BIOSYNTHESIS OF ALGINATE

PART III. TRITIUM INCORPORATION WITH POLYMANNURONIC ACID 5-EPIMERASE FROM *Azotobacter vinelandii*

BJØRN LARSEN AND ARNE HAUG
Norwegian Institute of Seaweed Research, 7034 Trondheim-NTH (Norway)
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ABSTRACT

Incubation of polymannuronic acid in tritiated water with the polymannuronic acid C-5-epimerase isolated from *Azotobacter vinelandii* led to incorporation of tritium into the glycuronan. Hydrolysis of the labelled glycuronan and separation of the uronic acids demonstrated that 92% of the activity was present in the guluronic acid produced by the epimerase. There was also a small, but significant, incorporation into the mannuronic acid. The results are discussed in relation to the present knowledge of the enzymic epimerisations, and it is suggested that the first step in the reaction is an abstraction of H-5.

INTRODUCTION

A number of enzymes capable of catalysing the epimerisation of nucleoside diphosphate sugars have been isolated[1,2]. These enzymes appear to be specific in their requirement for the monosaccharide to occur as a nucleotide, and they may conveniently be classified according to the position of their attack on the sugar molecule[1]. The 4-epimerases constitute the major group of these enzymes so far known, and the current concepts of the reaction mechanism are mainly derived from studies of the UDP-D-galactose 4-epimerase. This enzyme requires catalytic amounts of NAD[3]. The occurrence of protein-bound NAD in enzymes from certain sources[4,5] renders the detection of this requirement rather difficult with these enzymes. It is well established that neither ^{18}O, deuterium, nor tritium is incorporated from isotopically labelled water, and the same also applies to tritium-labelled NAD and NADH[3,5]. Bevill *et al.*[6] demonstrated that tritium in the 4-position was retained during enzymic epimerization of UDP-D-glucose-*4-t*. Reports concerning the kinetic isotope effects[6] seem to be consistent with its being a primary isotope effect where H-4 of the hexose participates directly in the reaction. In agreement with this, Glaser and Ward[7] demonstrated by mass spectrometry that the hydrogen transfer must be intramolecular. Thus, it appears necessary to assume the occurrence of enzyme-bound UDP-4-dehydro-D-glucose as an intermediate in the reaction, and a change in conformation to allow reintroduction of the same hydrogen atom with inversion of configuration at C-4.

Among the other 4-epimerases acting on nucleotides of hexose or hexose derivatives, a definite requirement for NAD has only been established for UDP-2-acetamido-2-deoxy-D-glucose 4-epimerase[8], while reports are conflicting concerning the UDP-D-glucuronic acid 4-epimerase[9,10]. No data are available concerning the incorporation of isotopic hydrogen or oxygen for any of these enzymes. A similar situation exists for the only enzyme, UDP-D-xylose 4-epimerase[11], known to catalyse 4-epimerization in a pentose nucleotide.

Enzymic epimerisation at C-5 has only been reported to occur with a hexuronic-acid nucleotide epimerase from rabbit skin[12], leading to the conversion of UDP-D-glucuronic acid into UDP-L-iduronic acid. The reaction was inhibited by NADH[8,12] and tritium was not incorporated from T_2O, thus indicating that the mechanism may be similar to the one responsible for the 4-epimerisation.

Recent studies[13] on the mechanism of action of UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase, which catalyses the reaction UDP-2-acetamido-2-deoxy-D-glucose→2-acetamido-2-deoxy-D-mannose+UDP, demonstrated the high degree of specificity of this enzyme. The enzymic reaction proceeded at the same rate in the presence or absence of NAD, and it led to incorporation of tritium at C-2 of the 2-acetamido-2-deoxy-D-mannose. The amount incorporated depended upon whether the glucose or the mannose derivative was used as substrate. The reaction is, however, not truly reversible, since the uridine pyrophosphate moiety is split off during the reaction. The mechanism of action is thus clearly different from those previously mentioned, and, as will be discussed later, the epimerisation reaction may not take place at the nucleotide level.

