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Hypothesis

Alginate-modifying enzymes

A proposed unified mechanism of action for the lyases and epimerases

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1. INTRODUCTION

A number of different uronic acid-containing polysaccharides undergo post-polymerization modification to produce the final biologically active structure [1,2]. These polysaccharides are widely distributed in nature and occur in bacteria, plants and animals. Recently, there has been considerable interest in the enzymes that catalyse the C5-epimerization of uronate residues at the polymer level [3,4] although little is known about the mechanism of the reaction. This post-polymerization modification of the polysaccharides has a profound effect on the secondary structure and consequently the biological role of these macromolecules. Therefore, a greater understanding of these epimerases would be of value.

An insight into the mechanism of the epimerases may be obtained by studying a closely related group of enzymes, the polysaccharide lyases (EC 4.2.2.). Polyuronides may be depolymerized by lyases which catalyse the eliminative cleavage of the 4-*O*-linked glycosidic bond with the production of unsaturated sugar derivatives (see, for example, [5]). The interrelationship between the lyases and epimerases is most clearly shown with the polyuronide, alginate. Alginate comprises 1→4-linked residues of β-D-mannuronate or α-L-guluronate arranged randomly or in contiguous blocks within the linear molecule [6]. There is a triad of enzymes (two lyases and an epimerase) that may modify the alginate structure (fig.1). A mannuronan C5-epimerase (no EC number has been designated – provisionally EC 5.1. .) is able

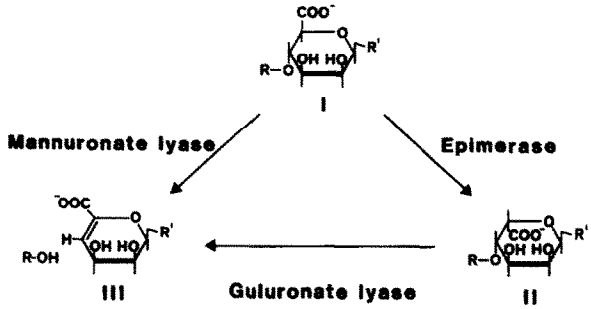

Fig.1. Reactions catalysed by alginate-modifying enzymes. The compounds are: I, D-mannuronate; II, L-guluronate, and III, 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate.

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to convert certain D-mannuronate residues in the initial polymeric material (poly(β-D-mannuronate)) to L-guluronate [7]. Also, mannuronate- [5] and guluronate-specific [8] lyases (EC 4.2.2.3) are able to degrade the polymer to produce oligosaccharides containing the unsaturated product, 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate at the non-reducing end. These types of enzymes are also common to other polyuronides.

In this paper it is proposed that both the lyases and the polymer-level epimerases have essentially a common mechanism of action and the implications of this hypothesis are considered with respect to the creation of novel enzyme activities.

2. THE PROPOSED MECHANISM

It is proposed that the enzymes utilize a three-step reaction to catalyse the appropriate transformation and it is only at the last stage that the mechanisms of the lyases and epimerases differ. The three steps may be considered to be: (i) the removal of the negative charge on the carboxylate anion; (ii) a general base-catalysed abstraction of the proton on C5 and finally; (iii) either a β-elimination of the 4-*O*-glycosidic bond (lyase) or the replacement of the proton at C5 (epimerization) (fig.2).

The basic rationale for the mechanism is derived from studies on the non-enzymic elimination reactions caused by the action of alkali on polyuronides. The effect of alkali on polyuronides is best demonstrated with the pectin/pectate system. Pectin (poly(6-*O*-methyl-D-galacturonate)) is rapidly depolymerized in mild alkali to give oligosaccharide fragments with terminally linked 4-deoxy-L-*threo*-hex-4-ene pyranosyluronate residues. The unsaturated oligosaccharides have a characteristic UV absorbance spectrum (λ_{max} = 235 nm) as a result of the conjugation between the double bond and the carboxylate group. However, pectate (poly(D-galacturonate)) is much less susceptible to alkaline depolymerization and much harsher conditions are required to produce the unsaturated oligosaccharide products [9]. These observations indicate that the most probable mechanism of alkaline depolymerization is the abstraction of the proton at C5 with resonance stabilization of the enolate anion intermediate (fig.3). Resonance stabilization of the intermediate will only occur if the negative charge on the carboxyl has been effectively removed or neutralized, e.g. by ester or salt bridge formation. Further

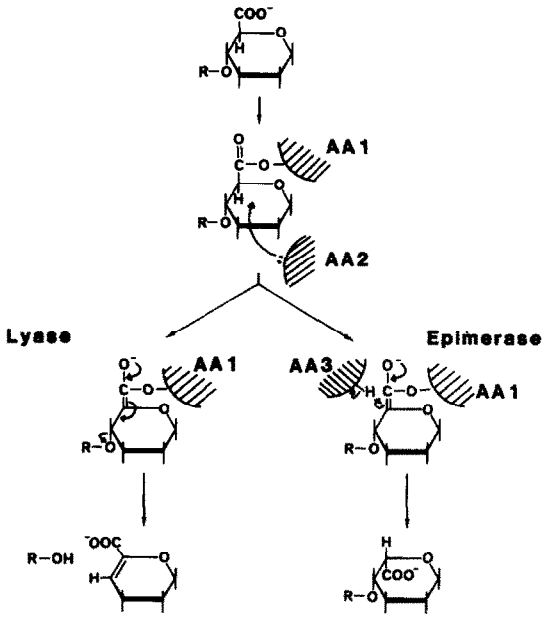

Fig.2. A proposed unified mechanism for alginate lyases and epimerases. To simplify the diagram the hydroxyl groups at positions 1–3 have been omitted. AA1 etc. refers to amino acid residues on the enzymes.

