Enzymatic Synthesis of Hyaluronic Acid with Regeneration of Sugar Nucleotides

Claudio De Luca,[†,‡] Manfred Lansing,[‡] Irene Martini,[‡] Fabiana Crescenzi,[‡] Gwo-Jenn Shen,[†] Michael O'Regan,[‡] and Chi-Huey Wong*[,†]

Department of Chemistry, The Scripps Research Institute 10666 North Torrey Pines Road, La Jolla, California 92037 Fidia Advanced Biopolymers, Via Ponte della Fabbrica 3/a 35031 Abano Terme, Italy

Received January 27, 1995

We report here a preparative enzymatic synthesis of hyaluronic acid (HA) from UDP–*N*-acetyl-D-glucosamine (UDP–GlcNAc) and UDP–glucuronic acid (UDP–GlcA) catalyzed by HA synthase coupled with regeneration of the sugar nucleotides (Scheme 1). HA is a linear high molecular weight ($>5 \times 10^5$ Da) glycosaminoglycan composed of β-1,4-linked repeating disaccharide units of GlcA β-1,3-linked to GlcNAc.[1] It possesses unique viscoelastic and rheological properties[2] and is involved in many important biological processes such as hemopoiesis,[3] angiogenesis,[4] and cell adhesion.[5] It has been used clinically for viscosupplementation in ophthalmic surgery[2,6] and for treatment of osteoarthritis. Due to its importance in biomedicine, various modifications of HA have been undertaken to improve its biological properties.[7] HA was obtained by extraction from rooster comb or umbilical cord, but lately mucoid streptococcal bacteria have become the most reliable source.[8] However, viral contamination is viewed as a potential problem, and excessive dispersed molecular weights are often encountered in these preparations. Development of enzymatic synthesis of HA may provide an alternative source of this important biopolymer and offers opportunities for the preparation of low molecular weight HA and analogs as HA receptors recognize short HA.[5c]

The biosynthesis of HA has been studied but not well understood.[1] It was not clear whether the polymerization process is primer dependent until the recombinant HA synthase from *Streptococcus pyogenes* was expressed in *Escherichia coli* and shown to catalyze the synthesis of HA from UDP–GlcNAc and UDP–GlcA in a radiolabeled assay.[9] To examine the feasibility of this enzymatic reaction for the synthesis of HA on large scales, we prepared the crude membrane-bound HA synthase from *Streptococcus equisimilis* strain D181[10] and tested its synthetic activity. The enzyme preparation was indeed found to catalyze the synthesis of HA from UDP–GlcNAc and UDP–GlcA, though the yield was only around 20%. In order to improve the yield and to scale up the process, both sugar nucleotides were then regenerated *in situ* from UDP as shown in Scheme 1 (it was shown that regeneration of sugar nucleotides in glycosyltransferase reactions would reduce the cost of sugar nucleotides and the problem of product inhibition and make the enzymatic synthesis practical for a large-scale process).[11] The enzymes required for the cofactor regenerations are commercially available except UDP–GlcNAc pyrophosphorylase (EC 2.7.7.23), which has been overexpressed in *E. coli* in this study.[12] The enzyme UDP-glucose dehydrogenase (EC 1.1.1.22) used in the regeneration of UDP–GlcA[13] is commercially available (from Sigma), but the preparation is impure and very expensive. We have therefore developed an overexpression system to produce this enzyme from *E. coli*. With these enzymes available, a preparative synthesis of HA was carried out. In a representative synthesis, to a HEPES buffer solution (0.1 M, pH 7.5, total volume = 10 mL) containing Glc-1-P (0.1 mmol), GlcNAc-1-P (0.1 mmol), phosphoenolpyruvate (PEP, 0.2 mmol), NAD (5 μmol), UTP (10 μmol), $MgCl_2 \cdot 6H_2O$ (0.1 mmol), dithiothreitol (40 μmol), and KCl (0.5 mmol) was added recombinant UDP-GlcNAc pyrophosphorylase (10 units), UDP-Glc pyrophosphorylase (20 units), UDP–Glc dehydrogenase (10 units), pyruvate kinase (200 units), lactate dehydrogenase (200 units), inorganic pyrophosphatase (50 units), and the membrane-bound HA synthase (0.4 unit). The mixture was gently stirred under argon at 25 °C for 48 h. The reaction was then stopped by digestion with proteinase-K (500 μg) for 60 min at 37 °C followed by addition of cold trichloroacetic acid to a final concentration of 5% to precipitate proteins. The solution was then centrifuged (14000*G*, 30 min, 4 °C), and the supernatant was passed through a Sepharose CL-4B column (50 × 0.65 cm) eluted with PBS buffer (0.01 M phosphate, 2.7 mM KCl, 137 mM NaCl). The fractions corresponding to HA were collected (68 mL) and dialyzed against 5 L of distilled water (repeated five times, 40 h each) using a dialysis tube with a molecular weight cutoff of 12000-14000 Da and then lyophilized to give 31 mg of HA sodium salt (90% yield). The turnover number for UTP, UDP–GlcNAc, and UDP–GlcA was 16. The HA prepared was further characterized by ^{1}H-NMR (D_2O, 500 Hz) and enzymatic digestion by hyaluronate lyase (EC 4.2.2.1) and hyalurono glucuronidase (EC 3.2.1.36), and the results were the same as that from an authentic HA. Analysis by multiangular laser light scattering indicates that the average molecular weight of the synthetic HA is $\sim 5.5 \times 10^5$, corresponding to a degree of polymerization of 1500.