The second group of enzymes comprises those not requiring the substrate to occur as a nucleotide. Two enzymes active in the pentose cycle belong to this group; L-*erythro*-pentulose 5-phosphate 4-epimerase[14], which catalyses the interconversion of L-*erythro*-pentulose and D-*threo*-pentulose 5-phosphates, and D-*erythro*-pentulose 5-phosphate 3-epimerase[15], which catalyses the interconversion of D-*erythro*-pentulose and D-*threo*-pentulose 5-phosphates. It is well documented that the action of L-*erythro*-pentulose 5-phosphate 4-epimerase is independent of the presence of free or bound NAD[16], which clearly distinguishes its action mechanism from that of the UDP-D-galactose 4-epimerase. On the other hand, both of these 4-epimerases conform to the same pattern with respect to failure to incorporate deuterium or tritium from isotopically labelled water, and to the preservation of tritium at position 4 of the specifically labelled sugar moiety. The 3-epimerase also seems to be insensitive to the action of NAD, but, in this case, the enzymic reaction leads[15] to an equimolecular incorporation of tritium from T_2O.

The first report on an enzymic epimerisation outside the monosaccharide domain involves[17] an extracellular 2-epimerase from *Ruminococcus albus*, which catalysed the interconversion of cellobiose and 4-*O*-*β*-D-glucopyranosyl-D-mannose. 4-*O*-*β*-D-Glucopyranosyl-D-fructose showed no reaction with the enzyme and is thus probably not an intermediate in the reaction. It is not clear whether NAD is required. Evidence was obtained by n.m.r. spectroscopy which demonstrated the incorporation

of deuterium at C-2 of the mannose moiety when the reaction was carried out with cellobiose and enzyme in heavy water[18].

We have previously reported the isolation and partial characterisation of an extracellular 5-epimerase from *Azotobacter vinelandii*, which catalyses the conversion of D-mannuronic into L-guluronic acid in a polymannuronic acid preparation[19,20]. This is the first report on an epimerase acting on a polymeric substrate and opens a new aspect in polysaccharide biosynthesis.

It is evident from the above summary that there are at least two different mechanisms for enzymic epimerisations. One important criterion for the allocation of the mechanism of the polymannuronic acid 5-epimerase to one of the existing groups is the eventual incorporation of deuterium or tritium from isotopically labelled water. The results now presented demonstrate that tritium is incorporated from T_2O into the enzyme-modified units during the enzymic reaction.

EXPERIMENTAL

Materials and methods. — The preparation of the enzyme and the methods used in conjunction with the enzymic epimerisation of polymannuronic acid have been described previously[19,20]. Hydrolysis of the labelled polyuronide and separation of the mannuronic and guluronic acids were carried out by our standard method[21].

Tritium was added to the reaction system as T_2O having a specific activity of 100 mCi/ml. Samples to be counted (0.1–0.5 ml) were pipetted into glass counting-vials and mixed with a sufficient quantity (1.0–2.0 ml) of Soluene-100 (Packard Instrument Company) to render all reactants, including the polyuronide, soluble in the scintillation solvent. The scintillation solvent consisted of PPO (4 g) and dimethyl-POPOP (0.25 g) (Packard Instrument Company) in analytical-grade toluene (1 litre). Countings were performed in a Packard Tri-Carb liquid scintillation spectrometer, Model 3375, using the ^{3}H-channel. All counting figures were corrected for quenching by means of the automatic, external standardisation provided in the scintillator.

Counting efficiency was estimated with samples of the same composition as that used in the actual epimerisation experiments, and was found to be 56%. This figure was not corrected for decay during the short storage time of the labelled water sample.

