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Characterisation of the conformational and quaternary structure-dependent heparin-binding region of bovine seminal plasma protein PDC-109

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Abstract PDC-109, the major heparin-binding protein of bull seminal plasma, binds to sperm choline lipids at ejaculation and modulates capacitation mediated by heparin. Affinity chromatography on heparin-Sepharose showed that polydisperse, but not monomeric, PDC-109 displayed heparin-binding capability. We sought to characterise the surface topology of the quaternary structure-dependent heparin-binding region of PDC-109 by comparing the arginine- and lysine-selective chemical modification patterns of the free and the heparin-bound protein. A combination of reversed-phase peptide mapping of endoproteinase Lys-C-digested PDC-109 derivatives and mass spectrometry was employed to identify modified and heparin-protected residues. PDC-109 contains two tandemly arranged fibronectin type II domains (a, Cys^{24}-Cys^{61}; b, Cys^{69}-Cys^{109}). The results show that six basic residues (Lys^{34}, Arg^{57}, Lys^{59}, Arg^{64}, Lys^{68}, and Arg^{104}) were shielded from reaction with acetic anhydride and 1,2-cyclohexanedione in heparin-bound PDC-109 oligomers. In the ^{1}H-NMR solution structures of single fibronectin type II domains, residues topologically equivalent to PDC-109 Arg^{57} (Arg^{104}) and Lys^{59} lay around β-strand D on the same face of the domain. In full-length PDC-109, Arg^{64} and Lys^{68} are both located in the intervening polypeptide between domains a and b. Our data suggest possible quaternary structure arrangements of PDC-109 molecules to form a heparin-binding oligomer.

Key words: Bovine seminal plasma; Glycoprotein PDC-109; Heparin-binding region; Chemical modification; Peptide mapping; Mass spectrometry

1. Introduction

Studies on mammalian reproductive physiology established that spermatozoa leaving the testis appear mature from a morphological standpoint but are unable to fertilise a metaphase II-arrested egg [1]. They gain their full fertilising capacity upon residence for a defined period of time in the female fallopian tube owing to a process termed capacitation. Although capacitation was discovered nearly half a century ago [2–5], the molecular and physiological bases of this event are still poorly understood. Ultrastructural and biochemical studies suggest that spermatozoa undergoing capacitation exhibit a membrane-remodelling process, including decrease in the molar ratio of cholesterol to phospholipids (membrane fluidity changes) and modification of the chemical and topographical composition of integral and peripherally bound membrane components [1,6,7].

Reports from several species indicate that the seminal plasma, the mixed secretions of the male accessory reproductive organs in which spermatozoa are suspended in semen, contains factors that influence the fertilising ability of spermatozoa. In the bull, Miller and co-workers [8] demonstrated a positive regulatory role of seminal plasma heparin-binding proteins in the in vitro capacitation of bull sperm. Acidic proteins PDC-109 (also termed BSP-A1/A2), BSP-A3, and BSP-30K, are secreted by the seminal vesicle epithelium and constitute the major heparin-binding proteins of bull seminal plasma [9–11]. PDC-109 and BSP-A3 bind specifically to choline phospholipids [12–14], mainly on the sperm head postacrosomal surface [15]. BSP-30K also binds to choline phospholipids but displays a much broader specificity [12]. Isolated PDC-109, BSP-A3, and BSP-30K each potentiate in vitro zona pellucida-induced acrosome reactions in the presence of heparin [16]. In addition to heparin, the bovine seminal plasma (BSP) proteins interact with a variety of ligands including different types of collagen, fibrinogen, apolipoprotein A1 (apoA1) and apoA1/high-density lipoprotein (HDL) complexes [16]. It has been proposed [13–15] that upon binding to sperm surface choline lipids, which comprise over 70% of the total bull sperm plasma membrane phospholipids [17], modification of the sperm plasma membrane composition by BSP proteins may underlie modulation of capacitation by HDL and heparin-like glycosaminoglycans that are present in the follicular and oviductal fluids of the cow at particularly high concentrations during the oestrus cycle [18].

