Glycoconjugate Journal (1997) **14:** 859–862

Capillary zone electrophoresis for the study of the binding of antithrombin to low-affinity heparin

Kerstin Gunnarsson[1]*, Ludmila Valtcheva[2] and Stellan Hjertén[2]

From the Departments of [1]Medical and Physiological Chemistry, PO Box 575 and [2]Biochemistry, Uppsala University, Biomedical Centre, PO Box 576, S-751 23 Uppsala, Sweden

When low-affinity interactions between glycosaminoglycans and precious proteins are studied, it is imperative to design an experimental set-up that consumes as little material as possible. To evaluate the applicability of the CZE technique to this problem, we explored the interaction between antithrombin and low-affinity heparin. In a series of CZE experiments we demonstrated that the mobility of antithrombin increases gradually as increased concentrations of low-affinity heparin were added to the electrolyte. The results were, as expected, consistent with the general algorithm for monovalent binding. The binding constant was estimated at 20±6 μM in excellent agreement with the value reported in the literature.

***Keywords*: reversible binding, capillary zone electrophoresis, low-affinity heparin, antithrombin**

Introduction

The glycosaminoglycans (GAGs), heparin and heparan sulphate (HS), are linear polymers composed of alternating glucosamine and hexuronic acid units carrying sulphate groups in various positions. They are highly heterogeneous compounds, both with respect to chain length and sulphate density and distribution. Heparin and HS are synthesized as proteoglycans in which the polysaccharide chains are covalently bound to a protein core. While heparin is found only in connective tissue mast cells, heparan sulphate can generally be found on cell surfaces. Heparin is more sulphated than HS. Most of the biological activities ascribed to heparin/HS are due to interactions with various proteins [1–3], for instance enzymes and enzyme inhibitors, cytokines including growth factors, and various matrix proteins. Such interactions range from highly specific, 'lock-and key' type binding, such as in the antithrombin-binding pentasaccharide region in heparin [4], to relatively non-specific, co-operative electrostatic interactions, for example in the interaction with platelet factor 4 or fibronectin [5], which depend on overall net surface charge density rather than on the specific disposition of the charged groups.

Most of the interactions between heparin/HS and proteins so far described in the literature are of relatively high affinity ($K_D = 10^{-8}$–10^{-10} M). For the study of high affinity interactions, affinity chromatography has been used as well as a conventional filter assay where the ligand binds to adsorbed protein [5, 6]. Methods based on adsorption are not applicable when $K_D > 1$ μM since such complexes are kinetically too labile to endure the experimental procedures at physiological conditions. Low-affinity interactions can, however, be of fundamental physiological importance. One example is the interaction of HS with the fibroblast growth factor receptor where K_D was approximated to 10^{-4} M [7]. It is not known whether there is a specific carbohydrate sequence involved in this interaction.

Our aim with this study was to establish a method that will allow us to detect and study low-affinity interactions and to determine binding constants and, possibly, sequence specificities. The availability of HS and well-defined fragments of heparin/HS as well as the access to interacting proteins are usually very limited. It is, therefore, important to design an experimental set-up that consumes as little material as possible. Earlier approaches for determining binding constants for low-affinity protein-ligand interactions all involve studies of equilibrium systems. In a series of experiments, differences in specific properties of free protein when compared to protein-ligand complex are monitored as the concentration of ligand is altered. Suitable monitoring techniques are, for example, gel filtration [8], fluorescence spectroscopy, NMR, differential scanning calorimetry, affinity gel-electrophoresis [9, 10], and ultracentrifugation. Most of these methods require considerable amounts of material. Capillary zone electrophoresis (CZE) is an attractive alternative for the investigation of interactions in free solution since only nanogram quantities of the protein to be

*To whom correspondence should be sent at present address. Dr Kerstin Gunnarsson, Biacore AB, Rapsg. 7, S-754 50 Uppsala, Sweden. Tel: +46-18-67 57 37; Fax: +46-18-15 01 14; E-mail: Kerstin.K.Gunnarsson@eu.biacore.com

0282–0080

analysed are needed, and the saccharide ligand consumption can be kept to a minimum. Since the ligand, heparin/HS, is heavily negatively charged, a pronounced difference in the electrophoretic mobility between the protein and the complex can be anticipated. Other attractive features are that neither the protein nor the ligand need to be labelled or immobilized, and that the experiments can be carried out under non-denaturing conditions at physiological pH and ionic strength. Another advantage is that the analysis is fast. Some studies of protein-ligand interactions utilizing CZE have been published, most of them connected with high affinity interactions between proteins and small ligands (for a review see Heegaard [11]).