Enzymic epimerisation. — The 0.25% aqueous solution (1.5 ml) of polymannuronic acid (93% of mannuronic acid) was mixed with collidine buffer (5.5 ml), 0.5% aqueous calcium chloride (1.05 ml), and T_2O (100 mCi/ml, 2.0 ml). A control sample was prepared by substituting neutralised 0.1N EDTA (pH 7, 1.05 ml) for the calcium chloride. The same amount of enzyme concentrate ($A = 7.5$, 0.2 ml)[20] was added initially to each sample, and a further amount of enzyme (0.2 ml) was added after 4 h. The reaction was allowed to proceed overnight at room temperature. The glycuronan was then precipitated by the addition of M potassium chloride (1.5 ml) and ethanol (12 ml), and collected by centrifugation. The precipitate was washed with aqueous ethanol (50% v/v, 10 ml) containing M potassium chloride (1.0 ml), collected by

centrifugation, and dissolved in distilled water (2 ml). The solution was dialysed exhaustively against distilled water (4 × 500 ml). Samples of the dialysed solution (5.0 ml) were taken for determination of the degree of conversion[20] (0.75 ml) and for counting (0.5 ml).

Hydrolysis of enzyme-modified alginate. — Prior to acid hydrolysis, algal alginate (30 mg, 92% of dry matter, 35.8% of mannuronic acid) was added to the dialysed solution (3.75 ml) of enzyme-modified alginate as a diluent. The alginate was then precipitated by the addition of M potassium chloride (0.25 ml) and ethanol (4 ml). The precipitate was washed with ethanol (5 ml) and then transferred to the hydrolysis tube. Hydrolysis and column chromatography were carried out as described previously[21].

The eluate from the ion-exchange column was collected in 7-ml fractions, and these were counted in the following way: 1.0 ml of each fraction was transferred to a counting vial and evaporated to dryness in a vacuum desiccator over potassium hydroxide. The residue was dissolved in 0.2 ml of distilled water followed by 1.5 ml of Soluene-100 and 10 ml of scintillation solvent. The counts were not corrected for quenching. After combination of appropriate fractions, acetic acid was removed by evaporation. The two uronic acids were redissolved in distilled water, and samples were counted as described above.

RESULTS

The activity of the enzyme preparation depends strongly upon the presence of calcium ions[20]. A control sample with EDTA added instead of calcium chloride was therefore examined in order to estimate the amount of adventitious tritium, *i.e.* tritium not incorporated in alginate but remaining in the dialysed solution for other reasons. The resulting data show (Table I) a more than 50-fold increase in activity upon the addition of calcium chloride and demonstrate that tritium is incorporated in the alginate as a result of the enzymic reaction. The accuracy of the quantitative data are not considered sufficiently high to justify a correction for the low level of adventitious tritium, and such a correction has therefore not been applied in the subsequent calculations.

After dilution with inactive alginate, the enzyme-modified product was hydrolysed, and the two uronic acids were separated on an anion-exchange column. The

TABLE I

INCORPORATION OF TRITIUM AND MEASURED CONVERSION OF D-MANNURONIC INTO L-GULURONIC ACID, WITH AND WITHOUT CALCIUM IONS

Sample	*Total c.p.m.*	*Conversion*[a] (%)
Enzyme + EDTA	6,500	+[b]
Enzyme + $CaCl_2$	315,000	37

[a]Given as increase in content of L-guluronic acid. [b]Analysis indicates a detectable, but not measureable, conversion.

fractions collected were assayed (Fig. 1) for uronic acid by the phenol method[22] and for tritium content. It is quite clear from Fig. 1 that the dominating part of the activity is associated with the first main peak eluted, the identity of which as guluronic acid was verified by paper electrophoresis[23]. The minor, radioactive peak occurring near the elution front was not identified.

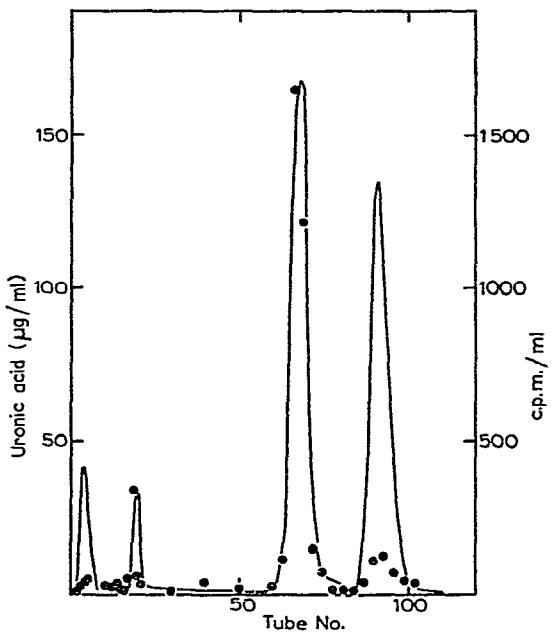

Fig. 1. Hydrolysis of tritium-labelled alginate. Separation of the two uronic acids on an ion-exchange column; ———, uronic acid; ●, tritium activity.

