Review of mutarotase in 'Metabolic Subculture' and analytical biochemistry: prelude to ^{19}F NMR studies of its substrate specificity and mechanism

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Abbreviations: NMR, nuclear magnetic resonance; RBC, red blood cell.

Abstract

This is the first paper in a sequential pair devoted to the enzyme mutarotase (aldose 1-epimerase; EC. 5.1.3.3). Here, the broader context of the physiological role of mutarotase, amongst those enzymes considered to be part of "metabolic structure", is reviewed. We also summarise the current knowledge about the molecular mechanism and substrate specificity of the enzyme, which is considered in the context of binding of fluorinated glucose analogues to the enzyme's active site. This was done as a prelude to our experimental studies of the anomerisation of fluorinated sugars by mutarotase that are described in the following paper.

Introduction

Anomerisation

Two crystalline forms of D-glucose with different plane-polarised light (optical) rotation were reported in 1895.[1] Yet, when the two forms are dissolved in water, the solutions have the same optical rotation after several minutes. This outcome led to the conclusion that there was interconversion between the two sugar forms (anomers) in solution. The surmised molecular mechanism of this phenomenon is the cleavage of a closed ring structure to give an open chain, and back again. For D-glucose, this amounts to the conversion of a cyclic hemiacetal (glucopyranose) to the open chain aldehyde, and back again (Fig. 1). The rotation around the C1-C2 bond allows access to both diastereomers at the C1 atom (‘anomers’) of the cyclic conformation that is formed when the C5-hydroxyl group closes the ring by a nucleophilic attack on the aldehydic C1.

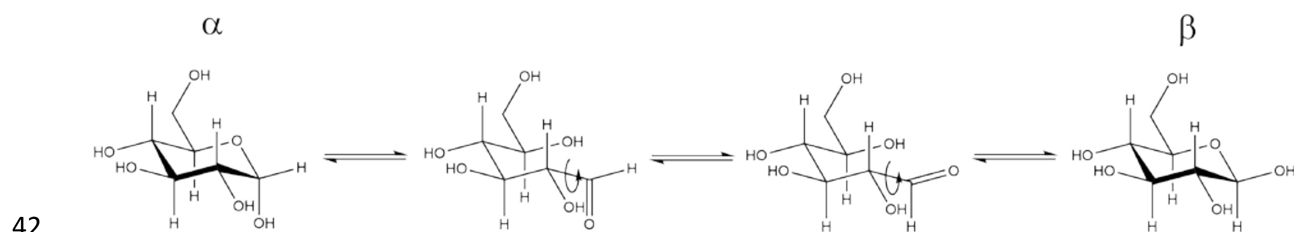

Fig. 1. Mechanism of spontaneous mutarotation (anomerisation) of D-glucose in aqueous solutions.

Mutarotase and metabolic subculture

Even though the anomerisation of glucose and other hexoses occurs spontaneously on the time scale of several minutes, mutarotase is found in most (if not all) organisms where it greatly enhances the rate of this reaction.[2-3] Thus, it belongs to a group of enzymes that catalyse reactions that are already spontaneously quite rapid in cells. For these enzymes, it might have been surmised, low evolutionary selection pressure would have been applied; however, they persist throughout phylogeny. These disparate reactions and enzymes have been colloquially referred to as the cell’s “metabolic subculture”.

The term “metabolic subculture” refers to reactions that occur with metabolites in metabolic pathways that are secondary to the mainstream biochemical transformations (such as the pentose phosphate pathway and glycolysis), but are nonetheless vital to the regulation/flux-control of the reactions in the mainstream pathways. An example of one such secondary reaction is hydration of CO_2 to H_2CO_3, which then dissociates to H^+ and HCO_3^-. This hydration reaction is known to occur rapidly in the absence of enzymes, but there is a good reason why

carbonic anhydrase (E.C. 4.2.1.1) is the second most abundant protein in the red blood cell [1-2 g (L RBC)$^{-1}$], with a turnover number of ~10^6 s^{-1}. The enzyme in these cells enhances the CO_2 hydration reaction to such an extent that it goes to completion in the transit time of an RBC through a lung capillary (~0.3 s). If the reaction were slower, there would be incomplete CO_2 release from the cells during a single passage through the lungs, so speeding up the hydration reaction has an important physiological outcome.

