Inhibition of hyaluronate synthesis

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(Received 2 August 1984/Accepted 9 October 1984)

UDP-GlcNAc (UDP-*N*-acetylglucosamine) and UDP-GlcA (UDP-glucuronic acid) were oxidized by periodate in the ribose ring and utilized as inhibitors for hyaluronate synthase in membrane fractions from the B6 cell line and in cell cultures of B6 cells and human fibrosarcoma HT 1080. Inhibition was irreversible and concentration-dependent and could be prevented in the case of periodate-oxidized UDP-GlcNAc by UDP-GlcNAc. Periodate-oxidized UDP-GlcNAc was shown to block chain elongation of hyaluronate. Introduction of periodate-oxidized UDP-GlcA into B6 cells by hypo-osmotic lysis of pinocytotic vesicles decreased hyaluronate synthesis by direct inhibition of the synthase. In HT 1080 cells the synthesis of hyaluronate, chondroitin sulphate and heparan sulphate was inhibited simultaneously.

Hyaluronate is synthesized at the inner side of the plasma membrane by alternate transfer of UDP-hyaluronate to the substrates UDP-GlcNAc and UDP-GlcA, and the growing chain is extruded through the membrane to the extracellular matrix (Prehm, 1983*a,b*, 1984). The synthesis of hyaluronate could not be inhibited by tunicamycin (Hart & Lennarz, 1978), xylosides (Hopwood & Dorfman, 1977) or other drugs (Mapleson & Buchwald, 1981; Mitchell & Hardingham, 1982), since neither lipid carriers nor protein primers are involved. The effect of hyaluronate on cellular behaviour could therefore only be studied indirectly by either hyaluronidase treatment of cultured cells or by comparison of cell lines exhibiting altered hyaluronate production (Toole, 1981). With the knowledge of the mechanism of hyaluronate synthesis it is possible to design inhibitors that interfere with the assembly of the hyaluronate chain. Appropriate inhibitors would be analogues of the precursors UDP-GlcNAc and UDP-GlcA selectively oxidized in the ribose moiety by periodate. Although periodate-oxidized nucleotides have been used as inhibitors and affinity labels for a variety of enzymes (Jacoby & Wilchek, 1977; Plapp, 1982; Colman, 1983), periodate-oxidized nucleotide sugars have not been applied to studies of glycosyltransferases. The present paper describes the inhibition of hyaluronate synthesis by periodate-

oxidized UDP-GlcNAc and UDP-GlcA in isolated membranes and in cell culture.

Materials and methods

Materials and growth of the B6 cell line were described previously (Prehm, 1984). The human fibrosarcoma cell line HT 1080 was obtained from the American Type Culture Collection. Periodate-oxidized UDP was obtained from Sigma. Particulate fractions of B6 cells containing high hyaluronate synthase activity were obtained from B6 cells grown to a density of 10^6 cells/ml and stimulated for 6 h by the addition of 5% (v/v) foetal-bovine serum. Cells were harvested by centrifugation at 1500*g* for 10 min, washed with phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.15 g of Na₂HPO₄, 0.2 g of KH₂PO₄ dissolved in 1 litre of water, pH 7.3) and suspended at 10^8 cells/ml in phosphate-buffered saline at 4°C. The cells were disrupted in a Parr-bomb for 10 min at 5.07 MPa (50 atm) (Evans, 1979). Nuclei were sedimented at 2000*g* for 10 min and the particulate fraction was obtained by centrifugation at 40000*g* for 20 min.

Preparation of o-UDP-GlcA and o-UDP-GlcNAc

UDP-GlcA (100 mg, 187 μ mol) was dissolved in 1 ml of 40 mM-sodium phosphate buffer, pH 6.8, at 0°C and 40 mg of sodium periodate was added. After 1 h at 0°C, 10 μ l of glycerol was added. *o*-UDP-GlcA was purified by gel filtration on a column (20 cm $\times$ 1 cm) of Sephadex G-10, with water as eluent. Fractions exhibiting absorbance at 260 nm were collected and freeze-dried. UDP-

Abbreviations used: SDS, sodium dodecyl sulphate; *o*-UDP-GlcA, periodate-oxidized UDP-glucuronic acid; *o*-UDP-GlcNAc, periodate-oxidized UDP-*N*-acetylglucosamine; DMEM, Dulbecco's modified Eagle's medium.

