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Demonstration of a heparin-binding site in serum amyloid P component using affinity capillary electrophoresis as an adjunct technique

Niels H.H. Heegaard[a,*], Hanne D. Mortensen[b], Peter Roepstorff[b]

[a]*Department of Autoimmunology, Statens Seruminstitut, DK-2300 Copenhagen S, Denmark*

[b]*Department of Molecular Biology, University of Odense, DK-5230 Odense M, Denmark*

Abstract

Linear heparin-binding sites in the DNA- and heparin-binding serum protein amyloid P component were investigated using affinity capillary electrophoresis and reversed-phase HPLC in conjunction with affinity chromatography. Peptide fragments were generated from amyloid P component by treatment with Glu-C and Asp-N endoproteinases. This peptide mixture was separated by HPLC before and after passage through a column of immobilized heparin. In addition, the proteolytic digest was separated by capillary electrophoresis in the presence of various amounts of heparin in solution. Migration shift patterns in the presence of heparin were in agreement with one of the components shown by HPLC to interact with immobilized heparin. The identity of this fragment was established by mass spectrometry after preparative HPLC and represents a novel heparin-binding sequence. The results illustrate the potential synergy in the combination of the two high-resolution separation techniques HPLC and CE. HPLC has the advantages of high recovery and preparative power while capillary electrophoresis is noted for highly efficient separations under physiological conditions. The possibility of using unmodified ligands in the study of biological activities of protein substructures while consuming very little material makes CE further attractive.

1. Introduction

Biomolecular interactions are often characterized using immobilized or otherwise modified (e.g., labelled) receptor proteins or ligands. In many cases, however, binding data may be obtained by means of various electrophoretic techniques using unmodified reactants [1]. The use of electrophoresis for the characterization of reversible binding is based on measurement of changes in electrophoretic parameters resulting from interactions that take place during electrophoresis. Accordingly, this type of interaction analysis is often called affinity electrophoresis [2]. Capillary electrophoresis (CE) has been used with advantage for affinity studies (affinity CE) in a growing number of specific cases [3–12] because of its merits of speed, versatility, low sample consumption and high resolving power.

Amyloid P component is a protein of unknown physiological function that is found in serum [serum amyloid P component (SAP)], from where it may readily be isolated by means of its Ca^{2+}-dependent affinity for DNA, heparin or residues in Sepharose. It seems to be an obligatory constituent of all amyloid lesions irrespec-

* Corresponding author.

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tive of the nature of the amyloid core protein [13,14]. Owing to the persistent association of SAP and heparan sulfate proteoglycans with amyloid deposits, including those of Alzheimer's disease [13], there is interest in elucidating the structure of binding sites on SAP for heparin. The three-dimensional structure of SAP at pH 5.5 has been determined and a binding site for small anionic carbohydrates requiring Ca^{2+} and contributions from non-contiguous parts of the molecule was delineated [15]. However, there may well be other binding sites for larger anionic carbohydrates such as heparan sulfate and heparin. SAP is structurally closely related to C-reactive protein (CRP) [16] but, unlike CRP, it is not an acute-phase protein in man and is glycosylated. It has been shown that bioactive peptides may be generated from CRP on incubation with proteolytic enzymes from polymorphonuclear leukocytes (PMN) [17], and it was recently demonstrated that SAP in addition to CRP binds to PMN receptors and that peptides generated by subjecting intact SAP to proteolytic enzymes from PMNs inhibited this binding [18].

Hence, to identify linear binding sites possibly mimicking the binding of heparin by the parent protein, SAP was digested with various proteolytic enzymes and subjected to analysis by HPLC after absorption with immobilized heparin and by affinity CE using heparin in solution. Here we report the results of experiments carried out with SAP fragments generated by treatment with Asp-N and Glu-C endoproteinases. The study identified a novel heparin-binding sequence in SAP and illustrated the usefulness of CE as a complementary technique for the identification and characterization of ligand-binding sites.

2. Experimental

2.1. Chemicals

Bovine serum albumin, chondroitin sulfate, type C, from shark cartilage (C-4384), dithiothreitol, heparin from porcine mucosa (H-7005), Tris [tris(hydroxymethyl)aminomethane] and 4-vinylpyridine were obtained from Sigma (St. Louis, MO, USA), *Staphylococcus aureus* V8 protease from Worthington (Freehold, NJ, USA), Asp-N endoproteinase from Boehringer (Mannheim, Germany), guanidinium hydrochloride, heparin-Eupergit C and trifluoroacetic acid (TFA) from Fluka (Buchs, Switzerland), DNA-agarose from Pharmacia–LKB (Uppsala, Sweden), formic acid from Aldrich (Steinheim, Germany) and HPLC-grade water and acetonitrile and all other chemicals (analytical-reagent grade) from Merck (Darmstadt, Germany).

