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Discussion Letter

Conformational aspects of the reaction mechanisms of polysaccharide lyases and epimerases

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1. INTRODUCTION

During the biosynthesis of some polysaccharides (e.g. alginic acid [1] and heparin [2]) pyranosyluronate residues undergo epimerization at C-5 after incorporation into the polymer chain. In the case of heparin, the enzyme heparosan-*N*-sulfate D-glucuronosyl 5-epimerase (EC 5.1.3.17) catalyzes the exchange of hydrogen atoms at C-5 with solvent protons. Hence, it was postulated that the initial step of the epimerization required abstraction of the C-5 proton by a nucleophilic group of the enzyme to form a carbanion; the carbanion could regain a proton either with retention or inversion of configuration [3,4].

These polysaccharides may also be modified by the action of lyases. Thus, in the modification of alginic acid, a 'mannuronate lyase' (alginate lyase, EC 4.2.2.3) degrades the polymer to oligosaccharides containing 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate at the non-reducing end. Gacesa [5] has proposed an extension of the carbanion mechanism to the lyase enzymes. For the lyase reaction, two amino acid residues (AA1, AA2) in the enzyme proteins are posited. AA1 neutralizes the carboxylate ion of uronate residues; AA2 catalyzes abstraction of the C-5 proton, forming an enolate carbanion which may be stabilized by resonance. Subsequently, there is a β-elimination of the C-4 substituent; the overall two step mechanism is formally an E_{1cb} process [6]. The same carbanion is formed in the epimerase reaction, but is discharged using another amino acid residue (AA3) to return hydrogen at C-5 with inversion of configuration. It is our purpose to explore whether these ideas are consistent with stereochemical considerations.

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2. CONFORMATIONS OF SUBSTRATES, PRODUCTS AND INTERMEDIATES

Although Gacesa [5] considered only Haworth structures, there is strong evidence that β-D-mannuronosyl residues assume the $^4C_1$ conformation and α-L-guluronosyl residues assume the $^1C_4$ conformation [7–9]. The enolate anion may be assumed to have any of the conformations that are possible for the normal pyranose ring. In the unsaturated product formed by lyase action, four atoms, O, C-5, C-4 and C-3, must lie in a plane with the molecule assuming one of two possible

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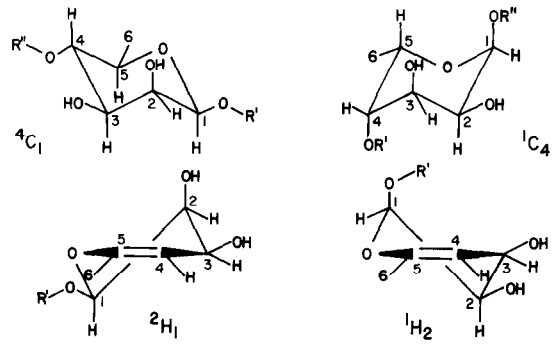
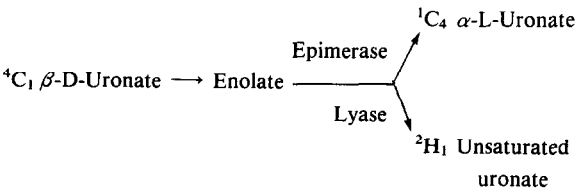


Fig.1. Substrate and product conformations in the alginate series. In this, and subsequent figures, the group at position 6 is the carboxylate anion neutralized by the residue, AA1, of the enzyme protein. This grouping is simply indicated as 6. R′ is the component in glycosidic linkage at C-1 and R′′ the component at C-4. (Top left) The β-D-mannuronosyl residue; (top right) the α-L-guluronosyl residue. The two structures at the bottom are the possible half-chair conformations for the unsaturated uronate residue, 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate.

half-chair conformations. These conformations of unsaturated carbohydrates have not been assigned specific descriptors; however, they would correspond to two of the normal pyranose half-chair conformations, namely, $^{2}H_{1}$ or $^{1}H_{2}$ [7,10]. For the mannuronate lyase product, the $^{2}H_{1}$ related possibility is most likely since it provides the favored equatorial alignment for the saccharide linkage (see fig.1); it also avoids the diaxial interaction for the oxygen atoms at C-1 and C-3 in the $^{1}H_{2}$ possibility.

