A sensitive assay for active link protein from cartilage

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A new assay for the activity of cartilage link protein is described. The method is based on the finding [Plaas, Sandy & Muir (1983) *Biochem. J.* **214**, 855–864] that addition of link protein to [³⁵S]sulphate-labelled proteoglycan aggregates from rabbit chondrocyte cultures resulted in the formation of link-stabilized aggregates. The percentage aggregate was found to be related linearly to the amount of purified bovine link protein added in the 20–120 ng range. The assay was used to monitor loss of link-protein activity during heat denaturation and to measure binding of link protein by purified proteoglycan monomer.

INTRODUCTION

Proteoglycan monomers of hyaline cartilages can interact with hyaluronic acid to form multimolecular aggregates (Hardingham & Muir, 1972; Gregory, 1973). The interaction, which has a K_d of about 5×10^{-8} M (Cleland, 1981; Nieduszynski & Sheehan, 1981), can be rendered essentially irreversible under physiological conditions by the addition of link protein (Hardingham, 1979; Franzén *et al.*, 1981), which stabilizes aggregates by specific interaction with both proteoglycan monomer and hyaluronate (Caterson & Baker, 1978; Tengblad, 1981; Lyon & Nieduszynski, 1983; Bonnet *et al.*, 1985).

Link proteins exist in two major native forms with apparent M_r values on polyacrylamide-gel electrophoresis of about 48000 and 44000 (Tang *et al.*, 1979), and the relative abundance of these forms varies among species (Baker & Caterson, 1979). The purification of link protein and production of monospecific antisera has enabled the accurate quantification of link protein by radioimmunoassay (Caterson *et al.*, 1979; Ratcliffe & Hardingham, 1983), Laurell immunoassay (Poole *et al.*, 1980) and enzyme-linked immunosorbent assay (Thonar *et al.*, 1982).

The finding that hyaluronate oligosaccharides can displace monomer from link-protein-free aggregates but not from link-protein-stabilized aggregates (Hardingham & Muir, 1973) established a method for determining the biological activity of link protein preparations. In this method the displacement of monomer was followed either by viscometry (Hardingham, 1979) or by sucrose-density-gradient centrifugation (Franzén *et al.*, 1981). Since these procedures depend on the presence of proteoglycan in rather concentrated solutions (0.5–5.0 mg/ml), they also require that link protein be added in relatively large amounts (1–10 µg).

In the present paper we describe a method for the determination of link-protein activity that is simple to perform and that can detect as little as 10 ng of native link protein.

EXPERIMENTAL

Materials

Materials were obtained as previously described (Plaas *et al.*, 1983; Plaas & Sandy, 1984). In addition,

chondroitin sulphate (type C) was from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of special materials

Proteoglycan monomer (A1A1D1) and aggregate (A1A1) were prepared from bovine nasal-septum cartilage as previously described (Sandy, 1979). Proteoglycan binding region and link protein were prepared from aggregate essentially as described (Ratcliffe & Hardingham, 1983). Binding region was purified by rechromatography on Sephacryl S-300, freeze-dried as the sodium salt and the concentration determined from $A_{275}^{0.1\%} = 0.65$ (Perkins *et al.*, 1981). Link protein was prepared by chromatography of A1A1D4 samples on Sephacryl S-300, stored in 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 6.8, and the concentration was determined from $A_{277}^{0.1\%} = 1.40$ (Tang *et al.*, 1979).

Methods

Chondrocyte culture. Chondrocytes were isolated from the articular cartilage of New Zealand White rabbits aged 6–8 weeks as previously described (Plaas *et al.*, 1983). About 10^6 cells were transferred to each well of Multiwell tissue-culture plates (growth area 2 cm²) and maintained for about 7 days before the addition of 25 µCi of [³⁵S]sulphate in 0.5 ml of medium. Incorporation was allowed to proceed for 24 h, after which the medium was removed, clarified by centrifugation and stored at –20 °C for analysis. This medium generally contained about 5×10^6 d.p.m. of ³⁵S-labelled proteoglycan monomer in the link-free monomer form (Plaas *et al.*, 1983)/ml. Only samples of medium containing less than 10% aggregate on Sepharose CL-2B chromatography were used for assay of link protein.