2.2. Serum amyloid P component fragmentation

Serum amyloid P component (SAP) was purified from human plasma by affinity chromatography on DNA-agarose followed by ion-exchange HPLC as described [19]. Aliquots of 1 mg of SAP were dialysed against water at 4°C and evaporated to dryness in a centrifugal evaporator (SpeedVac). Reduction and S-pyridylethylation was performed by adding 500 μl of 6 *M* guanidinium hydrochloride in Tris-buffered saline [TBS: 5 m*M* Tris–HCl (pH 7.4)–150 m*M* NaCl] to 1 mg of dried SAP followed by 2 mg of dithiothreitol. After stirring for 2 h under helium, 5 ml of 4-vinylpyridine was reacted with the sample at room temperature for 3 h, then 2 ml of formic acid were added and the solution was dialysed against water at 4°C overnight and dried.

The V8 protease was used for digesting the reduced and S-pyridylethylated SAP at a ratio of 10 μg of enzyme to 1 mg of SAP in 500 μl of 20 m*M* NH_4HCO_3–10% CH_3CN–1 *M* guanidinium hydrochloride. Under these conditions, the Glu-C endoproteinase activity of the enzyme predominates. The digestion was allowed to proceed at room temperature with stirring for 48 h. The digest was then mixed with an equal volume of formic acid and desalted on a C_{18} column (see below). All material that eluted with 70% CH_3CN in 0.1% TFA was collected, dried and resuspended in 400 μl of 45 m*M* phosphate (pH 7.5) containing 7.5% CH_3CN and 2 μg of Asp-N endoproteinase (corresponding to ca. 1% by mass of dried material). The sample was incubated at room temperature overnight with stir-

ring and then used for affinity chromatographic experiments. Chosen peptide fragments were collected manually from the HPLC system using a 150-μl injection volume. Another 150 μl were desalted on the HPLC column as described above. Purified material and desalted digest were dried by centrifugal evaporation.

2.3. Reversed-phase HPLC

HPLC was performed on an analytical C_{18} column (218TP54, 5 μm, 250 × 4.6 mm I.D.) from Vydac (Hesperia, CA, USA), eluted with a gradient at 1 ml/min of 5–65% acetonitrile in 0.1% TFA over 60 min. Detection took place at 210 and 280 nm.

2.4. Absorption with immobilized heparin

SAP-derived peptides in a volume of 50 μl in digestion buffer were mixed with 0.2 ml of heparin-Eupergit C matrix that had been previously washed with 10-ml volumes of water, tenfold concentrated TBS and TBS. After 10 min on the column at room temperature, the matrix was washed with 0.4 ml of TBS and all effluent was collected and analysed by reversed-phase HPLC as described above.

2.5. Plasma desorption mass spectrometry (PDMS) and amino acid sequencing

PDMS was performed on a BioIon 20 time-of-flight mass spectrometer (BioIon, Uppsala, Sweden). Peptide material was dissolved in 0.1% TFA–20% methanol to a concentration of ca. 50 pmol/μl and 2 μl were applied on a spin-cast nitrocellulose target [20].

Amino acid sequencing was done by Edman degradation using a Model 910 pulsed-liquid sequencer from Knauer (Berlin, Germany).

2.6. Capillary electrophoresis

The CE instrument was a P/ACE 2050 from Beckman Instruments (Fullerton, CA, USA) with data collection, storage, analysis and reporting performed by System Gold Software (Beckman Instruments).

Throughout, uncoated fused-silica 50 μm I.D. capillaries of 57 cm length (50 cm to the detector) and an electrophoresis buffer of 0.1 *M* phosphate (pH 7.5) (filtered through a 0.22-μm filter) were used. Sample injections took place by pressure at the anode end of the capillary. A 1-s injection corresponds to a sample volume of 0.9 nl according to the specifications of the manufacturer. Detection was effected at 200 nm and the field strength used for the separations was 15 or 20 kV as indicated. The liquid cooling system was set to 19°C.

2.7. Affinity capillary electrophoresis

The dried material was subjected to capillary electrophoresis experiments after resuspension in HPLC-grade water (purified fragments in 50 μl and the digest mixture in 100 μl). Electrophoresis took place as described above in phosphate buffers with various additions of heparin or chondroitin sulfate from stock solutions at 20 mg/ml of glycosaminoglycan in 0.05 *M* phosphate (pH 7.5). Identification of individual peptides took place by co-injection of SAP digests with purified peptide from a second injection vial.