The fractions were combined to provide pure fractions of D-mannuronic and L-guluronic acid, and the acid used as eluant was removed by evaporation. Analytical data for the two fractions are collected in Table II. The reasonable agreement between the recoveries for uronic acid and tritium activity again indicates that the main part

TABLE II

RECOVERY AND SPECIFIC ACTIVITY OF URONIC ACIDS AFTER HYDROLYSIS AND SEPARATION

	Tritium activity (c.p.m.)			*Uronic acid (mg)*		
	D-*Mannuronic*	L-*Guluronic*	*Total*	D-*Mannuronic*	L-*Guluronic*	*Total*
Before hydrolysis			236,000	11.7	19.1	30.8[a]
Eluate from column	6,100	70,000	76,100	5.3	6.9	12.2
Recovery (%)			32.3			39.5
Specific activity (c.p.m./mg)	8,000[b]	173,000[b]				

[a]After dilution with inactive alginate (28.0 mg). [b]Corrected for the dilution with inactive alginate.

of the activity must be incorporated into uronic acids. The recovery is in the normal range for this analysis[23]. Of the total tritium activity recovered, 92% was present in the guluronic acid.

Experiments were carried out to determine whether the activity of the epimerase was influenced by addition of NAD or NADH. Activity determinations were carried out in the presence of 3.4mM calcium chloride as described previously[20], and the A values obtained (control, 3.1; mM NAD, 2.8; mM NADH, 3.2) show no significant effect of either NAD or NADH on the enzyme activity.

DISCUSSION

In the presence of calcium ions, incubation of polymannuronic acid in tritiated water with the C-5 epimerase from *Azotobacter vinelandii* leads to the incorporation of tritium into the polymeric molecule (Table I). A small, but significant, amount was incorporated even when all of the calcium ions in the system were sequestered by the addition of EDTA. According to previous experience, this should lead to a rate of epimerisation that is very close to zero[20]. However, the data clearly show that this level of incorporation would result from a conversion of less than 1% of the available D-mannuronic acid into L-guluronic acid, the quantitative determination of which is beyond the capacity of our chemical method. Thus, it may safely be concluded that the incorporation of tritium results from the epimerisation reaction, and that the dominating part of the activity must be present in the glycuronan. Additional evidence to support this conclusion is provided by the similar yields obtained for uronic acids and tritium activity upon hydrolysis of the glycuronan and separation of the uronic acids (Table II).

Of the total amount of tritium supplied to the system, a fraction of 1.26×10^{-6} was incorporated into the glycuronan. According to results obtained for other enzymic epimerisations[15,18], it is reasonable to assume that only one tritium atom is introduced for every successful attack of the enzyme on a uronic acid unit. Clearly, the maximum incorporation allowed is then equal to the molar ratio between the actual reactants, *i.e.* "anhydro uronic acid" and water in our system. The observed ratio (3.22×10^{-5}) of incorporation was well below this limit. According to the data of Tables I and II, it is a fair approximation to set the total conversion at 40%, leading to an expected ratio of incorporation of 1.29×10^{-5}. The measured ratio was close to one tenth of this value. This is quite probably due to a slower rate of reaction for tritium compared to hydrogen.

Acid hydrolysis of the enzyme-modified alginate, followed by column separation of the uronic acids (Fig. 1), unequivocally demonstrates that the enzymic conversion of D-mannuronic into L-guluronic acid is accompanied by an incorporation of tritium. Of the total tritium activity recovered in the uronic acids, 92% was present in L-guluronic acid (Table II). Calculated on the basis of the L-guluronic acid remaining from the original, enzyme-modified alginate (by using the recoveries actually determined in the experiment), this corresponds to a specific activity of 173,000 c.p.m./mg.