PDC-109, a 109-amino acid residue polypeptide, is the most abundant protein of bull (*Bos taurus*) seminal plasma, where its concentration reaches 15–20 mg/ml [19]. PDC-109 coats to the sperm surface at ejaculation and around 8 million PDC-109 have been quantitated on the surface of a single bull spermatozoon after incubation for 24 h in capacitation medium [19]. Structurally, PDC-109 belongs to the same protein family as BSP-A3 and BSP-30K. Each of these proteins displays a mosaic architecture, which consists of N-terminal distinctly *O*-glycosylated polypeptide extensions of variable length followed by two tandemly arranged and highly conserved fibronectin type II domains ([11] and references therein). Glycoprotein PDC-109 has been crystallised in complex with *o*-phosphorylcholine (oPC) [20]. The resolution of the

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Abbreviations: CHD, 1,2-cyclohexanedione; DHCH-Arg, N^7,N^8-(dihydroxy-1,2-cyclohexylidene) arginine

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crystal structure of the PDC-109/oPC complex will serve to model the binding mechanism of the seminal plasma protein to sperm choline lipids. Here, we show that heparin binding by PDC-109 is dependent on its aggregation state. A heparin protection assay and selective chemical modification in combination with peptide mapping and mass spectrometry were applied for the identification of basic residues of PDC-109 forming part of its conformational heparin-binding region.

2. Materials and methods

2.1. Isolation and characterisation of PDC-109

PDC-109 was purified from the seminal plasma of healthy and reproductively active Holstein bulls (a generous gift of Rinder Produktion Niedersachsen, Verden, Germany) by affinity chromatography as previously described [21]. Isolated PDC-109 was pure as judged by SDS-polyacrylamide electrophoresis, N-terminal sequence analysis (using an Applied Biosystems Procise instrument), and matrix-assisted laser-desorption ionisation (MALDI) mass spectrometry (using a Kratos MALDI-I spectrometer and 3,5-dimethoxy-4-hydroxycinnamic acid (Aldrich) saturated in 2:1 (v/v) acetonitrile/0.1% trifluoroacetic acid as matrix) [19,21]. It consisted of a roughly equimolar mixture of non-glycosylated (12 788 Da) and *O*-glycosylated (13 443 Da) [19,22] molecules.

2.2. Affinity chromatography

5 mg of isolated PDC-109 in 50 mM Tris-HCl, pH 7.4, was bound at room temperature to a 5 ml heparin-Sepharose (Pharmacia) column equilibrated in this buffer. In successive experiments, the protein was eluted with gradients of column buffer (solution A) and column buffer containing either 150 mM *ortho*-phosphorylcholine, 150 mM EDTA, 125 mM $CaCl_2$, and 350 mM NaCl.

2.3. Selective chemical modification of arginine residues and amino groups

PDC-109 (~1 mg/ml in 16.6 mM Tris-HCl, 50 mM NaCl, 1.6 mM EDTA, 0.025% NaN_3, pH 7.4) was applied to 5 ml heparin-Sepharose (Pharmacia) equilibrated in the same buffer. After washing out unbound material, the column was recycled overnight at room temperature with either 1,2-cyclohexanedione (0.04–6-fold molar excess over arginine residues) or acetic anhydride (25–1600-fold molar excess over lysine residues) in the same buffer. Bound material was eluted with column buffer containing 1 M NaCl. N^7,N^8-(Dihydroxy-1,2-cyclohexylidene) arginine (DHCH-Arg-) and aminoacetylated derivatives of isolated PDC-109 (~1 mg/ml in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.025% NaN_3, pH 7.4) were prepared overnight at room temperature by reaction with a 6-fold molar excess over arginine residues of 1,2-cyclohexanedione and a 800-fold molar excess over lysine residues of acetic anhydride, respectively. The same labelling protocol was carried out using free PDC-109 in solution. The degree of derivatisation was assessed by MALDI mass spectrometry.