GlcNAc (100mg, 153 μ mol) was oxidized by the same procedure with 33mg of sodium periodate. The purity of the products was determined by ion-exchange chromatography on DEAE-cellulose with a linear gradient of 0–0.2M-NaCl in 10mM-Tris/HCl, pH8.4, and t.l.c. on cellulose, with ethanol/1M-ammonium acetate, pH5.5 (13:7, v/v), as solvent. The concentrations were determined by measurements of the absorbance at 260nm, an absorption coefficient of 10000 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ being assumed.

Inhibition of hyaluronate chain elongation

A particulate fraction (0.5ml) from B6 cells containing 5mg of protein was digested with 1 NF unit (7.5 μ mol of GlcNAc/min) of bacterial hyaluronidase for 10min at 37°C, centrifuged for 3min at 10000g and washed twice with 1ml of 40mM-phosphate buffer, pH6.8. The pellet was dissolved in 200 μ l of 40mM-phosphate buffer, pH6.8, containing 0.1mg of hyaluronate as a scavenger for residual hyaluronidase and 8 μ M-UDP-[¹⁴C]GlcA/166 μ M-UDP-GlcNAc/10mM-MgCl₂/4mM-dithiothreitol as the substrate for hyaluronate synthesis, and incubated at 37°C for 30min. The particulate fraction was sedimented for 3min at 10000g, washed with 1ml of 40mM-phosphate buffer, pH6.8, containing 1mg of hyaluronate, and suspended in 400 μ l of 200 μ M-o-UDP-GlcNAc in the same buffer. After incubation at 37°C for 30min the particulate fraction was sedimented for 3min at 10000g and washed with 40mM-phosphate buffer, pH6.8. The pellet was suspended in 10 μ M-UDP-[³H]GlcNAc/10 μ M-UDP-GlcA/10mM-MgCl₂/4mM-dithiothreitol and incubated at 37°C for 60min. The enzymic reaction was stopped by addition of 20 μ l of 10% (v/v) SDS, maintained at 100°C for 3min and subjected to gel filtration on a column (0.8cm $\times$ 96cm) of Sephacryl S-1000 with phosphate-buffered saline containing 0.1% SDS as eluent at a flow rate of 5.6ml/h. Fractions (0.7ml) were collected and 0.5ml aliquots were withdrawn for the determination of radioactivity.

Inhibition of hyaluronate synthesis in B6 cells

B6 cells (4 $\times$ 10⁶) were suspended in 150 μ l of DMEM containing 10% foetal-bovine serum, 0.5M-sucrose, 10% poly(ethylene glycol) 1000 and various concentrations of o-UDP-GlcA at 37°C for 10min. The suspension was diluted with 15ml of a mixture of DMEM and water (3:2, v/v) and centrifuged for 3min at 1500g. The sediment was again subjected to the same incubation sequence and finally incubated in 5ml of DMEM containing 5% foetal-bovine serum. After 1h, 100 μ Ci of [³H]-glucosamine was added to each culture. The cells

were grown for 18h, sedimented for 5min at 1500g, washed with phosphate-buffered saline and frozen in liquid N₂. After thawing, their hyaluronate synthase activity was determined as described by Prehm (1980). Macromolecules from the culture medium were precipitated by addition of 20ml of ethanol. The precipitate formed after 1h was sedimented by centrifugation at 9000g for 10min, suspended by sonication in acetone, and again centrifuged at 9000g for 10min. The pellet was dissolved in 1ml of 10mM-Tris/HCl, pH7.8, containing 5mg of Pronase. After overnight incubation at 37°C, 200 μ l of the solution was withdrawn for determination of radioactive hyaluronate. A 20 μ l portion of hyaluronate (10mg/ml) was added as carrier, and the glycosaminoglycans were precipitated by addition of 10 μ l of 10% (w/v) cetylpyridinium chloride at 37°C for 1h. The precipitate was centrifuged for 3min at 10000g, washed with 1ml of water containing 0.1% cetylpyridinium chloride, suspended in 100 μ l of water and the radioactivity determined.

