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Kinetic and structural analysis of two transferase domains in *Pasteurella multocida* hyaluronan synthase

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ABSTRACT

Pasteurella multocida hyaluronan synthase (PmHAS) encompasses two transferase domains that elongate a growing hyaluronan (HA) oligosaccharide chain by addition of either GlcNAc or GlcUA residues from a corresponding UDP-sugar. Initial velocity studies of single-step elongations were conducted for both domains by independently varying the concentrations of the HA oligosaccharide and the UDP-sugar. Two-substrate models were discriminated by their goodness-of-fit parameters and by dead-end inhibition studies. A mechanistic shift from a steady-state ordered bi-bi to rapid equilibrium ordered bi-bi mechanism was observed at the NAc-site between the HA_6 and HA_8 elongation. This shift was invoked by a minor reduction in turnover number k_{cat}. Both NAc- and UA-transferase domains follow a sequential kinetic mechanism, most likely an ordered one in which the UDP-sugar donor binds first, followed by the HA oligosaccharide. After transfer of the sugar moiety, both products are released, first the elongated HA oligosaccharide and then the UDP sugar. This mechanism was visualized with a structural model of PmHAS that presented two flexible loops, one in each transferase domain; these loops form a bridge above the active site. Based on structural similarities between PmHAS with α1,3-galactosyltransferase (α3GT) and β1,4-galactosyltransferase (β4Gal-T1), these flexible loops are seemingly involved in a conformational change upon binding the UDP-sugar, inducing the ordered mechanism. Kinetic analyses have demonstrated significant differences in dissociation constant $K_{D,UA}$ and $K_{M,NAc}$ of 124 ± 31 μM and 28 ± 8.8 mM, respectively, suggesting that the variance in the binding affinity of the two UDP-sugars controls the molecular weight of hyaluronan.

1. Introduction

Hyaluronan (HA) is an unbranched polysaccharide composed of two alternating sugars, *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA). HA with a molecular weight exceeding 500 kDa is abundant in mammalian tissues, such as skin, eyes, cartilage and extracellular matrix, where elasticity and viscosity of the chain serves as a biological lubricant or as a flexible structural component. HA with molecular weight below 500 kDa is involved in a number of cell biological phenomena, including cell motility, cell proliferation, cell differentiation and cell–cell interactions [1]. HA plays an ambiguous role in cancer treatment, where long HA chains promote cell proliferation and short HA oligosaccharides inhibit cancer growth [2]. HA chain length therefore determines its function; for pharmaceutical applications, it is thus essential to produce homogeneous chains. Elsewhere, the theoretical relationship between elongation kinetics and the distribution of polymer chain length is investigated [3].

Production of monodisperse HA oligosaccharides has been achieved with a stepwise enzymatic [4–7] or chemical [8] addition or removal of sugar units, but remains limited in the number of steps and often requires extensive separation procedures. The most promising technique is to control enzymatic synthesis of HA by *Pasteurella multocida* hyaluronan synthase (PmHAS). Such control, however, requires a heightened comprehension of the biological characteristics of the enzyme.

Abbreviations: α3GT, α1,3-galactosyltransferase; β4Gal-T1, β1,4-galactosyltransferase; EAB, complex of enzyme and its substrates; GlcUA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; HA, hyaluronan; hT2, UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase T2; PmHAS, *Pasteurella multocida* hyaluronan synthase.

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PmHAS contains two transferase domains that alternatingly transfer a sugar moiety of UDP-GlcNAc or UDP-GlcUA to a growing HA chain.

The current study is the first to investigate the two-substrate kinetics of a hyaluronan synthase (HAS). Unlike Class I HAS enzymes, PmHAS possesses the abilities to bind, extend and release the HA oligosaccharide [9], which calls for a description of enzyme state kinetics by a two-substrate model. Our results indicate that both transferase domains elongate HA through a sequential mechanism. Furthermore, a significant difference in binding affinities of UDP-GlcNAc and UDP-GlcUA may control the hyaluronan molecular weight. For the NAc-transferase domain, a shift in mechanism was observed for longer HA oligosaccharides. These mechanistic results are further analyzed, employing a structural model of PmHAS that was based on a crystal structure of a related glycosyltransferase enzyme [10].

2. Experimental

2.1. Enzyme expression and activity measurements

Purified PmHAS was provided by Schering-Plough (formerly Organon N.V.). PmHAS represents the soluble PmHAS$^{1-703}$ enzyme, as described by Jing and DeAngelis [11], cloned and expressed in a pET101/D-TOPO expression vector (Invitrogen) with an additional V5 epitope and polyhistidine (6× His) region at the C-terminal end of the enzyme. PmHAS was purified from the crude extract by affinity chromatography on Ni-NTA columns (Qiagen).