2.4. Endoproteinase Lys-C peptide mapping and peptide characterisation

DHCH-Arg-PDC-109 and aminoacetylated PDC-109 derivatives (5–10 mg/ml in 100 mM Tris-HCl, pH 8.6, 6 M guanidine hydrochloride) were reduced with 10% (v/v) β-mercaptoethanol for 2 min at 100°C, alkylated with a 4.5-fold molar excess of iodoacetamide over reducing reagent, dialysed against 100 mM NH_4HCO_3, pH 8.6, 1 M guanidine hydrochloride, and digested in this buffer with endoproteinase Lys-C (substrate:enzyme ratio of 100:1, w/w) overnight at 37°C. Endoproteinase Lys-C peptides of DHCH-PDC-109 (termed R-) and aminoacetylated PDC-109 (termed K-) were separated by reversed-phase HPLC (250×4 mm Lichrospher RP100 C18, 5 μm) eluting at 1 ml/min with a mixture of 0.1% TFA in water (A) and acetonitrile (B), first 5 min isocratic (5% B), followed by a linear gradient of 5–50% in 135 min, and 50–70% B in 20 min. Peptides were subjected to amino acid analysis (after hydrolysis with 6 N HCl, 18 h, 110°C) using a Pharmacia-LKB AlphaPlus instrument, N-terminal sequencing (using an Applied Biosystems Procise instrument), and their mass was determined by electrospray ionisation mass spectrometry using an Sciex API III LC/MS/MS triple quadrupole mass spectrometer.

3. Results and discussion

3.1. Correlation between the heparin-binding capability and the quaternary structure of PDC-109

Initially, we sought to characterise the heparin-binding region of PDC-109 using the approach employed for mapping the heparin-binding domain of boar spermadhesins [23]. Thus, PDC-109 was bound to a heparin-Sepharose column and the conjugated protein was proteolytically degraded overnight. The rationale behind this approach is that those regions interacting with heparin are protected from proteolysis and will be recovered in the affinity matrix-bound fraction. However, using various enzymes displaying diverse cleavage specificities (α-chymotrypsin, elastase, subtilisin, endoproteinase Glu-C), we always obtained non-heparin-bound peptides and intact PDC-109 bound to the affinity column. This strongly suggested that the heparin-binding ability of PDC-109 was dependent on a polypeptide conformation which is only retained by the native protein. In addition, we noticed that PDC-109 could be eluted from heparin-Sepharose by including in the column buffer NaCl, $CaCl_2$, EDTA, or oPC (Fig. 1). The concentration of each of these solutes needed for the quantitative elution of PDC-109 from the affinity column was the same concentration which caused oligomer dissociation of polydisperse PDC-109 molecules [13]. Thus, the PDC-109 peaks obtained with the different eluents had maxima centred at 190 mM NaCl, 48 mM $CaCl_2$, 39 mM EDTA, and 18 mM oPC (Fig. 1). At these concentrations of solutes, the average molecular mass of PDC-109, determined by equilibrium sedimentation at room (20–22°C) temperature, was decreased from 70 kDa to 47, 45, 19, and 20 kDa, respectively [13]. These results indicated a correlation between the aggregation state of PDC-109 and its heparin-binding affinity, suggesting that the minimal size of the heparin-binding PDC-109 oligomer may display a tetrameric or greater quaternary structure. Moreover, PDC-109 did not bind to the heparin-Sepharose column when the chromatography was carried out at 36°C. In a previous study we reported that PDC-109 displayed two calorimetric transition at 36°C and 55°C. The 36°C melting transition was assigned to the dissociation of the 70-kDa PDC-109 oligomer [13], which further supports our hypothesis that the heparin-binding region of PDC-109 is conformational

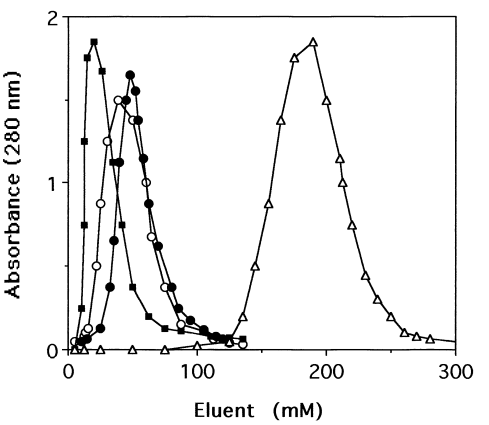

Fig. 1. Elution patterns of PDC-109 from a heparin-Sepharose column as a function of the solute concentration in the elution buffer: □, *ortho*-phosphorylcholine; ○, EDTA; ●, $CaCl_2$; and ▲, NaCl.

and dependent on the quaternary arrangement of more than four PDC-109 monomers.