To assess the applicability of the CZE technique in studies of low-affinity interactions of GAGs and proteins, we explored the interaction between antithrombin (AT) and low-affinity heparin [12]. AT is known to have one unique binding site for heparin. The heparin-derived pentasaccharide sequence involved in the high-affinity interaction with antithrombin ($K_D \approx 19$ nM) is present only in one third of the heparin chains. Heparin chains lacking this sequence still bind to AT, but this interaction is of much lower affinity ($K_D \approx 19 \pm 6$ μM [12]). This heparin fraction will hereafter be referred to as low-affinity heparin (LAH).

Materials and methods

Heparin was prepared from pig intestinal mucosa (state 14; Inolex Pharmaceutical Division, US) purified as described previously [13]. This material was further purified by affinity chromatography on a bed with immobilized AT. The break-through material is LA-heparin. The average size of this fraction is ≈15 kDa. Human antithrombin was a gift from Dr L.-O. Andersson (Pharmacia Sweden). *N*,*N*,*N*′,*N*′,-tetramethylethylenediamine (TEMED), ammonium persulphate and acrylamide of electrophoresis purity (for coating of the capillaries) were from Bio-Rad Laboratories, US. Bind silane was from Pharmacia, Sweden. α-naphthylacetic acid (α-NAA) was a gift from Professor K.-G. Wahlund, Lund, Sweden.

The HPCE system is laboratory designed and has been described elsewhere [14]. In order to minimize the consumption of LAH, the instrument was equipped with small buffer reservoirs with a volume of 50 μl.

Fused silica tubing of 0.05 mm i.d. and 0.375 mm o.d. was from MicroQuartz, Germany. Tubing pieces of 10 cm length (7 cm to the detector) were used for the experiments. A 2–3 mm zone of the outside polyimide coating was removed at the detection point by burning on an electrically heated Kantal wire [15]. The interior surface of the capillaries was coated with linear polyacrylamide as described [16]. The coating eliminates the interaction between the protein and the silica wall as well as the electroendoosmotic flow thus increasing the reproducibility of the migration rate and the accuracy in the determination of binding constants.

The migration rates of antithrombin were determined in a series of experiments where the concentration of LA-heparin in the electrolyte, PBS, pH 7.3, was varied (0.1 μM–1 mM). The capillary and the cathodic buffer reservoir were filled with the ligand-containing electrolyte and the anodic buffer reservoir with PBS alone. The applied sample consisted of AT ($2\ mg\ ml^{-1}$) and α-NAA ($0.1\ mg\ ml^{-1}$, internal standard) in PBS. The protein zone was detected at 230 nm.

Electrophoresis experiments were performed as follows. A sample zone of about 1 mm was applied by means of the capillary forces at the cathodic end of the tube. A short agarose gel plug was introduced right behind the sample zone (for details about sample application and agarose gel preparation, see [14]). The agarose gel contained LA-heparin at the same concentration as in the running buffer. Its function was to prevent hydrodynamic flow in the capillary caused by level differences in the buffer vessels. Prepared in this way, the capillary was placed into the instrument and the electrophoresis was carried out with constant current (60 μA) giving a voltage of approximately 1 kV. The advantage of using constant current instead of constant voltage is that the migration velocity is independent of the temperature of the buffer [17]. The migration times of AT and the reference were measured. Three determinations were made at each concentration.

Data treatment

The relative mobility of AT was defined as the ratio of the migration time for α-NAA and AT in each experiment. The dissociation constant for the 1:1 complex was calculated from the relative mobility for AT as a function of heparin concentration:

$$m = m_P + (m_{PL} - m_P)\frac{[L]}{K_D + [L]}$$

where [L] = ligand concentration
m = relative mobility of the protein at the actual [L]
m_P = relative mobility of free protein
m_{PL} = relative mobility of protein-ligand complex
K_D = dissociation constant

The parameters m_P, m_{PL} and K_D were treated as variables in the regression analysis.

Results and discussion

The technique described above for the determination of the binding constant for the rather weak interaction between AT and LAH is based on the difference in electrophoretic mobility of a protein-GAG complex and that of the corresponding free protein. The migration rate of a protein is determined in a series of experiments in which the concentration of ligand, in this case LAH, in the background electrolyte is varied. With the recorded differences in

mobility, binding curves can be constructed and the binding constant estimated.