Inhibition of glycosaminoglycan synthesis on HT 1080 cells

HT 1080 cells were grown on 2cm² culture dishes to a density of 4 $\times$ 10⁴ cells/cm². They were washed with phosphate-buffered saline and incubated with 300 μ l of DMEM containing 10% foetal-bovine serum, 1M-sucrose, 50% poly(ethylene glycol) 1000 and various concentrations of UDP-GlcA or o-UDP-GlcA for 10min at 37°C. The above solution was withdrawn and the cells were incubated at 37°C with DMEM/water (3:2, v/v) for 2min. The cells were twice subjected to this incubation sequence and then grown further in 1ml of DMEM. After 1h the medium was supplemented with 20 μ Ci of [³H]glucosamine/ml. The medium was harvested after 18h and macromolecules were precipitated by addition of 5ml of ethanol. The resulting precipitate was washed with 5ml of acetone and digested with Pronase as described above. The Pronase-treated solution was subjected to ion-exchange chromatography on a column (0.8cm $\times$ 3cm) of DEAE-cellulose. Glycosaminoglycans were eluted with a 50ml linear gradient of 0–0.6M-NaCl in 10mM-Tris/HCl, pH8.4. Fractions (1.25ml) were collected and aliquots (0.5ml) were taken for determination of radioactivity. For the determination of the effect of the inhibitors on protein synthesis the cells were labelled with 8 μ Ci/ml of [³H]leucine after hypo-osmotic lysis of pinocytotic vesicles. The medium was harvested after 18h and proteins were precipitated by addition of 100 μ l of 20% (w/v) trichloroacetic acid. The precipitate was left at 4°C overnight and centrifuged for 3min at 10000g. It was washed twice with 1ml of 2% (w/v) trichloro-

acetic acid, suspended in 1 ml of water and 0.5 ml was withdrawn for determination of radioactivity.

Results

Periodate oxidation of UDP-GlcNAc and UDP-GlcA

UDP-GlcNAc and UDP-GlcA were oxidized with periodate under mild conditions. The periodate-oxidized products were analysed by ion-exchange chromatography and t.l.c. The contents of GlcNAc and GlcA was determined by the carbazole method (Bitter & Muir, 1962) and with dimethylaminobenzaldehyde (Reissig *et al.*, 1956) respectively. In each case the periodate-oxidized products were chromatographically homogeneous and gave quantitatively the predicted values for intact carbohydrates. The n.m.r. spectra of o-UDP-GlcNAc and o-UDP-GlcA gave proton signals for the sugar moieties similar to those of the reference compounds GlcNAc and GlcA respectively. However, free aldehyde groups were not found, because adjacent aldehydes form cyclic acetals by addition of water, as shown for periodate-oxidized uridine (Hansske & Cramer, 1977).

Inhibition of hyaluronate synthesis

Hyaluronate synthesis by the particulate fraction of B6 cells was strongly inhibited in the

Fig. 1. Inhibition of hyaluronate synthase by o-UDP-GlcNAc

The particulate fraction (200 μg) from B6 cells was incubated in 500 μl of 8 μM-[¹⁴C]UDP-GlcA (264 mCi/mmol), 166 μM-UDP-GlcNAc, 10 mM-MgCl₂, 4 mM-dithiothreitol in 40 mM-phosphate buffer, pH 6.8, in either the presence (O) or absence (Δ) of 100 μM-o-UDP-GlcNAc at 37°C. At the times indicated, 100 μl was withdrawn; 10 μl of 10% SDS was added to inactivate the synthase and the solution was subjected to paper chromatography with ethanol/aq. 1M-ammonium acetate, pH 5.5 (13:7, v/v), as solvent. After 18 h the radioactivity of [¹⁴C]hyaluronate at the origin was determined.

presence of 100 μM-o-UDP-GlcNAc (Fig. 1). A Dixon plot of the initial velocity of hyaluronate synthesis showed non-linearity, indicating that the inhibition is irreversible (Fig. 2). The time course of hyaluronate synthase inactivation is shown in Fig. 3. The inactivation was biphasic, with a rapid decrease during the initial 30 min, followed by a slower phase. The rate of inactivation of hyaluronate synthase by o-UDP-GlcNAc and o-UDP-GlcA was concentration-dependent, and a plot of the initial inactivation rates versus reagent concentration gave hyperbolic curves tending towards a maximum (Fig. 4). Fig. 3 illustrates that hyaluronate synthase is partially inactivated by incubation of the particulate fraction with 100 μM-o-UDP-GlcA and 100 μM-UDP-GlcNAc for 30 min. The natural substrate, UDP-GlcNAc, could protect the enzyme only against o-UDP-GlcNAc, but not against o-UDP-GlcA. It was, however, not protected against o-UDP-GlcA by UDP-GlcNAc or UDP-GlcA (Table 1). Highest inactivation occurred in the simultaneous presence of both inhibitors. The percentage inactivation after 30 min by increasing concentrations of o-UDP-GlcNAc, o-UDP-GlcA and o-UDP is shown in Fig. 5. A plateau of 50–60% enzyme inactivation at increasing o-UDP-GlcNAc and o-UDP-GlcA concentrations was obtained. o/UDP was approx. 10 times less effective.