3. STEREOCHEMICAL ASPECTS OF THE UNIFIED MECHANISM

The elimination, therefore, requires conversion of a normal $^{4}C_{1}$ pyranosyl structure to an unsaturated product with a half-chair conformation. The epimerization reactions, which generally appear to be reversible [4,11], require three interconnected events: (i) the configurational change at C-5, D → L; (ii) the anomeric change at C-1, $\beta \rightarrow \alpha$; and (iii) the conformational change, $^{4}C_{1} \rightarrow {}^{1}C_{4}$. For the hypothesis to be credible, it must be possible to assign to the enolate anion a conformation in harmony with the considerations just outlined. In schematic form, the reactions are as follows:



For a pyranosyl moiety to undergo the conformational change from $^{4}C_{1}$ to $^{1}C_{4}$, a specific 'itinerary' of non-chair conformations must be followed. The simplest possible itinerary for this interconversion requires the intervention of three other recognisable conformations: two half-chair and one skew [7,10].

Chair ⟷ Half-chair ⟷ Skew ⟷ Half-chair ⟷ Chair

With twelve half-chair (H) and six skew (S) conformations, there are six possible itineraries for the $^{4}C_{1} \longleftrightarrow {}^{1}C_{4}$ interconversion. Other possible pathways arise by the addition of a boat conformation.

Chair ⟷ Half-chair ⟷ Skew ⟷ Boat ⟷ Skew ⟷ Half-chair ⟷ Chair

These, however, will not be considered as major contributors since they involve more movements than the previously outlined itineraries. Only the following itinerary accommodates the required half-chair structure necessary for the lyase reaction:

$$^{4}C_{1} \longrightarrow {}^{2}H_{1} \longrightarrow {}^{5}S_{1} \longrightarrow {}^{5}H_{4} \longrightarrow {}^{1}C_{4}.$$

We interpret the reactions in the alginate case as follows (see also figs 2 and 3). Proton removal at C-5 (with the aid of AA2) from β-D-mannuronosyl residues in the $^{4}C_{1}$ conformation occurs from 'below' the plane of the pyranose ring. The anion, initially in a $^{4}C_{1}$ related conformation, changes to the $^{2}H_{1}$ related arrangement. For the epimerase reaction, AA3 now donates a proton at C-5 to this conformational state of the anion from 'above' the plane of the pyranose ring; using Hanson's nomenclature [12], to the *si* face of the C-5 atom. Hence, an α-L-guluronosyl moiety is initially formed in the $^{2}H_{1}$ conformation and the itinerary shown below leads to the final, stable, $^{1}C_{4}$ conformation. Although the protonation could involve any of the half-chair or skew intermediates, the $^{2}H_{1}$ conformation of the anion appears, from inspection of models, to be least hindered for the

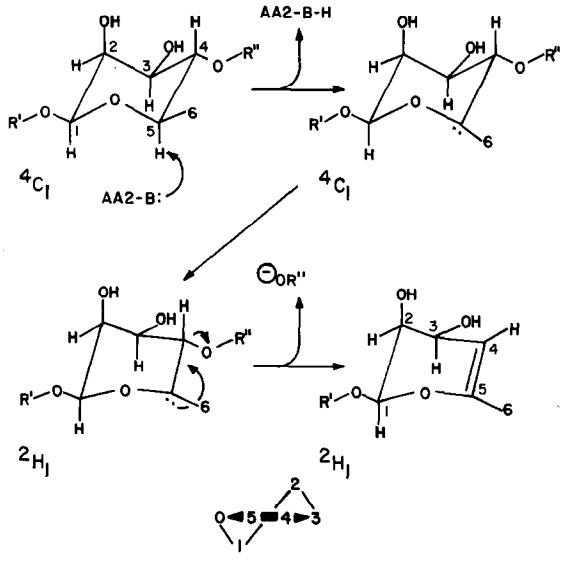
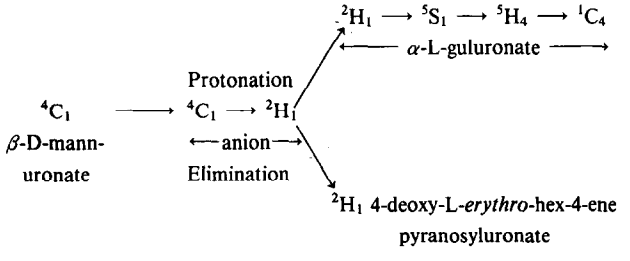


