THE BINDING OF CHONDROITIN SULPHATE TO COLLAGEN*

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1. Introduction

The binding of connective tissue polysaccharides to collagen has been the subject of a number of investigations – most recently by one of us [1,2]. By means of light-scattering it could be shown that at physiological pH and ionic strength, there is a weak interaction between collagen and chondroitin 4-sulphate and that only a limited number of polysaccharide molecules could bind to each collagen molecule [2]. In the present communication we have investigated the binding of chondroitin sulphate to collagen which was coupled covalently to agarose beads. The use of collagen-substituted agarose gels in interaction studies with glycosaminoglycans has been reported earlier by us [3] and most recently by Greenwald et al. [4]. With this technique, experiments may be performed over a large range of ionic strengths in spite of the low solubility of collagen at low salt concentrations and neutral pH. We have found that there are at least three binding sites for chondroitin 4-sulphate on each collagen. The number of sites is constant but the binding affinity decreases with increasing ionic strength.

2. Materials and methods

Monomeric, lathyritic collagen was prepared as described by Öbrink [5]. All experiments involving collagen were carried out at 0–4°C. Lyophilized collagen was disolved in 0.1 M acetic acid at a concentration of 0.3% (w/v). After centifugation at 42 000 rev./min for 5 hr in a Spinco Model L ultracentrifuge, rotor 50, the supernatant was dialysed against 0.01 M phosphate buffer pH 7.4 containing NaCl to an ionic strength of 0.15. The solution was then recentrifuged as above. The clear supernatant contained 0.12–0.15% (w/v) of collagen as determined by the biuret method with human serum albumin as a standard [5,6], and the molecular weight was close to that of monomeric collagen as determined by light scattering as previously described [5].

The collagen was coupled to agarose (Sepharose-4B, Pharmacia Fine Chemicals, Uppsala) activated by cyanogen bromide [7,8]. Equal volumes of collagen solution and activated gel, suspended in the aforementioned buffer, were mixed and stirred for 16 hr at 4°C. After addition of 5 ml ethanolamine per 100 ml solution and stirring for another 4 hr, the gel was washed repeatedly with the original buffer and then transferred to 2 mM phosphate pH 7.4. Concentration determinations before and after the coupling procedure indicated that approximately 90% of the added protein was bound to the gel. The exact amount of protein bound to the gel was determined from amino acid analyses on a Biocal amino acid analyzer after hydrolysis for 24 hr at 110°C in 6 M HCl. In six different batches it varied between 0.86 and 1.42 mg of collagen per g wet gel which corresponds to between 2.7 and 4.5 nmol per g gel if the mol. wt of collagen is assumed to be 315 000 [5]. For each experiment a control gel was prepared. Only ethanolamine was coupled to this gel. The free liquid in the gel-suspensions was removed by filtration under suction before the wet gels were used for binding experiments.

A sample of unfractionated chondroitin 4-sulphate from bovine nasal septum with a weight-average mol. wt of 25 300 and a number-average mol. wt of 20 800

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was used in this investigation [9]. A stock solution was carefully dialysed against distilled water and its weight-concentration was determined by the carbazole method [10] using glucuronolactone as standard and assuming a mol. wt of 503 for the disaccharide unit of sodium chondroitin sulphate. The mol. wt of 20 800 was used to obtain the molar concentration of the polysaccharide.

The binding experiments were carried out by mixing 0.5–1 g portions of wet gel with appropriate volumes of the chondroitin sulphate solution and 1 M NaCl. The total volume of liquid added to the gel was adjusted with distilled water to equal the gel volume which made the phosphate buffer 1 mM. The mixtures were stirred for 48 hr at 4°C and centrifuged. The supernatants were analysed for uronic acid by the carbazole method [10]. The pH of each sample was checked and varied between 7.30 and 7.14 at NaCl concentrations from 0 to 0.1 M.

When a macromolecule is added to a gel, it becomes distributed within a volume which is smaller than the total volume of the system due to steric exclusion [11]. The distribution volume of chondroitin sulphate in agarose was calculated in experiments with the gels suspended in 0.5 M NaCl (at this ionic strength no ionic binding between collagen and chondroitin sulphate occurs) from the known amount of polysaccharide added and its final concentration. The distribution volume was on average 0.9 of the total volume. From this distribution volume and the supernatant concentrations the total amount of free chondroitin sulphate could be calculated. The amount of bound polysaccharide was then obtained by subtracting the amount of free compound from the total amount added. At the lowest ionic strengths, the binding to the control gel (ethanolamine coupled gel) was sometimes significant. This unspecific binding was subtracted before the specific binding to the collagen gel was calculated.

3. Results and discussion

The binding of chondroitin sulphate to collagen was found to be strongly dependent on the ionic strength of the medium. It was evident that with the experimental technique used, further studies were best performed at sodium chloride concentration between 0.01M and 0.05M. A low degree of binding

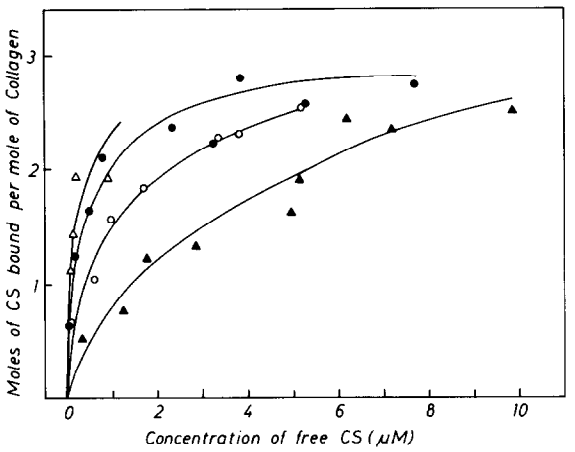

Fig.1. Binding of chondroitin 4-sulfate to collagen as a function of polysaccharide concentration. The experiments were performed as described in the text and made in 1 mM phosphate buffer pH 7 containing 0.0125 M (△), 0.025 M (●), 0.036 M (○) and 0.05 M (▲) NaCl.

was, however, also apparent at physiological ionic strength.