3.2. Mapping the heparin-binding site by selective chemical modification

For the identification of amino acid residues presumably forming part of the heparin-binding region of quaternary structure-intact PDC-109 oligomers, we chose an indirect approach. Isolated PDC-109 was bound at room temperature to a heparin-Sepharose column and the accessible amino groups or arginine residues of the bound protein were alternatively labelled by reaction with acetic anhydride (AcA) or 1,2-cyclohexanedione (CHD). We argued that those lysine and arginine residues involved in binding heparin will be essentially protected by the ligand from chemical labelling and will be identified by peptide mapping and mass spectrometric characterisation as unmodified amino acids. The same amino group- and arginine-modification protocols were carried out with free protein in solution to assess whether the failure to modify a particular residue could be attributed to a ligand protection effect or might be due to protein structural constraints. A similar approach has been successfully applied for mapping the heparin-binding site of mucus proteinase inhibitor [24].

The molecular mass of free PDC-109 aminoacetylated with a 800-fold molar excess over lysine residues was 13 174 ± 11 Da, which may correspond to non-glycosylated PDC-109 modified by nine acetyl moieties (mass increase of 42 Da per acetylated amino group; calculated isotope-averaged molecular mass of 13 167 Da). Since PDC-109 contains a free N-terminal α-amino group and seven lysine ε-amino groups (Fig. 2), the result indicated that all lysine residues were solvent-exposed and that additional acetylation took place at the reagent concentration used. On the other hand, treatment of free PDC-109 with a 6-fold molar excess of 1,2-cyclohexanedione over arginine residues yielded a modified protein sample with molecular masses of 13 235 ± 14 Da and 13 905 ± 6 Da. These molecular masses were assigned to non-glycosylated and glycosylated PDC-109 molecules each containing four DHCH-Arg residues (mass increase of 112 Da per DHCH-Arg [25]; calculated isotope-averaged molecular masses of 13 244 Da and 13 899 Da, respectively). This result clearly indicated that four out of five arginine residues of PDC-109 (Fig. 1) were accessible to form DHCH-Arg derivatives.

Columns containing 5 mg of PDC-109 bound to heparin-Sepharose were incubated with a continuous flow of column buffer containing either a 800-fold molar excess of acetic anhydride over lysine residues or a 6-fold molar excess of 1,2-cyclohexanedione over arginine residues. After washing, the protein was eluted, dialysed, degraded with endoproteinase Lys-C, and the resulting peptides were isolated by reversed-phase HPLC and characterised by amino acid analysis and mass spectrometry (Fig. 2). CHD-treated PDC-109 peptide R26 displayed three ions at 3955, 4320, and 4611 Da, which corresponded to *S*-carbamoylmethylated (CM) polypeptide 1–34 non-glycosylated (M_{calc}: 3956 Da), glycosylated with GalNAcβ(1-3)-Gal [21] (M_{calc}: 4321 Da), and glycosylated with the trisaccharide GalNAcβ(1-3)-Galα(2-3)-Neu5Ac [22] (M_{calc}: 4612 Da), respectively. Peptides R28 and R29 showed in addition to the ions of K26 an ion at 4180 Da, which was assigned to CM-(1–33) with two DHCH-Arg residues. CHD-treated PDC-109 peptides R38a (3056 Da), R38b (4581 Da), R5 (1176 Da), R6 (676 Da) and R10 (547 Da) corresponded