3. Results and discussion

After treatment of reduced and S-pyridylethylated SAP with Glu-C and Asp-N endoproteinases, a mixture of peptide fragments was obtained. The analysis of this by reversed-phase HPLC is shown in Fig. 1A. The digest was examined for heparin-binding components by absorption on immobilized heparin followed by HPLC (Fig. 1B). Affinity chromatography was performed using heparin immobilized on acrylamide instead of Sepharose because SAP is known to bind to components of the latter matrix [21]. Several fragments decreased in amount after incubation with immobilized heparin, indicating retention on the matrix. The main heparin-interacting components are peaks

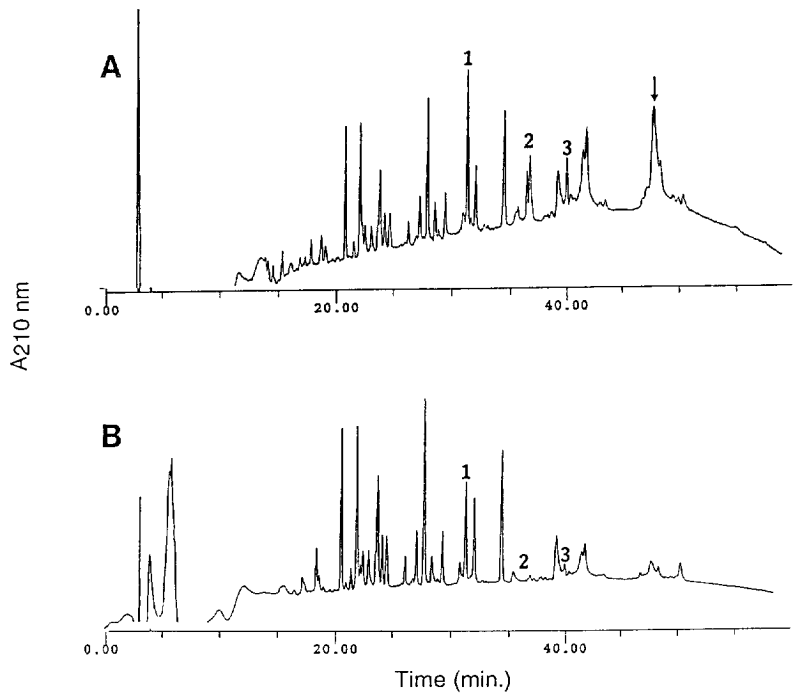

Fig. 1. HPLC analysis (detection at 210 nm) of an endoproteinase Asp-N-treated Glu-C digest of SAP. Heparin-binding SAP fragments characterized by absorption on immobilized heparin followed by HPLC. (A) Control (10 μl of material injected); (B) mixture after passage through a heparin-Eupergit C column (a volume of 400 μl was injected). Peaks 1–3 were collected separately and analysed further. Arrow marks a late complex that probably represents incompletely digested protein.

1–3 in Fig. 1A. The late complex observed after 45 min in Fig. 1A (arrow) probably represents intact and incompletely digested protein which is also retained on the heparin column. No material was eluted with 1.5 *M* NaCl (not shown). The late component was not investigated further but peaks 1–3 were isolated for characterization.

Analysis of the digest by CE after drying and resuspension in water is shown in Fig. 2. To characterize heparin binding under circumstances close to physiological conditions, the CE separations were performed using 0.1 *M* phosphate buffer at neutral pH as the electrophoresis buffer. Hence a 100% recovery of all the peptides seen in the HPLC separation was not expected. Approximately 20 components were separated by CE (Fig. 2A), and changes in the migration pattern were observed on incorporation of heparin into the electrophoresis buffer (Fig. 2B). Even through the whole population of peptides had an increased migration time when heparin (Fig. 2B) or chondroitin sulfate (Fig. 2C) was added (due to a decrease in the electroosmotic flow), it was evident that the marked peptide peak (asterisks in Fig. 2) had a much more pronounced migration shift than the bulk of peptides in experiments with heparin (Fig. 2B), whereas this was not the case in experiments with additions of chondroitin sulfate to the electrophoresis buffer (Fig. 2C). Other, smaller, changes also occurred specifically in the heparin experiment, but these have not yet been investigated further. In accordance with these results, the parent protein, SAP, has been shown to exhibit a lower affinity for chondroitin 4-sulfate and chondroitin 6-sulfate than for heparin [22].