Mechanism of inhibition

If the inhibitors were covalently bound to the active site of the enzyme, chain elongation of

Fig. 2. Dixon plot of hyaluronate synthesis

A 100 μl portion of a particulate fraction from B6 cells (5 mg/ml) were incubated for 1 h at 37°C with increasing concentrations of o-UDP-GlcNAc (as indicated) and 8 μM-[¹⁴C]UDP-GlcA, 166 μM-UDP-GlcNAc, 10 mM-MgCl₂ and 4 mM-dithiothreitol (Δ), or with 2.4 μM-[¹⁴C]UDP-GlcA, 66.4 μM-UDP-GlcNAc, 10 mM-MgCl₂ and 4 mM-dithiothreitol (O). Hyaluronate synthesis was determined as described in Fig. 1.

Fig. 3. Kinetics of inactivation of hyaluronate synthase

The particulate fraction (50 μg) from B6 cells was incubated at 37°C for the times indicated in 300 μl of phosphate buffer (Δ), or phosphate buffer containing 200 μM α-UDP (◇), α-UDP-GlcA (△), α-UDP-GlcNAc (□) or α-UDP-GlcA + α-UDP-GlcNAc (○). The hyaluronate synthase activity was sedimented by centrifugation for 3 min at 10000g and the remaining activity was determined as described in Fig. 1.

Fig. 4. Initial rates of hyaluronate synthase inactivation

Inactivation of hyaluronate synthase was determined as described in Fig. 3 with increasing inhibitor concentrations. Reciprocal values of radioactivity of [¹⁴C]hyaluronate synthesized in 15 min were plotted against the inhibitor concentration: Δ, α-UDP-GlcA; □, α-UDP-GlcNAc; ○, α-UDP-GlcA + α-UDP-GlcNAc.

hyaluronate should be blocked. This was tested on a particulate fraction of B6 cells digested with hyaluronidase to remove endogenous hyaluronate

Table 1. Protection of hyaluronate synthase by substrates

The particulate fraction (100 μl) from B6 cells was incubated at 37°C with either 40 mM-phosphate buffer, pH 6.8, or phosphate buffer, pH 6.8, containing the listed compounds (100 μM) for 30 min. The hyaluronate synthase activity was sedimented at 10000g for 3 min, washed with 0.5 ml of phosphate buffer and determined as described in Fig. 2.

Substrate(s)	Activity (%)
Phosphate buffer	100
Phosphate buffer + α-UDP-GlcNAc	69
Phosphate buffer + α-UDP-GlcA	67
UDP-GlcNAc + α-UDP-GlcNAc	97
UDP-GlcNAc + α-UDP-GlcA	69
UDP-GlcA + α-UDP-GlcNAc	53
UDP-GlcA + α-UDP-GlcA	58
α-UDP-GlcA + α-UDP-GlcNAc	36

chains. It was incubated with UDP-GlcNAc and UDP-[¹⁴C]GlcA for 30 min so that the [¹⁴C]-hyaluronate chromatographed in the included volume of a Sephacryl S-1000 column. The preparation was then treated with the inhibitor α-UDP-GlcNAc for 30 min to partially inactivate the synthase. The residual active enzyme was

allowed to complete the chains with UDP-[³H]-GlcNAc and UDP-GlcA. The elution profile of hyaluronate on Sephacryl S-1000 showed that part of the [¹⁴C]hyaluronate was not elongated further and another part was elongated to form hybrid chains labelled with [³H]GlcNAc and [¹⁴C]GlcA, which were eluted in the excluded volume of the column (Fig. 6).

Inhibition of cellular glycosaminoglycan synthesis

o-UDP-GlcA was introduced into the cytosol of B6 cells by hypo-osmotic lysis of pinocytotic vesicles (Okada & Rechsteiner, 1982). Table 2 demonstrates the concentration-dependent inhibition of the production of hyaluronate, which is shed into the medium for 24h after uptake of the inhibitor. At this time the hyaluronate synthase activity was determined from the same cells (Table 2). Since B6 cells produce hyaluronate as the only detectable glycosaminoglycan, the effect of o-UDP-GlcA on proteoglycans was studied on the human fibrosarcoma cell line HT 1080. In the same experiment the natural substrate UDP-GlcA was also introduced into the cytosol. Proteoglycan production was measured by metabolic labelling of glycosaminoglycans in the medium with [³H]GlcN

Fig. 5. *Inhibition of hyaluronate synthesis*
Particulate fractions from B6 cells were incubated with the periodate-oxidized nucleotide sugars, and after 30min the remaining hyaluronate synthase activities were determined as described in Fig. 4. ○, o-UDP-GlcA + o-UDP-GlcNAc; △, o-UDP-GlcA; □, o-UDP-GlcNAc; ◇, o-UDP.