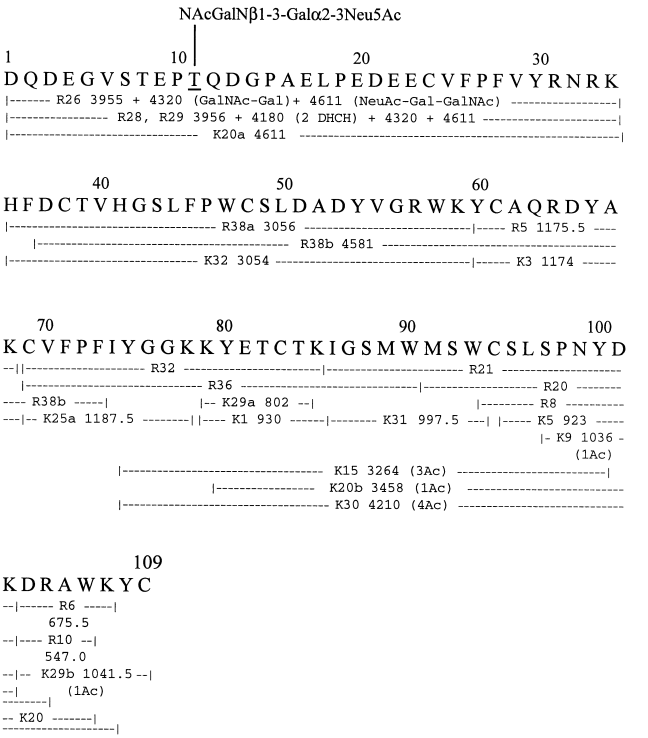

Fig. 2. Identification of reversed-phase HPLC-purified endoproteinase Lys-C-derived peptides of PDC-109 eluted from a heparin-Sepharose column after incubation with acetic anhydride (K) or 1,2-cyclohexanedione (R-). Disulphide bonds were reduced and cysteine residues were carboxymethylated before proteolysis. Molecular masses determined by electrospray ionisation mass spectrometry are shown in parentheses. Peptides containing aminoacetylated (Ac) groups and DHCH-Arg residues are indicated.

to CM polypeptides 34–59, 37–73, 60–68, 103–107 and 103–106 with unmodified arginine residues 57, 64, and 104. These results showed that only the N-terminal arginines residues 30 and 32 (Fig. 2) were modified by 1,2-cyclohexanedione.

Characterisation of endoLys-C degradation peptides from aminoacetylated PDC-109 identified major peptide K20 ($M+3^+ = 1538$ Da, $M = 4611$ Da) as non-modified residues 1–34 with threonine 11 *O*-glycosylated with the trisaccharide NeuAc-Gal-GalNAc (Fig. 2). Peptide K32 (3055 Da), which corresponded to the carboxymethylated polypeptide 35–59 (Fig. 2), was produced by cleavage after Lys^{33} further indicating that this residue was not acetylated. Peptide K3 (1174 Da) was identified as non-modified polypeptide stretch 60–68 showing that Lys^{68} was not modified. Comparing peptides K1 (930 Da) and K29a (802 Da), which were characterised as non-acetylated polypeptides 79–85 and 80–85, with peptides K15 (3264 Da, residues 75–100 including aminoacetylated lysines 78, 79, and 85), and K30 (4208 Da) encompassing residues 75–108 with aminoacetylated lysine residues 78, 79, 85, and 103, also indicated that modification of lysine residues 78 and 79 was partial. The ratio of non-modified to modified residue was estimated from the height of the HPLC chromatogram to be roughly equimolar. Peptide K9 corresponded to residues 97–104 including aminoacetylated lysine 102, and peptide K20 (3458 Da) was identified as residues 80–106 with aminoacetylated Lys^{85}. However, the fact that minor peptides K31 (86–93, 998 Da) and K5 (95–102, 923 Da) were produced by cleavage after lysine residues 85 and 102,

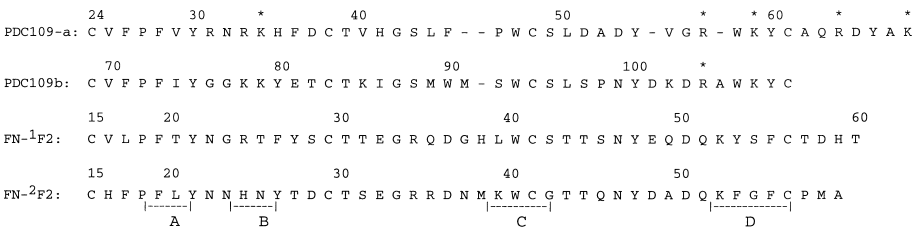

Fig. 3. Alignment of the amino acid sequences of the fibronectin type II (FnII) domains a and b of PDC-109 and the first and second FnII modules of human fibronectin (labelled PDC109-a, PDC109-b, FN-^{1}F2, and FN-^{2}F2, respectively). The four β-strands of the NMR solution structures of the latter three FnII domains [26–28] are labelled A–D. The six basic residues of PDC-109 protected by bound heparin from chemical modification are identified with asterisks.

respectively, also indicated that acetylation of these residues was partial (40–50%). Finally, lysine 108 was found as its aminoacetylated derivative in peptide K29b (residues 103–109, 1042 Da) (Fig. 2).

As a whole, the results of selective chemical modification indicate that six basic residues (Lys^{34}, Arg^{57}, Lys^{59}, Arg^{64}, Lys^{68}, and Arg^{104}) were protected from reaction with acetic anhydride and 1,2-cyclohexanedione in heparin-bound PDC-109 oligomers. The lysine residues may be truly shielded by bound heparin because all lysine residues of free PDC-109 were labelled by acetic anhydride. On the other hand, since only four out of five PDC-109 arginines were accessible to 1,2-cyclohexanedione labelling, at least two of the three non-modified arginine residues of heparin-bound PDC-109 may reside within the heparin-binding region. The third non-labelled arginine residue could form part of the intersubunit surface.