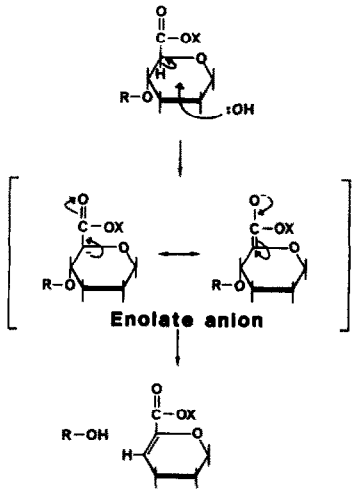

Fig.3. The base elimination of uronates. To simplify the diagram the hydroxyl groups at positions 1–3 have been omitted.

proof of this mechanism was obtained using model compounds such as 6-*O*-methyl(methyl α-D-galactopyranosyluronate). As the 4-hydroxyl is unsubstituted and therefore a poor leaving group, fairly vigorous reaction conditions are required to effect elimination [10].

It is generally accepted that the lyases operate by the chemical mechanism described above. Early work on the bacterial hyaluronate lyase (EC 4.2.2.1) established that ^{18}O from $H_2{}^{18}O$ was not incorporated into the oligosaccharide products [11], thus confirming that the depolymerization was not simply a hydrolysis step followed by elimination of water. Clearly, those lyases that utilize non-esterified polyuronates as substrates must be able to neutralize the charge on the carboxylate group and it seems likely that a lysine residue would perform this role by the formation of a salt bridge. The other positively charged amino acids, arginine and histidine, are not normally involved in this sort of mechanism. An alternative approach to neutralize the charge would be to form an ester linkage between the carboxylate and either a serine or threonine residue. However, this is unlikely as the energetics of the reaction would be unfavourable. There are a number of candidate amino acids that could act as the general base for the abstraction of the proton at C5. For example, aspartate, glutamate, histidine, lysine and cysteine are all able to act as general bases but as yet there is no direct evidence for the involvement of any one of these amino acids. Finally, one assumes that a different amino acid acts as a proton donor and protonation of the leaving group occurs although it is possible that the proton is derived directly from the solvent.

Preliminary mechanistic evidence for the mannuronan C5-epimerase reaction supports the concept of an aborted β-elimination. In other words, the enolate anion is formed and stabilized in the same way as for the lyases but instead of an elimination of the 4-*O* substituent the stereoselective replacement of a proton at C5 aborts the lytic reaction. Studies with (5-^{3}H)-labelled alginate have shown that the ^3H is released as a result of epimerization [11]. This indicates that the proton is abstracted from C5 and furthermore that it is not the same proton that is replaced in the final step of the mechanism. The implication of this is that the amino acid which acts as the general base is not the same residue that acts as the proton donor. In fact, for the epimerization of D-mannuronate to L-guluronate to occur the proton at C5 must be abstracted from below the plane of the sugar ring whereas the replacement must occur from above. The abstraction of the proton at C5 has also been confirmed by NMR studies [12]. Similar results have been obtained with an analogous polymer-level epimerase heparosan-*N*-sulphate-glucuronate 5-epimerase (EC 5.1.3.17) [13] and an enolate anion intermediate has been proposed for this enzyme mechanism [14]. Therefore, it is highly probable that the lyases and epimerases are working by essentially the same mechanism (fig.2). Further evidence in support of a common mechanism is that the polymer-level epimerases do not require NAD^+/NADH as is the case with other epimerases [7]. Some reversal of the epimerase reactions has been noted and it is interesting to speculate whether this is a result of random replacement of the proton directly from the solvent and not from AA3.

The key to the difference in the two mechanisms is the accurate stereochemical donation of a proton by the enzymes. In the lytic reaction the proton must be donated to the leaving group and not to the carbanion at C5, whereas for the epimerase the converse is true. It seems likely from thermodynamic considerations that the epimerase would possess a residual lytic activity and that the lyase would cause some epimerization. However, there is no experimental evidence to support this supposition.

At present there is no real indication of which amino acids are involved in the mechanisms of either the lyases or the epimerases. The only amino acid that has been shown possibly to be essential for the activity of both types of enzyme is cysteine. This conclusion is based on the observation that *p*-chloromercuribenzoate is an inhibitor although the experimental protocol is lacking essential details [15,16]. Therefore, at the moment it seems premature to assign a central role to a cysteine residue in the catalytic mechanism of either enzyme.

3. IMPLICATIONS OF THE UNIFIED MECHANISM

There are two significant consequences of a

unified mechanism for the lyases and epimerases. Firstly, selective modification of the proton donor amino acid (AA3 in fig.2) of the epimerase should result in conversion of the enzyme into a lyase-type of activity albeit with low activity as the protonation of the leaving group would have to be mediated by the solvent. Secondly, it should be possible, although more difficult, to use site-directed mutagenesis to convert lyases into novel epimerases. This would involve a substitution at an appropriate position in the primary structure with a suitable amino acid that could act as a proton donor and therefore effectively abort the β-elimination reaction. This latter option is particularly significant as the ability to alter uronate residues at the polymer level may have important commercial implications. At present only the mannuronan C5-epimerase is available in significant quantities. Also, there are polyuronides that are degraded by lyases yet do not have a corresponding epimerase system. Thus the ability to produce novel epimerase activities may provide the opportunity to create new polysaccharide products.

4. CONCLUSION

In conclusion it is proposed that polymer-level uronate epimerases should be regarded as having an 'aborted β-elimination' type of mechanism and that the only difference from the lyases is in the final step of the reaction. The similarities in the reaction mechanisms should allow for the conversion of lyases into epimerases and vice versa if this hypothesis is correct.

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