By spiking with purified components (not shown), the heparin-binding component identified by affinity CE was found to correspond to fragment 3 in Fig. 1A. In contrast, the material of peak 2 of Fig. 1A was not recovered under the CE conditions used in this study, and the materi-

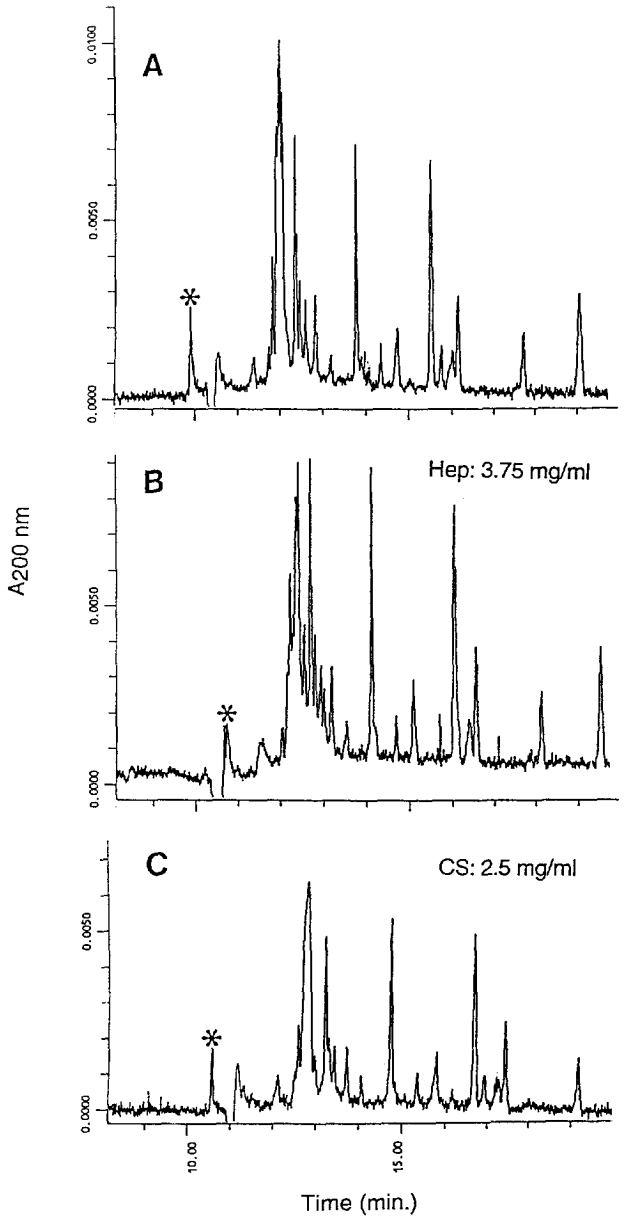

Fig. 2. Screening by affinity CE for interaction of SAP peptides with heparin in solution. An endoproteinase Asp-N-treated Glu-C digest of SAP solubilized in water was injected for 12 s and subjected to CE at 15 kV (detection at 200 nm) in the presence of heparin (Hep) (B) or chrondroitin sulfate (CS) (C) added to the electrophoresis buffer (0.1 *M* phosphate, pH 7.5) at the concentrations indicated. The peptide marked with asterisks was identified by spiking with HPLC-purified fragments and corresponds to fragment 3 in Fig. 1.

al of peak 1 yielded two components that did not seem to be influenced by heparin in affinity CE (not shown).

Fragment 3 was subjected to mass spectrometry and amino acid sequencing. The first four residues were found to be H–Ile–Arg–Gly–Tyr and this and the measured mass of 1555.7 (expected, 1556) corresponded to a C-terminal

peptide: -Ile-Arg-Gly-Tyr-Val-Ile-Ile-Lys-Pro-Leu-Val-Trp-Val-OH (amino acids 192–204 in the SAP structure [23]). This peptide, purified from the SAP digest, was analysed and examined for heparin binding in affinity CE (Fig. 3). As can be seen, there was a concentration-dependent decrease in the mobility of the peptide with increasing concentration of heparin in the electrophoresis buffer without apparent changes in peak shape or size. This is taken to indicate binding of the peptide to heparin with a dynamic equilibrium existing in the course of the electrophoretic process between non-complexed and heparin-complexed peptide (with a lower mobility than the free peptide). The equilibrium shifts towards longer times spent in the complexed form (and thus a more slowly migrating but homogeneous peak) when more heparin is present [1,5].