The method was applied to the interaction between AT and LAH. When the concentration of LAH in the system was varied from 0 to 0.1 mM, a gradual difference in migration time for the protein was observed (Figure 1). α-NAA was included in all the experiments to monitor the stability of the system. An appropriate reference compound must not interact with the ligand. This prohibits *eg* albumin as a reference, since it has been shown to bind weakly to heparin [9]. It is not known whether there are proteins that do not interact with heparin at higher concentrations, so we chose a small organic compound, α-NAA as the reference substance. The migration time of α-NAA was about 30% of that of AT in PBS. For ligand concentrations up to 100 μM,

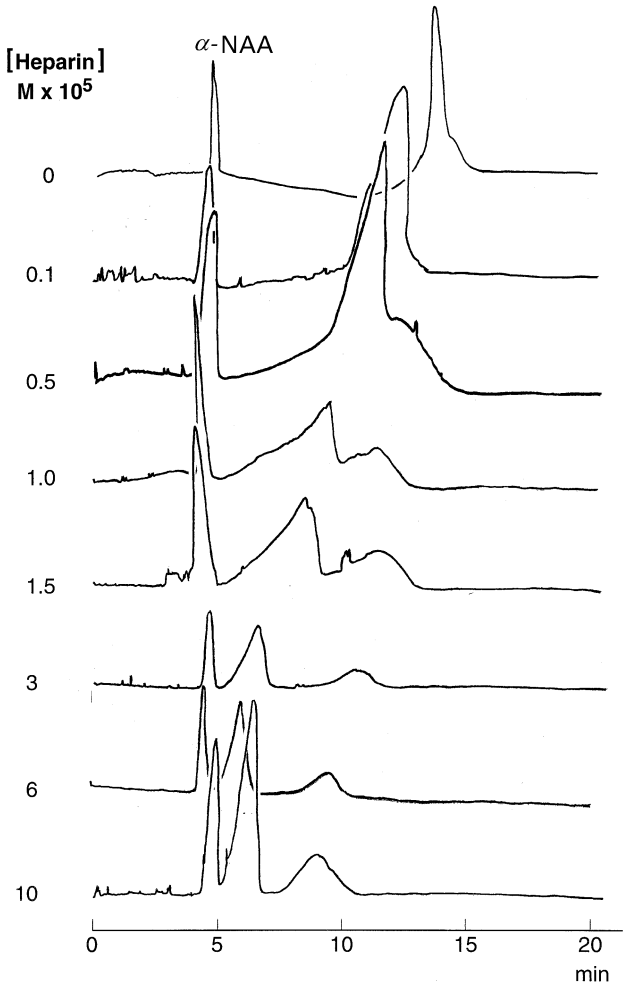

Figure 1. Representative electropherograms from experiments carried out in PBS, pH 7.3, or in the same buffer containing heparin at concentrations varying from 1 μM to 0.1 mM. The applied sample consisted of AT (2 mg ml^{-1}) and *a*-NAA (0.1 mg ml^{-1}) as internal standard in PBS. The protein zone was detected at 230 nm. The electrophoresis was carried out at constant current (60 μA) giving a voltage of approximately 1 kV.

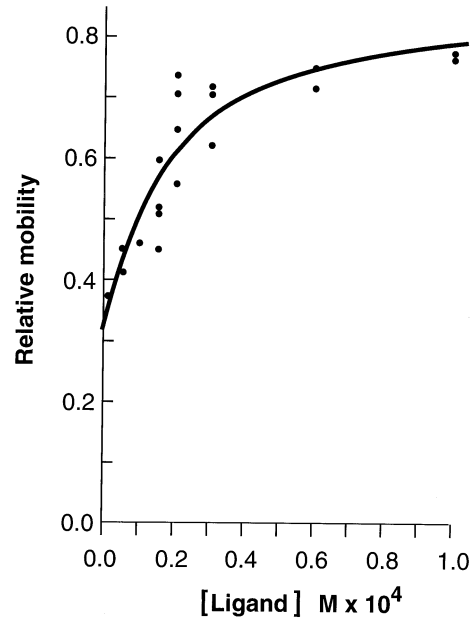

Figure 2. Relative mobility of AT as a function of ligand concentration. The solid line shows the function obtained by regression analysis.

the migration time of the reference compound was essentially the same in all experiments, ranging from 4.2 to 4.7 min, showing that the experimental conditions were relatively stable and reproducible. The reference compound appeared earlier at higher concentrations. The results from these concentrations were omitted in the data processing. When the LAH concentration was $5 \cdot 10^{-6}$ M and higher, a second slower component could be seen, probably the dimer of AT which is known to form slowly upon storage. The binding of the dimer to heparin is weaker than binding of the monomer (Ingemar Björk, personal communication).