Binding curves obtained at different ionic strengths are shown in fig.1. The experiments were carried out with a gel containing 1.0 mg of collagen per g of wet gel. Essentially the same results were obtained with the other gel preparations. The highest affinity for the polysaccharide was found at low ionic strength. However, at all ionic strengths the gels appeared to be saturated when approximately three chondroitin sulphate molecules were bound to each collagen molecule.

The binding constant and the stoichiometry of the reaction can be analysed according to Scatchard [12] using the expression

$$\bar{\nu}/c = K(n-\bar{\nu})$$

where $\bar{\nu}$ in this case is the molar ratio of bound chondroitin sulphate to collagen, K is the association constant, n is the number of binding sites on collagen and c is the molar concentration of free chondroitin sulphate. Examples of $\bar{\nu}/c$ plotted vs. $\bar{\nu}$ at different ionic strengths are shown in fig.2. Data from experiments on two different gels are incorporated in the figure. The scatter of the experimental points makes the curve-fitting uncertain. It is, however, clear that the plots are non-linear. They extrapolate to an intercept on the $\bar{\nu}$-axis of approximately 3 (or possibly more) indicating at

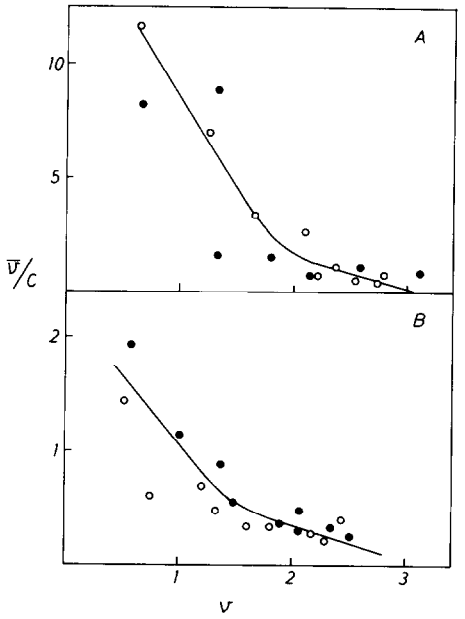

Fig.2. Scatchard plots of the binding of chondroitin 4-sulfate to collagen. The symbols (•, o) indicate experiments with two different batches of collagen coupled to agarose. (A) Experiments in 0.025 M NaCl. (B) Experiments in 0.05 M NaCl.

Table 1
Apparent affinity constants for the binding of chondroitin 4-sulphate (number average mol. wt 20 800) to lathyritic collagen at different ionic strengths

NaCl (M)	Binding constants (M^{-1}) Average of the two strongest binding sites	Third site
0.0125	30×10^6	3×10^6
0.025	8×10^6	2×10^6
0.036	5×10^6	1×10^6
0.050	1×10^6	0.3×10^6

The experiments were performed in 1 mM phosphate buffer pH 7.2 and the sodium chloride concentrations indicated.

least three binding sites. The non-linearity implies that the three sites have different binding strengths. A determination of the binding constants from the plots will therefore be very approximate and the values given in table 1 only indicate the order of magnitude. The binding constant for the two stronger sites was obtained from the initial slopes of the Scatchard plots and that of the third site from the terminal slopes.

The results agree with earlier light scattering measurements at physiological ionic strength which indicated 1.9 binding sites for an essentially monodisperse preparation of chondroitin sulphate of mol. wt 21 000 [2]. It was also found that chondroitin sulphate of mol. wt 32 000 had 4 binding sites on collagen [2]. This observation indicates the presence of two classes of binding sites with different association constants. The chondroitin sulphate preparation used in the present investigation is polydisperse containing polysaccharides ranging in mol. wts from 12 000 to 40 000 [9]. Thus, it seems likely that the two binding sites with high affinity can bind chondroitin sulphate chains of all the mol. wts whereas the third site only might be available for molecules of higher mol. wts. It is conceivable that more than three binding sites could be found if chondroitin sulphate of higher mol. wt and of a narrower mol. wt range was tested. It is also possible that the low affinity sites are available for relatively small chondroitin sulphate molecules but that this cannot be be detected at physiological ionic strength, where the affinity is too low.

The present technique appears to have great potential for studying the interaction between collagen molecules and other compounds. The coupling to agarose enables the experiments to be performed at low ionic strengths where the solubility of collagen prohibits experiments in solution. The fact that the number of binding sites seems to be the same at different ionic strengths makes it possible to determine the number of sites under physiological conditions in spite of the weak interactions in this region. The association constant can also be obtained more readily by this technique than by any other available method. The simplicity of the technique also allows more explicit experiments than e.g. by light-scattering.

It may be argued that the coupling of collagen to agarose may obstruct some binding sites. However, in view of the large number of lysine and hydroxylysine residues in the molecule (approx. 100 [5]) there is less likelihood that coupling will occur at a binding site. Further the size of collagen will not favour coupling to a part of the gel which is unavailable to chondroitin sulphate.

Acknowledgements

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