Kinetic studies of the two PmHAS transferase domains were performed with a coupled-enzyme assay under stationary conditions[10]. Coupled-enzyme assay components included 60 u PK/ml, 75 u LDH/ml, 2 mM phosphoenolpyruvate, 0.4 mM NADH, 15 mM $MgCl_2$, 112.5 mM KCl, 1 M ethylene glycol, 50 mM Tris·HCl at pH 8.0. Purified HA oligosaccharides HA_4, HA_5, HA_6 and HA_8 (consisting of 4, 5, 6, or 8 sugar units) were all purchased from Hyalose, L.L.C (USA). The even numbered templates (HA_4, HA_6 and HA_8) are elongated with UDP-GlcNAc, and the odd numbered template HA_5 is elongated with UDP-GlcUA. Initial rates were measured in duplicate at 35 °C for each single-step elongation at varying HA oligosaccharide concentrations of 0.1, 0.5, 1, 2, 4, or 6 mM, and a fixed UDP-sugar concentration ranging from 1-10 mM for UDP-GlcNAc and 0.025-40 mM for UDP-GlcUA. For the dead-end inhibition study with UMP, the fixed concentration of UDP-GlcNAc was set close to saturation (40 mM); HA_4 concentrations were varied at 0.1, 0.5, 1, 2, 4 or 6 mM. PmHAS inhibition was measured at fixed UMP concentrations (0, 100 and 150 mM). Following a 5 min pre-incubation period, reactions were initiated by theaddition of 5 μg/ml PmHAS and the HA oligosaccharide. Reactions were monitored for 20 min in 150-μl 96-well UV star microplates (Greiner Bio-One, Germany) with a temperature-controlled Safire spectrophotometer (Tecan, Switzerland).

2.2. Kinetic analysis

Initial velocity data were fitted with kinetic models [12,13] for sequential mechanisms random bi-bi (1) and ordered bi-bi (2), and the substrate-substituted mechanism ping-pong bi-bi (3):

$$v = \frac{v_{max}AB}{K_{D,A}K_{M,B} + K_{M,B}A + K_{M,A}B + AB} \quad (1)$$

$$v = \frac{v_{max}AB}{K_{D,A}K_{M,B} + K_{M,B}A + AB} \quad (2)$$

$$v = \frac{v_{max}AB}{K_{M,B}A + K_{M,A}B + AB} \quad (3)$$

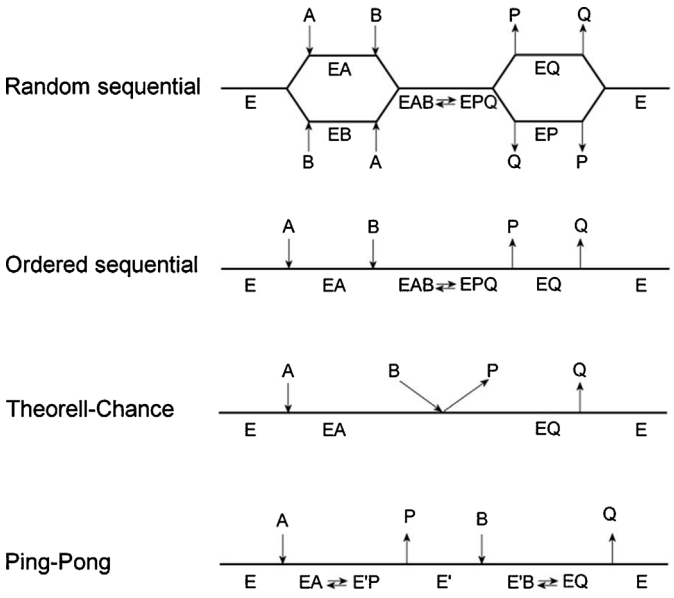

Fig. 1. Cleland notation of random sequential, ordered sequential, Theorell–Chance, and substrate-substituted ping-pong mechanisms. A and B represent substrates, Q and P the products from A to B, respectively. E is the enzyme, E′ the enzyme intermediate.

where v_{max} is the maximum velocity by volume (the product of enzyme concentration and turnover number k_{cat}), $K_{M,A}$ and $K_{M,B}$ are the apparent Michaelis constants for the substrates, $K_{D,A}$ is the dissociation constant for substrate A, and A and B are the concentrations of substrates A and B. Further details such as the order of substrate binding and product release are indicated in Fig. 1. For random and ping-pong models, substrate A can be either an UDP-sugar or an HA oligosaccharide. For the ordered bi-bi mechanism, the sequential binding of the substrates is relevant to the model, and was thus utilized twice: first, Model 2A, with the UDP-sugar (substrate A) binding before the HA oligosaccharide (substrate B), and then again, Model 2B, with HA oligosaccharide (substrate A) binding before the UDP-sugar (substrate B).

For these models, it was presupposed that the complex of enzyme and substrates (EAB) is in rapid equilibrium with the free substrates and free enzyme and that product formation is the rate-limiting step [12]. Other models employ the steady state assumption, i.e. the concentration of EAB is considered constant and the disintegration of the EAB complex and product formation proceed at comparable rates. This shift in rate-limiting steps in the mechanism results in different equations for the steady state models. Details about these equations can be found elsewhere [12,13], but the result is that Model 1, based on the rapid-equilibrium random bi-bi mechanism, also expresses the steady state ordered bi-bi and the Theorell–Chance mechanisms. Theorell–Chance is an ordered mechanism, where, at saturating substrate concentrations, the only slow step within the overall reaction is the release of the second product (Fig. 1). All models were employed to identify the kinetic mechanism of the two transferase domains of PmHAS, except for the steady-state random mechanism, which is rarely observed and difficult to distinguish from the rapid equilibrium random mechanism.

In dead-end inhibition studies, the inhibition is measured at several inhibitor concentrations, whereas the concentration of one substrate is varied and the other substrate being kept constant, resulting in Michaelis Menten kinetics as described below. Models (4)–(6) for competitive, noncompetitive, and uncompetitive