Fig.2. Proposed stereochemistry for the D-mannuronate lyase reaction. The β-D-mannuronosyl residue is attacked by AA2 leading to proton removal. The initial $^4C_1$ related enolate structure rearranges to the $^2H_1$ related conformation prior to loss of $R''O^-$. The diagram at the bottom of the figure is a conventional skeletal representation of the $^2H_1$ conformation.

proton approach to C-5. These molecular movements are represented by Newman projections for C-4 and C-5 in fig.4. The lyase reaction proceeds by elimination of the C-4 leaving group from the $^2H_1$ conformational state of the anion.



Since the D-mannuronosyl $\longrightarrow$ L-guluronosyl reaction is demonstrably reversible [11], and there is also a L-guluronosyl specific lyase [5], the question arises whether a common intermediate is possible for all of these processes. There is a formal stereochemical difference for the elimination reaction with D-mannuronate and L-guluronate, at least when the two chair conformations are considered [6,13]. In $^4C_1$ D-mannuronate, the C-5 proton is axial, whereas the leaving group at C-4 is equatorial; thus the elimination is of the axial-equatorial (*cis*) type. However, in $^1C_4$ L-guluronate, the C-5 proton and the C-4 leaving group are both axial. For this diaxial (*trans*) elimination a concerted reaction mechanism would be likely; hence, an anion with a finite half-life might not participate in the L-guluronate lyase reaction. However, since the same product is formed by the D-mannuronate and the L-guluronate lyase reactions, the L-guluronate lyase might form the $^2H_1$ anion postulated for the D-mannuronate lyase, but not by a concerted mechanism. This would be possible if the conformational change $^1C_4 \longleftrightarrow {}^2H_1$ took place in the L-guluronosyl moiety followed by the E_{1cb} deprotonation.

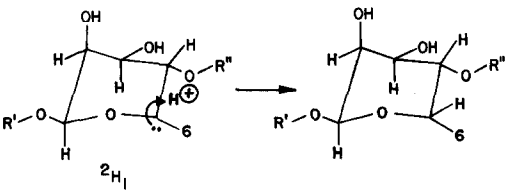


Fig.3. The protonation step for the mannuronan C-5 epimerase. The enolate anion in the $^2H_1$ related conformation adds the proton at C-5 from 'above' the plane to form the $^2H_1$ conformation of α-L-guluronate. The subsequent changes, $^2H_1 \longrightarrow {}^5S_1 \longrightarrow {}^5H_4 \longrightarrow {}^1C_4$, are not diagrammed but are indicated, in part, in fig.4.

$^1C_4 \longrightarrow {}^5H_4 \longrightarrow {}^5S_1 \longrightarrow {}^2H_1 \longrightarrow {}^2H_1 \longrightarrow {}^2H_1$ 4-Deoxy-L-
$\longleftarrow$ α-L-Guluronate $\longrightarrow$ anion -*erythro*-
hex-4-ene
pyranosyl-
uronate

