of the different forms of PRP

Amino Acid	PRP eluted in peak					
	I	II	III	IV	V	VI
Cysteine	N.D.*	N.D.	N.D.	N.D.	N.D.	N.D.
Aspartic acid	6.0	7.0	5.8	7.1	5.5	5.4
Threonine	11.6	10.5	10.7	10.9	9.9	10.7
Serine	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Glutamic acid	8.2	6.8	6.3	6.4	6.0	5.9
Proline	18.1	20.0	20.0	18.6	19.6	17.5
Glycine	4.0	5.8	7.7	5.9	8.1	8.2
Alanine	4.5	3.1	3.5	2.7	2.7	2.5
Valine	3.8	3.8	2.9	2.9	3.6	3.4
Methionine	N.D	N.D	N.D	N.D.	N.D.	N.D.
Isoleucine	2.4	4.4	4.9	4.7	4.1	4.1
Leucine	11.1	8.7	8.5	8.6	8.3	8.1
Tyrosine	6.0	5.5	5.2	5.4	5.3.	5.2
Phenylalanine	11.0	8.1	7.9	8.1	7.9	7.8
Histidine	3.8	5.7	5.8	7.5	7.8	10.1
Lysine	9.0	7.6	7.9	8.3	7.4	8.2
Arginine	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Tryptophan	N.D.	3.2	3.0	3.1	3.6	3.5

* N.D. : not detectable

group (e.g. an imidazole), with a pK situated between the pH of the ion exchange experiment (pH 5) and the isoelectric point of the polypeptide. In fact, as shown below, the histidine content of peaks III, V and VI (and of peaks II and IV) increases in parallel with their elution position from the CMC column.

Further characterization of the different forms of PRP. As shown in Table I the different forms of PRP produce a similar response in the immunoassay of PRP and their extinction coefficient at 280 nm is comparable. Only peak I shows a lower extinction, probably due to the absence of tryptophan (see below). The 6 PRP-forms also have identical N- and C-terminal sequences (Table I) except for the N-terminus in peak I.

The amino acid composition of peaks II to VI shows a general similarity (Table II) although some important differences are observed. In-

deed, peaks II and IV contain more aspartic acid and less glycine than the other peaks, whereas the higher histidine content of peaks IV, V and particularly of peak VI, is compensated by a lower proline content. The amino acid composition of peak I, on the other hand, differs more markedly. With respect to the other peaks, this form of PRP is characterized by the absence of tryptophan, a lower glycine, isoleucine and histidine content and a relative increase of most other amino acids. In view of its composition, its different N-terminus and its smaller size this form of PRP most probably corresponds to a large fragment of PRP, which lacks several amino acids (glycine, tryptophan, ...) on its N-terminus, possibly as a result of endogenous protease activity. Such a mechanism, however, can hardly explain the differences in amino acid composition between the other forms of PRP, since their size and their N and C-terminal sequences are identical. For this reason, we believe that these differences are due to internal changes of their amino acid sequence by substitution of one or a few amino acids (e.g. an aspartic acid-glycine or a proline-histidine interchange). If this hypothesis could be confirmed by sequence determination, this would imply that the different forms of PRP differ at the genomic level. The finding of multiple forms of PRP in individual rats would then indicate that PRP is encoded by multiple genes, as described for other androgen-regulated secretory proteins (12).

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