In summary, this study has demonstrated that high molecular weight HA can be synthesized enzymatically from relatively

† The Scripps Research Institute.

‡ Fidia Advanced Polymers.

(1) (a) Markovitz, A.; Cifoneli, J. A.; Dorfman, A. *J. Biol. Chem.* **1959**, *234*, 2343. (b) Sugahara, K.; Schwartz, N. B.; Dorfman, A. *J. Biol. Chem.* **1979**, *254*, 6252. Prehm, P. *Biochem. J.* **1983**, *211*, 191.

(2) (a) Rosen, E. S. *Viscoelastic Materials: Basic Sciences and Clinical Applications*; Pergamon: New York, 1989. (b) Holmbeck, S. M. A.; Petillo, P. A.; Lerner, L. E. *Biochemistry* **1994**, *33*, 14246.

(3) Siczkowski, M.; Amos, T. A. S.; Gordon, M. Y. *Exp. Hematol.* **1993**, *21*, 126.

(4) Brown, J. J. G.; Papaioannou, V. E. *Differentiation* **1992**, *52*, 61.

(5) (a) Toole, B. P. *Curr. Opin. Cell Biol.* **1990**, *2*, 839. (b) Hardwick, C.; Hoare, K.; Owens, R.; Hohn, H. P.; Hook, M.; Moore, D.; Cripps, V.; Austen, L.; Nance, D. M.; Turley, E. A. *J. Cell. Biol.* **1992**, *117*, 1343. (c) Underhill, C. *J. Cell. Sci.* **1992**, *103*, 293.

(6) Goa, K. L.; Benfield, P. *Drugs* **1994**, *47*, 536.

(7) For a leading reference, see: Pouyani, T.; Harbison, G. S.; Prestwich, G. D. *J. Am. Chem. Soc.* **1994**, *116*, 7515.

(8) van Brunt, J. *Bio/Technology* **1986**, *4*, 780. O'Regan, M.; Martini, I.; Crescenzi, F.; De Luca, C.; Lansing, M. *Int. J. Biol. Macromol.* **1994**, *16* (6), 283.

(9) DeAngelis, P. L.; Weigel, P. H. *Biochemistry* **1994**, *33*, 9033. For another work on cloning and sequencing of the HA gene, see: Lansing, M.; Lellig, S.; Mausolf, A.; Martini, I.; Crescenzi, F.; O'Regan, M.; Prehm, P. *Biochem. J.* **1993**, *289*, 179.

(10) Obtained from Fidia, Italy. The membrane-bound HA synthase was isolated according to the procedure described by Prehm with a slight modification: Prehm, P.; Mausolf, A. *Biochem. J.* **1986**, *235*, 887.

(11) Wong, C.-H.; Haynie, S.; Whitesides, G. M. *J. Org. Chem.* **1992**, *47*, 5416. Ichikawa, Y.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 4698. Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G.-J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, S.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 9238.