Determination of link-protein activity. Link protein samples in 0.4 M-guanidinium chloride/1 M-sodium acetate buffer, pH 6.8, were added to chondrocyte culture medium (about 5×10^4 d.p.m. of ³⁵S-labelled proteoglycan in 10 µl) and 0.5 M-sodium acetate buffer, pH 6.8, in a total volume of 0.20 ml. After 1 h at room temperature mixtures were applied to columns (95 cm × 0.6 cm) of Sepharose CL-2B in 0.5 M-sodium acetate buffer, pH 6.8, and fractionated at about 2 ml/h. Fractions (0.7 ml) were

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collected into Mini-vials and scintillation fluid (3 ml) was added for determination of radioactivity. From the plot of radioactivity against fraction number the percentage aggregate present was calculated from the summation of aggregate (V_0) and monomer peaks assuming the monomer elution profile to be symmetrical (see Fig. 1). A low- M_r non-aggregating proteoglycan (K_{av} , about 0.75) was also present, and this generally accounted for about 8% of the total macromolecular radiolabel; this species was excluded from calculations of percentage aggregate. Samples containing link protein were as a routine assayed in triplicate at a range of concentrations producing between 20% and 65% aggregation. The results were plotted as percentage aggregate against amount of protein added, and from the slope of the line a specific activity of the link protein (defined here as the percentage increase in aggregate produced by the addition of 100 ng of protein) was calculated.

Inhibition of aggregate formation. This was calculated from:

$$\text{Percentage inhibition} = \frac{100 \times \text{Percentage aggregate (without inhibitor)} - \text{Percentage aggregate (with inhibitor)}}{\text{Percentage aggregate (without inhibitor)} - \text{Percentage aggregate (without link protein)}}$$

RESULTS

Assay method

In a previous study (Plaas *et al.*, 1983) we described the secretion by rabbit articular chondrocytes of proteoglycan aggregates deficient in active link protein. This deficiency was confirmed by biosynthesis studies with [^3H]serine and by showing that the addition of purified link protein to the medium generated a high percentage of link-stabilized aggregates. In more-detailed studies with this medium we have now shown (Fig. 1) that the percentage aggregate recovered from Sepharose CL-2B is directly proportional to the amount of link protein added. In the absence of exogenous link protein (Fig. 1a) about 6% of the aggregatable monomer was eluted as aggregate and most was eluted as free monomer (K_{av} , at peak about 0.28). The addition of increasing amounts of bovine nasal-septum-cartilage link protein (10–200 ng) resulted in a concentration-dependent increase in percentage aggregate up to a maximum of about 71% (Figs. 1a–1f). The plot of percentage aggregate against amount of link protein (Fig. 1g) showed good linearity between 20 ng (18% aggregate) and 120 ng (63% aggregate) of protein added.

The maximum percentage aggregate obtained was generally between 70% and 75%, and this was not increased by the addition of up to 5 μg of link protein. The maximum percentage formed (Fig. 1g) was also not altered by the addition of hyaluronate in amounts equivalent to 1% or 5% (w/w) of the proteoglycan, assuming the proteoglycan to be present at 10 $\mu\text{g}/\text{ml}$ (Plaas *et al.*, 1983); it therefore appears that the hyaluronate present in the medium, presumably from the serum and from chondrocyte synthesis, was sufficient for maximal aggregate formation under these conditions. Samples fractionated on Sepharose CL-2B immediately after addition of link protein resulted in a similar percentage aggregate to equivalent samples stored at 4 °C for 24 h before chromatography, suggesting that link-stabilized aggregates are formed very rapidly in these experiments. The reproducibility of the assay was tested

with link protein added at both 50 ng and 100 ng levels. Seven samples were prepared at each concentration and each was fractionated on a different Sepharose column. The percentage aggregate obtained was 33.4 ± 1.6 (mean $\pm$ s.d.) for 50 ng and 58.1 ± 2.1 (mean $\pm$ s.d.) for 100 ng.