There is a small, but significant incorporation of tritium also into the D-mann-

uronic acid, corresponding to 8% of the total activity recovered. The specific activity of this monomer was less than 5% of that found for L-guluronic acid and may indicate that the reaction is reversible, but with a great difference in rate constant between the forward and the backward reaction. This is in good agreement with our previous observation[20] that the end-point of the reaction in the presence of calcium ions corresponds to an alginate rich in L-guluronic acid. As suggested previously, the position of the equilibrium in the direction of a polymer rich in L-guluronic acid when calcium ions are present may be caused by the higher affinity of calcium ions for the L-guluronic acid residues in the polymer. That no reversion of the reaction in the direction of polymers rich in mannuronic acid was observed in the absence of calcium ions may be due to a lack of enzymic activity under these conditions.

The information so far available seems to suggest that enzymic epimerisations requiring NAD as a cofactor and proceeding without the incorporation of hydrogen or oxygen from water may be restricted to sugar nucleotides only. Evidence has been presented[24] to suggest that the nucleoside stabilises the substrate in a secondary structure essential for the enzymic reaction to occur.

The 4-epimerisation of pentulose phosphate, *i.e.* L-*erythro*-pentulose 5-phosphate, also proceeds without incorporation. The mechanism of this reaction is, however, different from the one operating on the nucleotide level by lacking the requirement for NAD[16].

In addition to the enzyme discussed in this work, three enzymes are known that catalyse reactions leading to the incorporation of tritium from isotopically labelled water: UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase[13], D-*erythro*-pentulose 5-phosphate 3-epimerase[15], and cellobiose 2-epimerase[18], none of which appears to require NAD. The three reactions have one distinct feature in common: the epimerisation takes place at a carbon atom adjacent to an existing, or potential, carbonyl group. Moreover, they represent all three classes of substrates involved: sugar nucleotide, free or phosphorylated sugar, and oligo- or polymers. It is possible that there may be a common epimerisation mechanism.

The incorporation of tritium as well as the failure of both NAD and NADH to affect the rate of epimerisation suggest that the polymannuronic acid C-5-epimerase belongs to this group of epimerases. Although the rate is very much higher when the uronic acid units are esterified, it has been demonstrated that β-alkoxy-elimination may take place in the free glycuronan[25]. It is also well known that the enzymic degradation of (1→4)-linked glycuronans often occurs as an elimination reaction, indicating that this type of reaction is facilitated by the existence of a neighbouring carboxyl group, possibly by a transient esterification taking place on the enzyme.

Based on this evidence, it seems reasonable to assume that the mechanism common to all these reactions is an abstraction of the hydrogen atom adjacent to the carbonyl or carboxyl group, as proposed by Amein and Leatherwood[18] for the C-2-epimerisation of cellobiose.

It is interesting to note that Bohak and Katchalski[26] have demonstrated the chemical racemisation of poly-L-serine at the polymeric level without linkage scission.

The enzymic C-5-epimerisation of UDP-D-glucuronic into UDP-L-iduronic acid proceeds without the incorporation of tritium[12]. The mechanism of this reaction is thus clearly different from that operating with the polymannuronic acid C-5-epimerase, demonstrating that there are at least two mechanisms available for the C-5 epimerisation of uronic acids. The epimerisation of UDP-D-glucuronic acid may require catalytic amounts of NAD[12], and it seems reasonable to assume that the mechanism of this reaction is related to the one described for the 4-epimerisation of sugar nucleotides.

Since the action of the UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase is accompanied by the incorporation of tritium, this reaction would seem to be at variance with the proposed relationship among enzymic epimerisations of sugar nucleotides. As pointed out by Salo and Fletcher[13], however, the first step in this reaction is quite probably an irreversible scission of the glycosidic linkage. The actual epimerisation may thus involve the free sugar, in contrast to the other sugars of this substrate group where the nucleotide is retained during the reaction.

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