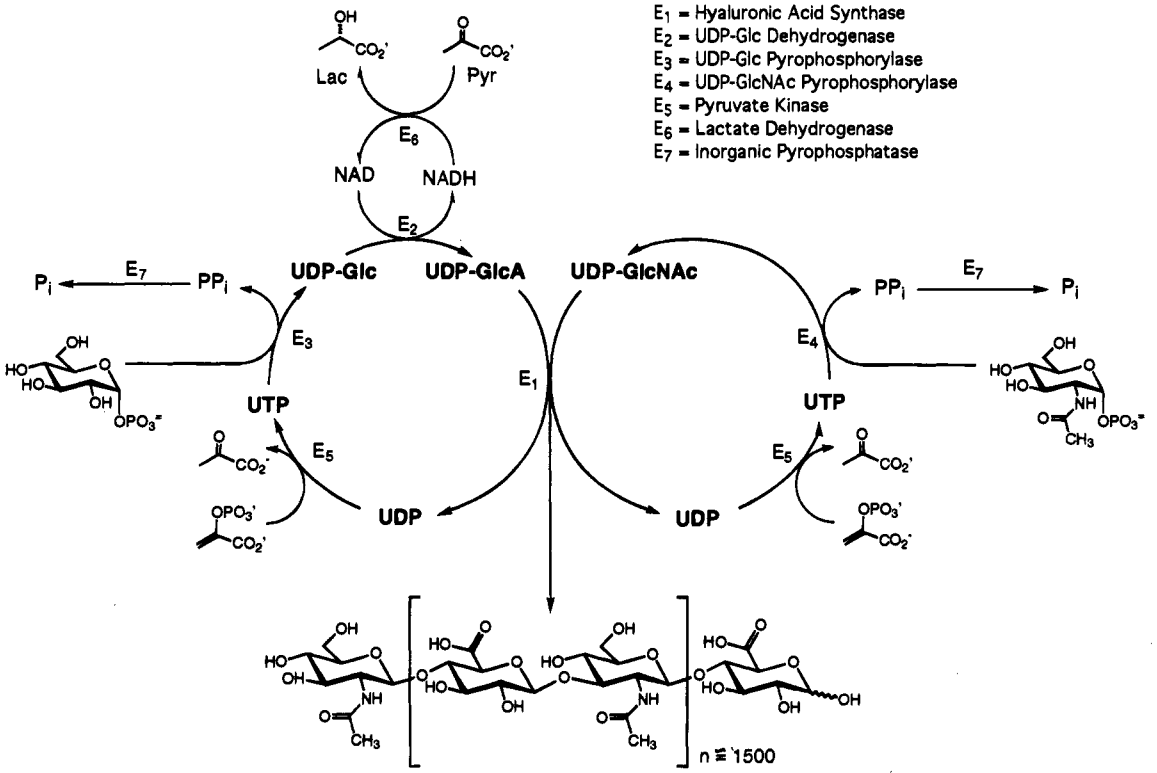
(12) The *E. coli* gene *glmU* coding from the enzyme was amplified by PCR using the 5′-primer ATATTGGATCCTTGAATAATGCTATG and the 3′-primer GCGCGAATTCTTACTTTTTCTTACCGGACG digested with *Bam*HI and *Eco*RI inserted to pTRC-His-A vector and transformed into supercompetent epicurean *E. coli* XL1 blue MRF cells for overexpression of the enzyme (300 units/L).

(13) UDP-Glc dehydrogenase was used in the regeneration of UDP–GlcA (Gygax, D.; Spies, P.; Winkler, T.; Pfarr, U. *Tetrahedron* **1991**, *28*, 5119) and in the synthesis of UDP–GlcA (Toone, E. J.; Simon, E. S.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 5603). To overexpress the enzyme, the gene *kfa*C from *E. coli* strain K5 was amplified by PCR using the 5′-primer ATATTGAGCTCTTCGGAACACTAAAAAA and the 3′-primer GCGCAAGCTTTTAGTCACATTTAAACAAATC, digested with *Sac*I and *Hin*dIII, inserted into PTrc-His-A vector and transformed into supercompetent epicurean *E. coli* XL1 blue MRF cells for overexpression of the enzyme (40 units/L).

(14) The membrane-bound enzyme was found to be more stable than the solubilized enzyme. Immobilization may be necessary to improve the enzyme stability.

0002-7863/95/1517-5869$09.00/0

Scheme 1. Enzymatic Synthesis of Hyaluronic Acid with Regeneration of Sugar Nucleotides



inexpensive substrates: Glc-1-P and GlcNAc-1-P. All the enzymes were quite stable except HA synthase,[14] which exhibited a half-life of 24 h at 25 °C. Work is in progress to improve the enzyme stability and to prepare HA analogs with controlled molecular weight.

Supplementary Material Available: Procedures for the preparation of HA synthase, recombinant UDP-GlcNAc pyrophosphorylase, and UDP-Glc dehydrogenase and assays and analysis of the enzymatic reactions (19 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA950280W