The recovery of proteoglycan in the monomer position in the absence of added link (Fig. 1a) did not depend on the addition of hyaluronate oligosaccharides. Although oligosaccharides were added previously (Plaas *et al.*, 1983) to ensure the dissociation of monomer from link-protein-free aggregates, we have now shown this to be unnecessary; it therefore appears that at the concentration of monomer present in the medium, probably about 5 nM, the link-protein-free aggregates are almost fully dissociated. Such dissociation would be promoted by the further dilution of samples during chromatography, and would also be predicted from the

measured K_d of the proteoglycan–hyaluronate interaction. Poor aggregation in the absence of link protein might also be related to the finding (A. H. K. Plaas & J. D. Sandy, unpublished work) that the radiolabelled proteoglycan used in these studies exhibits a much lower affinity for hyaluronate than does proteoglycan monomer (A1A1D1) from bovine nasal-septum cartilage.

Loss of link-protein activity during heat denaturation

The effect of heat treatment on the activity of link protein from bovine nasal-septum cartilage was determined (Fig. 2). The half-life (defined as the time taken for a 50% reduction in the specific activity) was found to be about 55 min at 60 °C and about 7 min at 100 °C. There was no significant loss of activity for up to 24 h on storage at 4 °C or 37 °C under these conditions. The relatively rapid loss of link activity at 60 °C contrasts with the stability of hyaluronate-binding region at this temperature, a difference also suggested by viscometric analysis of heat-treated link-protein-stabilized aggregates (Hardingham, 1979).

Assay of link-protein-binding activity of proteoglycan monomer

Since the assay described above (Fig. 1) is dependent on the availability of exogenous free link protein to bind the radiolabelled proteoglycan, it can also be used as an inhibition assay to determine the capacity of molecules to interact with link protein and so prevent link-protein stabilization. Thus the addition of proteoglycan (A1A1D1) from bovine nasal-septum cartilage produced a concentration-dependent inhibition of aggregate formation between 2 μg and 20 μg of monomer (Fig. 3). This inhibition was presumably due to the binding of link protein by monomer at the hyaluronate-binding region, since purified hyaluronate-binding region was also inhibitory, whereas free chondroitin sulphate chains were ineffective (Fig. 3). The finding that both proteoglycan monomer and purified binding region were strongly

Fig. 1. Assay of link-protein activity

Purified link protein solution (see the Experimental section) was diluted in 1 M-sodium acetate buffer, pH 6.8, portions were added to ³⁵S-labelled chondrocyte culture medium and the samples were fractionated on Sepharose CL-2B as described in the Experimental section [(a) 0 ng; (b) 10 ng; (c) 20 ng; (d) 50 ng; (e) 100 ng; (f) 200 ng]. The percentage of proteoglycan in link-protein-stabilized aggregate (hatched area), plotted against the amount of link protein added, is shown in panel (g).

inhibitory on addition of 5 pmol is consistent with each being capable of binding in a 1:1 molar ratio with the 150 ng (3.4 pmol) of protein added to each assay.

DISCUSSION

The only material required for the present assay that is not readily available is the chondrocyte culture medium

Fig. 2. Heat denaturation of link protein

Link protein (30 $\mu\text{g}/\text{ml}$ in 0.4 M-guanidinium chloride/1 M-sodium acetate buffer, pH 6.8) was incubated at 4 °C ($\square$), 37 °C ($\blacksquare$), 60 °C ($\bullet$) or 100 °C ($\blacktriangle$), and portions were removed to an ice bath at 5, 10, 30, 60, 120 and 240 min. The specific activity was determined by triplicate assay of each sample (see the Experimental section), and log(specific activity of link protein) was plotted against time of incubation.

Fig. 3. Assay of link-protein-binding activity

Proteoglycan monomer ($\blacksquare$), hyaluronate-binding region ($\bullet$) and chondroitin sulphate ($\circ$) were added to 0.05 ml of ³⁵S-labelled chondrocyte culture medium and 0.5 M-sodium acetate buffer, pH 6.8, in a total volume of 0.2 ml. Link protein (150 ng in 20 μl of 0.4 M-guanidinium chloride/1 M-sodium acetate buffer, pH 6.8) was then added, and after 16 h at 4 °C samples were fractionated on Sepharose CL-2B to determine percentage aggregate. The results were expressed as percentage inhibition of aggregate formation (see the Experimental section) and plotted against the amount of inhibitor added.

containing link-protein-free proteoglycan. In recent studies (Plaas & Sandy, 1985) we have found that the loss of link-protein secretion in culture is most rapid when cells are plated at a high initial density (5×10^5 cells/ cm^2) and under conditions where greater than 80% attachment

is obtained. In such cultures, dense confluent monolayers are obtained within 3 days, and we have invariably obtained medium containing greater than 90% link-protein-free monomer at day 7 of culture. The radiolabelled medium obtained from a single well of a Multiwell plate (about 10^6 cells) is sufficient for 100–200 assays; further, this medium remains useful when stored for up to 3 months at -20°C .