3.3. *On the topology of the heparin-binding region of PDC-109 oligomers*

The three-dimensional structure of full-length PDC-109 has not been solved. However, several groups have reported the NMR solution structure of individual Fn type II modules, including PDC-109 domain b (residues 65–109) [26] and the first and second human fibronectin type II modules [27,28] (Fig. 3). These studies showed that each FnII domain contains two double-stranded antiparallel β-sheets, oriented almost perpendicular to each other, which enclose a cluster of conserved solvent-exposed aromatic residues possibly involved in the binding of hydrophobic ligands. In each of these structures residues topologically equivalent to PDC-109 Arg^{57} (Arg^{104}) and Lys^{59} are solvent-exposed and located around β-strand D on the same face of the domain, and Arg^{64} and Lys^{68} are located in the polypeptide stretch between domains a and b (Fig. 3). The side chain of Lys^{68} is likely also to form part of the same cluster of positively charged residues (Fig. 4). However, lysine 11, which is topologically equivalent of Lys^{34} (Fig. 3), is oriented towards the opposite site of the domain. It is possible that this residue forms part of another cluster of basic residues in full-length PDC-109 or PDC-109 oligomers. Alternatively, Lys^{34} may be protected from chemical modification because it is hidden in the oligomeric association of PDC-109.

The arrangement of the conformational and quaternary structure-dependent heparin-binding site of PDC-109 resembles the topology of glycosaminoglycan-binding sites of other heparin-binding proteins, which are typically extended clusters or bands of positively charged amino acids distributed along the surface of the protein, or in a shallow groove [29]. Residues important for binding heparin are often remote in the amino acid sequence but are brought together in the folded protein. Moreover, many heparin-binding proteins display non-contiguous cationic clusters distant from each other by 16–24 Å. The fact that oligomeric, but not monomeric, PDC-109 possesses heparin-binding activity indicates that the PDC-109 monomer lacks the strategically positioned cationic resi-

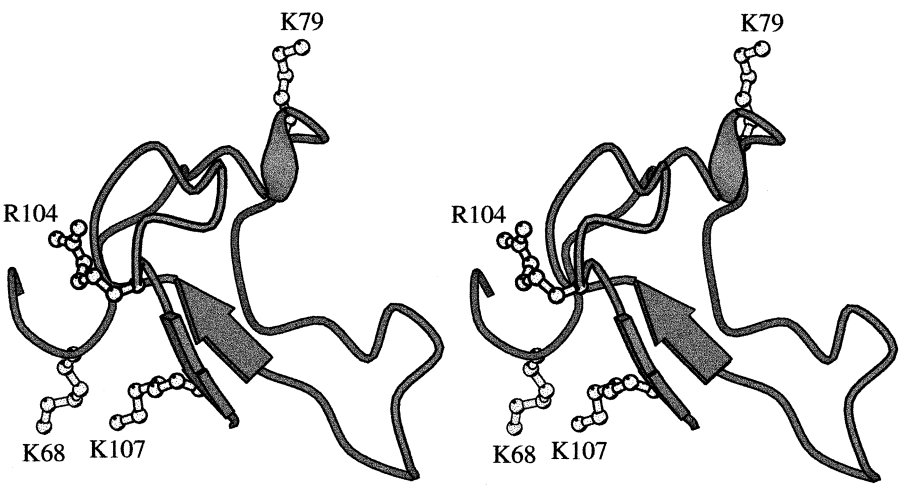

Fig. 4. Stereo drawing of the α-carbon backbone of the PDC-109 domain b structure showing the relative orientation of residues Lys^{68}, Lys^{79}, Arg^{104}, and Lys^{107}. The latter three residues are topologically equivalent to domain b residues Lys^{34}, Arg^{57} and Lys^{59}, respectively (Fig. 3), which are shielded from cheminal modification in heparin-bound PDC-109 oligomers.

dues that are necessary for tight binding to heparin. A similar example is platelet factor 4, where the clustering of four lysine residues contributed by each monomer is enhanced through self-association of the protein to form dimers and tetramers with progressively higher affinity for heparin [30].

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