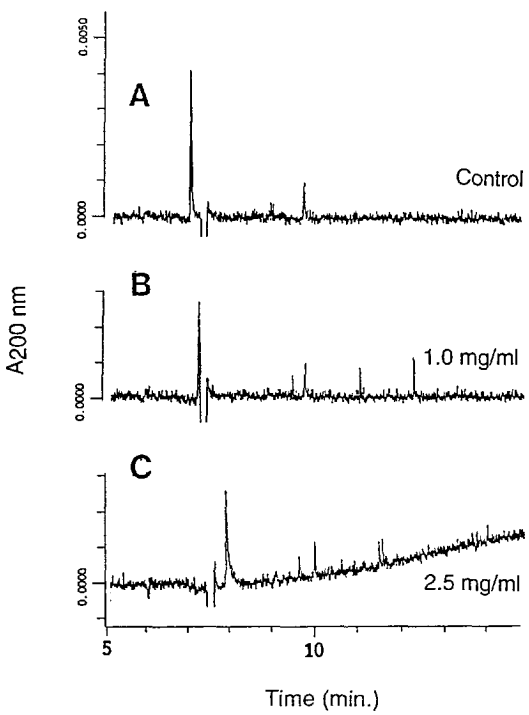

Fig. 3. Purified heparin-binding fragment in affinity CE. Demonstration of interaction with heparin. Fragment 3 (cf., Fig. 1) was analysed at 20 kV (detection at 200 nm) after 5-s injection in the presence of the indicated concentrations of heparin in the electrophoresis buffer (0.1 *M* phosphate, pH 7.5).

This pattern of behaviour is consistent with affinity interactions of intermediate strength characterized by fast association and dissociation rates [4,24].

A fundamental demand for the use of affinity CE to measure binding interactions is that complexed molecules must differ from unbound, free species in mobility. In the present study this was expected because heparin, by virtue of its high sulfation, is highly negatively charged. Thus, heparin-binding components would be predicted to reveal themselves in affinity CE by changing to a lower overall mobility or, for stronger interactions where the time for the electrophoresis is too short to ensure equal participation of all peptide molecules in complexes and in a free state, by decreases in peak size or by peak broadening or splitting [24]. For the peptide identified in this study, addition of heparin in sufficient amounts actually results in a reversal of its electrophoretic mobility from being towards the cathode to being towards the anode (Fig. 3B and C). In both cases, the observed mobility is due to the addition of the electroosmotic flow to the electrophoretic mobility and the consequence is that the molecule crosses the gap (water) representing the movement of neutral components in the system. Thus, on incorporation of heparin, the mobility of the peptide changes much more than the minor alterations in the electroosmotic flow account for.

4. Conclusions

A novel heparin-binding peptide from SAP has been demonstrated by a combination of HPLC and affinity CE methods. It does not contain the usual stretches of positively charged residues that are found in concensus sequences for glycosaminoglycan-binding peptides [25].

The observations in CE were supported by the results of reversed-phase HPLC separations of the same fragments incubated before chromatography with immobilized heparin, which demonstrated binding of this and other peptides.

Additional SAP-derived heparin-binding peptides generated utilizing other endoproteinases

and also synthetic peptides based on the activities discovered are now being characterized and related to the functions of the parent protein. This will be the subject of a forthcoming publication.

The problem of the recovery of potentially interesting molecules is illustrated in this study by fragment 2, which could not be analysed by CE at pH 7.5. The advantage of not being restricted to using an immobilized ligand was exemplified by peptide 1, which appeared to be retained on the heparin-Eupergit column but where neither of the two components that appeared in the CE analysis (not shown) interacted with free heparin in solution. Thus, the retention on the column for this peptide fraction may not be ligand specific or is due to a change in the ligand as a consequence of the coupling chemistry. The avoidance of artifacts due to modified ligands is one of the general advantages of methods such as affinity CE that use ligands in solution.

The solubilization and recovery of molecules are generally less problematic in reversed-phase HPLC than in CE. On the other hand, binding studies in solution are feasible in CE experiments in contrast to the meager opportunities to do this in the acidic organic environment of reversed-phase HPLC.

A final advantage of affinity CE analyses for the characterization of ligand-binding activities of components of proteolytic digests of proteins is the low consumption of material in comparison with solid-phase and equilibrium dialysis procedures, where the demands for material normally make it impossible to use components purified from enzyme digests. The present study is, to our knowledge, the first example of the use of CE to map directly binding sites in protein digests.

Thus, in summary, the potential for high-performance resolutions together with the preparative purification and the non-denaturing functional characterization of small amounts of material afforded by HPLC and CE, respectively, make the combination of the two techniques a potentially highly valuable tool in elucidating correlations between structure and function in proteins and other biomolecules. Peptides with binding activity may be demonstrated, identified in the sequence and characterized to an extent that none of the individual techniques can afford on its own.

Acknowledgements

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