The major advantage of the present assay is its sensitivity. The ability to quantify link-protein activity with as little as 20–50 ng of protein should make it possible to compare the functional properties of link proteins from a variety of sources. When used in combination with immunossays to determine total link protein, the present method may also allow analysis of the possible effects of aging (Mort *et al.*, 1983), tissue source (Tengblad *et al.*, 1984) or pathology on the ability of link proteins to stabilize proteoglycan aggregates.

The present method requires that the link protein samples to be assayed are essentially free of proteoglycan monomer or hyaluronate-binding region, since these inhibit the assay at very low concentrations (Fig. 3). Although standard preparations of active link proteins (A1D4 samples) can contain significant amounts of low-buoyant-density proteoglycan, the purification of link protein on affinity columns substituted with link-protein-specific antibodies should overcome this problem. Finally, the ability to quantify the link-protein-binding activity of very small amounts of proteoglycan monomer (Fig. 3) should also make it possible to determine whether the capacity of aggregating monomers to bind link protein changes with age, cartilage source or in articular disease.

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REFERENCES

- Baker, J. R. & Caterson, B. (1979) *J. Biol. Chem.* **254**, 2387–2393
- Bonnet, F., Dunham, D. G. & Hardingham, T. E. (1985) *Biochem. J.* **228**, 77–85
- Caterson, B. & Baker, J. (1978) *Biochem. Biophys. Res. Commun.* **80**, 496–503
- Caterson, B., Baker, J. R., Levitt, D. & Paslay, J. W. (1979) *J. Biol. Chem.* **254**, 9369–9372
- Cleland, R. L. (1981) *Arch. Biochem. Biophys.* **210**, 565–572
- Franzén, A., Björnsson, S. & Heinegård, D. (1981) *Biochem. J.* **197**, 669–674
- Gregory, J. D. (1973) *Biochem. J.* **133**, 383–386
- Hardingham, T. E. (1979) *Biochem. J.* **177**, 237–247
- Hardingham, T. E. & Muir, H. (1972) *Biochim. Biophys. Acta* **279**, 401–405
- Hardingham, T. E. & Muir, H. (1973) *Biochem. J.* **135**, 905–908
- Lyon, M. & Nieduszyński, I. A. (1983) *Biochem. J.* **213**, 445–450
- Mort, J. S., Poole, A. R. & Roughley, P. J. (1983) *Biochem. J.* **214**, 269–272
- Nieduszyński, I. A. & Sheehan, J. K. (1981) *Biochem. Soc. Trans.* **9**, 502–504
- Perkins, S. J., Miller, A., Hardingham, T. E. & Muir, H. (1981) *J. Mol. Biol.* **150**, 69–95
- Plaas, A. H. K. & Sandy, J. D. (1984) *Biochem. J.* **220**, 337–340
- Plaas, A. H. K. & Sandy, J. D. (1985) *Trans. Annu. Meet. Orthop. Res. Soc.* **10**, 52
- Plaas, A. H. K., Sandy, J. D. & Muir, H. (1983) *Biochem. J.* **214**, 855–864
- Poole, A. R., Reiner, A., Tang, L.-H. & Rosenberg, L. (1980) *J. Biol. Chem.* **255**, 9295–9305
- Ratcliffe, A. & Hardingham, T. (1983) *Biochem. J.* **213**, 371–378
- Sandy, J. D. (1979) *Biochem. J.* **177**, 569–574
- Tang, L.-H., Rosenberg, L., Reiner, A. & Poole, A. R. (1979) *J. Biol. Chem.* **254**, 10523–10531
- Tengblad, A. (1981) *Biochem. J.* **199**, 297–305
- Tengblad, A., Pearce, R. H. & Grimmer, B. J. (1984) *Biochem. J.* **222**, 85–92
- Thonar, E. J. M., Kimura, J. H., Hascall, V. C. & Poole, A. R. (1982) *J. Biol. Chem.* **257**, 14173–14180

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