The number of data points allowed us to treat not only K_D but also m_P and m_{PL} as variables in the non-linear regression analysis, thus eliminating the need for extreme accuracy of these parameters. The result was consistent with the general algorithm for monovalent binding. The stability of the result was confirmed by omitting points in the regression analysis (data not shown).

For this study, only 20 μg of AT and less than 3 mg of LAH were required. The consumed protein amount is less than 1% of this, but our application routine requires at least 10 μl of solution, and when relying on UV-detection, a protein concentration of >0.2 mg ml^{-1} is desirable.

By using laser-induced fluorescence detection, the concentration of the protein can be reduced considerably. This technique requires, however, labelling of the protein which may affect its structure and consequently the binding constant. More attractive is to use wavelengths in the range 190–200 nm, although the detection limit is higher than in laser-induced fluorescence. Only non-UV absorbing buffers can be used in this approach, such as phosphate and borate

buffers. By obvious modifications of the present application method the sample volume can easily be reduced from 10 to 2 μl. In many experiments the available sample volumes are in the range 0.1–1 ml. We have developed both off- and on-line methods for 100- to 1000-fold enrichment for such volumes [19–22].

Acknowledgements

This study was supported by grants from the Swedish Medical Research Council (grant no. 2309), Polysackaridforskning AB, Uppsala, Sweden, and the Swedish Natural Science Research Council. K.G. is much indebted to Professor Ulf Lindahl for support and valuable advice.

References

1 Kjellén L, Lindahl U (1991) *Annu Rev Biochem* **60**: 443–75.
2 Lindahl U, Lidholt K, Spillmann D, Kjellén L (1994) *Thromb Res* **75**: 1–32.
3 Spillmann D, Lindahl U (1994) *Curr Opn Struct Biol* **4**: 677–82
4 Lindahl U, Thunberg L, Bäckström G, Riesenfeld J, Nordling K, Björk I (1984) *J Biol Chem* **259**: 12368–76.
5 Maccarana M, Lindahl U (1993) *Glycobiology* **3**: 271–7.
6 Maccarana M, Casu B, Lindahl U (1993) *J Biol Chem* **268**: 23898–905
7 Pantoliano MW, Horlick RA, Springer BA, Van Dyk DE, Tobery T, Wetmore DR, Lear JD, Nahapetian AT, Bradley JD, Sisk WP (1994) *Biochemistry* **33**: 10229–48.
8 Hummel JP, Dreyer WJ (1962) *BBA* **63**: 530–2.
9 Lee MK, Lander AD (1991) *PNAS* **88**: 2768–72.
10 Witt DP, Lander AD (1994) *Curr Biol* **4**: 394–400.
11 Heegaard NHH (1994) *Appl Theor Electrophor* **4**: 43–63.
12 Streusand VJ, Björk I, Gettins PGW, Petitou M, Olson ST (1995) *J Biol Chem* **270**: 9043–51.
13 Lindahl U, Cifonelli A, Lindahl B, Rodén L (1965) *J Biol Chem* **240**: 2817–20.
14 Valtcheva L, Mohammad J, Pettersson G, Hjertén S (1993) *J Chromatogr* **638**: 263–7.
15 Lux JA, Hänsig U, Schomberg G (1990) *J High Resolut Chrom* **13**: 373.
16 Hjertén S (1985) *J Chromatogr* **347**: 191–8.
17 Hjertén S (1967) *Chromatogr Rev* **9**: 122–219.
18 Nordenman B, Björk I (1978) *Biochemsitry* **17**: 3339–44.
19 Hjertén S, Valtcheva L (1994) *J Cap Electr* **1**: 83–9.
20 Hjertén S, Liao JL, Zhang R (1994) *J Chromatogr A* **676**: 421–30.
21 Liao J-L, Zhang R, Hjertén S (1994) *J Chromatogr A* **676**: 409–20.
22 Zhang R, Hjertén S (1997) *Anal Chem* **69**: 1585–92.

Received 25 April 1996; revised 26 March 1997