For heparin, labilization of the C-5 hydrogen atom parallels epimerization with microsome bound intermediates such as heparosan *N*-sulfate and *N*-acetyl heparosan ('endogenous' substrates); however, D-glucuronosyl residues of 'exogenous' substrates not bound to microsomes can lose the C-5 hydrogen without epimerization [4]. Whatever the explanation for this difference, both lines of experimentation support carbanion formation. The D-glucuronosyl residues are clearly in the stable $^4C_1$ conformation but the situation may be more complex for L-iduronate. Unsubstituted L-iduronate is often assigned the $^1C_4$ conformation [7–9] while sulfated L-iduronate residues in

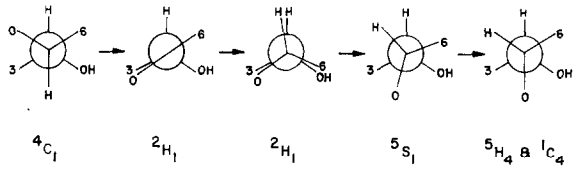


Fig.4. Newman projections along the C-5 to C-4 axis for the structures involved in epimerization. The C-5 atom is at the front, and C-4 to the rear. A structure has not been drawn for the $^4C_1$ anion. The angles for $^2H_1$ and $^5S_1$ are approximations derived from models; the $^5H_4$ and $^1C_4$ conformations are indicated by a single structure since the bond angles are very similar in these two cases.

heparin, may contain an equilibrium mixture of both chair conformations, and the skew conformation, $^2S_0$ [14,15]. This skew conformation actually participates in one itinerary for chair-chair interconversion:

$$^4C_1 \longleftrightarrow {}^2H_3 \longleftrightarrow {}^2S_0 \longleftrightarrow {}^5H_0 \longleftrightarrow {}^1C_4.$$

The unsaturated uronate product from the lyase reaction is, in this case, 4-deoxy-L-*threo*-hex-4-ene pyranosyluronate. As for the *erythro* product, only the two half-chair conformations, $^2H_1$ and $^1H_2$, are possible for this compound. Of these, the $^2H_1$ possibility seems to be clearly preferred, not only because the glycosidic bond is equatorial, but because this conformation also places the C-2 OH in an equatorial alignment. Hence, one possible unified pathway for the epimerase and lyase uses the same conformational sequence as that discussed for the alginate case. This would lead to the $^1C_4$ conformation for the epimerized product, L-iduronate; the equilibrium mixture of chair and skew forms would be formed by the itinerary noted above. Alternatively, a 'branching' of the conformational itinerary at the skew conformational level could occur with the participation of a boat conformation.

While the conformational considerations are consistent with a carbanion intermediate common to both lyase and epimerase reactions, other aspects of these reactions need clarification. In particular, some epimerase reactions are very dependent on the presence of Ca^{2+}. For instance, Ca^{2+} influences both reaction rate and epimerization pattern for the mannuron C-5 epimerase from *Azotobacter vinelandii* [16]. It appears unlikely that Ca^{2+} would be bound specifically by any of the carbohydrate conformations involved in epimerization; presumably there is some specific action on the enzyme. If a common carbanion is formed by both lyases and epimerases, Ca^{2+} would be expected to be required for all lyase reactions as well. However, this does not appear to be the case, with the exception that Ca^{2+} inhibits an endopolyguluronide lyase from a *Pseudomonas* species with slight stimulation at low concentrations [17].

There is, moreover, a complicating duality with respect to the effects of Ca^{2+}, since certain polyuronates interact strongly with Ca^{2+} to form ordered gels. The best known example [8] is the packing of polyguluronate chains with the interstices filled by Ca^{2+} (the 'egg box' model). Although formation of such structures could drive a reaction, it is more likely that the action of Ca^{2+} on the epimerases results in a conformational change of the protein. While the transition state intermediate for the reactions catalyzed by the individual lyase and epimerase enzymes is the same, and while the respective active sites may be similar, the two enzyme types are probably distinguished by other characteristics such as protein folding and specific binding of Ca^{2+}. It is of interest that poly-D-mannuronate specific alginate lyases from *Turbo cornutus* have been shown recently to contain a single -SH group which apparently maintains the enzyme in the specific conformation required for activity [18].

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