Table 2. *Concentration-dependent inhibition of hyaluronate production and synthase*

Details are given in the Materials and methods section.

[o-UDP-GlcA] in pinocytotic medium (mM)	[³ H]Hyaluronate production (c.p.m.)	[¹⁴ C]Hyaluronate synthase activity (c.p.m.)
0	100494	3950
16	85268	1156
32	6845	238
64	7569	178

Fig. 6. *Inhibition of chain elongation*
○, [¹⁴C]hyaluronate; ●, [³H]hyaluronate. Details are given in the Materials and methods section.

Fig. 7. Modulation of glycosaminoglycan synthesis on HT 1080 cells

Details are given in the Materials and methods section. ○, Protein; △, chondroitin sulphate; □, heparan sulphate; ◇, hyaluronate.

(Fig. 7). Protein synthesis was barely affected by UDP-GlcA or o-UDP-GlcA, as measured by the incorporation of [^3H]leucine into trichloroacetic acid-precipitable material (Fig. 7).

Discussion

When periodate oxidation is performed under mild conditions with equimolar amounts of NaIO_4 , *vicinal* hydroxy groups in *cis* positions are oxidized faster than those in the *trans* positions (Bobbitt, 1956; Guthrie, 1961). In UDP-GlcNAc and UDP-GlcA the ribose ring is therefore more susceptible than are the GlcNAc or GlcA moieties. Quantitative analysis of the GlcA and GlcNAc content in the periodate-oxidized nucleotide sugars indicated that GlcA and GlcNAc remained intact and the ribose was altered to cyclic acetals. These compounds were applied as inhibitors of hyaluronate synthase.

Inhibition was irreversible and, in the case of o-UDP-GlcNAc (but not o-UDP-GlcA), could be prevented by the natural substrate. A 10-fold higher concentration of o-UDP is needed to inactivate hyaluronate synthase to the same extent as the periodate-oxidized nucleotide sugars, indicating that the enzyme specifically recognized the sugar moieties. The initial inactivation rates of

hyaluronate synthase reached a maximum with increasing inhibitor concentrations (Fig. 5). Such behaviour indicates that inactivation occurs in two steps, in which reversible binding of the inhibitor to the enzyme precedes covalent binding (Meloche, 1967).

The observation that UDP-GlcNAc could protect the enzyme from o-UDP-GlcNAc and that o-UDP-GlcNAc and o-UDP-GlcA inactivated the enzyme to about 50% in the initial rapid phase (Figs. 4 and 6) could be interpreted as follows. Hyaluronate synthase contains active sites for each of the nucleotide sugars, one of which is alternately occupied by the end of the hyaluronate chain. Thus 50% of the hyaluronate synthase would be accessible to the inhibitors for inactivation. If transfer of the chain to the inhibitor molecule occurred at a low rate, then the active site for the other inhibitor becomes susceptible. The inhibition studies (Figs. 2–6) suggested that the periodate-oxidized nucleotide sugars behave as affinity labels for the site at which the nucleotide sugars bind to hyaluronate synthase.

o-UDP-GlcA blocked chain elongation of hyaluronate (Fig. 7), which may serve as additional evidence that hyaluronate is elongated at the reducing end by alternate addition of the chain to new substrate. If hyaluronate chains were elongated at the non-reducing end, the residual active

synthase would be expected to elongate all chains further, although at a lower rate. These inhibitors can therefore discriminate between these two chain-elongation mechanisms.

Since these inhibitors are the first documented compounds to interfere directly with hyaluronate synthesis, they provide valuable tools for inhibition of hyaluronate synthesis in cell culture. Introduction of α -UDP-GlcA into the cytosol of living cells by hypo-osmotic lysis of pinocytotic vesicles inhibited hyaluronate synthesis by inactivation of the synthase (Table 2). In HT 1080 cells hyaluronate and proteoglycan syntheses were simultaneously inhibited by α -UDP-GlcA. Introduction of the natural substrate, UDP-GlcA, into HT 1080 cells also inhibited the synthesis of heparan sulphate and chondroitin sulphate, whereas hyaluronate synthesis was further stimulated. The compounds had no influence of protein synthesis.

With these periodate-oxidized nucleotide sugars it may now be possible to study the function of hyaluronate on cellular behaviour directly.

I thank Miss D. Weigand for technical assistance and Mrs. G. Schild for performing the n.m.r. spectroscopy. This work was supported by the 'Deutsche Forschungsgemeinschaft'.

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