Another example of these secondary reactions is the spontaneous hydration of ketones and aldehydes, e.g., pyruvic acid exists in aqueous solution as both a free ketone and a *gem*-diol. However, since lactate dehydrogenase (E.C. 1.1.1.27) operates only on the ketone form of the pyruvate,[4] for the redox reaction to yield lactic acid, dehydration of the *gem*-diol must occur first. This is generally seen to happen so rapidly that the overall rate of glycolysis is not decreased by any limitations in the rate of pyruvate hydration/dehydration. It is, however, likely that the reaction is catalysed in the cytoplasm by an enzyme such as carbonic anhydrase,[5] which seems to engage in 'moonlighting' hydration-dehydration reactions with aldehydes such as acetaldehyde, and ketones including pyruvate and dihydroxyacetone phosphate.

Another "metabolic subculture" enzyme is 6-phosphogluconolactonase (E.C. 3.1.1.31) - it is a hydrolase that catalyses a rapidly reversible reaction in the oxidative pentose phosphate pathway. In terms of the reactions of the pathway, it lies between the two dehydrogenases. The reaction is sufficiently fast without the enzyme that, again, it was not known to exist until quite late in the emerging understanding of the biochemistry of the pentose phosphate pathway.[6]

Overall, enzymes including those above have evolved to enhance reaction rates, which in some cases are already quite rapid. These enhancements take the reaction rate into a new time regime where the reaction exerts almost no control over the main metabolic or transport flux. This leaves other reactions in a metabolic pathway to be the sites of pathway regulation. As introduced above, mutarotase is one such member of this metabolic subculture.[7]

Mutarotase in cell physiology

Many carbohydrate-processing enzymes have a significant sugar-anomer specificity, thus leaving one of the anomers unreacted in the case of slow mutarotation. For example, using ^{19}F NMR magnetisation-transfer experiments, it has been shown that glucose transporter GLUT1 (in human RBCs) transports the α-anomer faster than the β-anomer, and this is likely to hold in other tissues.[8-10] Glucose freely enters the glomerular filtrate via GLUT1, GLUT4 and GLUT8 and passes into the convoluted tubules. From there it enters the lining cells of the

90 tubules, via the sodium dependent glucose transporters SGLT1 and SGLT2. It then passes out
91 into the capillaries of the cardiovascular circulation via the basolateral membrane that contains
92 GLUT1 and GLUT2.[11] This glucose recycling has been naturally selected to be fast and
93 efficient and not dependent on the rate of glucose anomerisation. As transmembrane exchange
94 of glucose via the GLUT1 transporters is α-anomer preferred, rapid intracellular equilibration
95 between the anomers, catalysed by mutarotase, reduces the potential flux limitation that would
96 otherwise be imposed by this reaction, if it were only spontaneous. In mammals, the highest
97 activity for mutarotase has been reported in the cortex of the kidney. Thus, in the absence of
98 mutarotase, accumulation of these sugars in the kidney might have occurred.

99 **Mutarotase as a reagent**

100 Another important biochemical aspect of mutarotase is its use in metabolic/metabolomic
101 analysis as a type of (bio)chemical reagent. Specifically, when glucose oxidase (EC 1.1.3.4
102 from *Aspergillus niger*) is used in the clinical biochemical assay of glucose in blood plasma, it
103 is usual to add exogenous mutarotase, because the glucose oxidase is highly specific for the β-
104 anomer of glucose.[12] Plasma levels of mutarotase may be elevated in patients with renal
105 disease, so adding an excess of mutarotase in the assay solution obviates any enhancing effect
106 on the amount of β-anomer that is generated by the endogenous enzyme, had only the β-anomer
107 been detected in the assay.[13] In other words, addition of mutarotase ensures that the reaction
108 rate is not restricted by the relatively slow spontaneous anomerisation reaction.

109 The anomer-exchange property of mutarotase was also used in an experiment to quantify the
110 transmembrane exchange of ^{13}C-labelled glucose.[14] The method exploited the finding that
111 human RBCs display little activity of endogenous mutarotase, but it can be added in abundance
112 (as a *reagent*) outside the RBCs in a suspension. Thus, ^{13}C-labelled glucose underwent rapid
113 anomerisation outside the RBCs but not inside. For [1,^{13}C]D-glucose, well-resolved resonances
114 appear in the ^{13}C NMR spectrum at 92.9 ppm for the α-anomer and 96.7 ppm for the β-anomer.
115 Selective irradiation of the sample at the resonance frequency of the α-anomer (92.9 ppm)
116 brings about partial suppression of the intensity of the resonance from the β-anomer. The extent
117 of peak suppression (due to the transfer of the saturation-of-magnetisation) is smaller when the
118 main glucose transport protein of the RBCs, GLUT1, is inhibited by phloretin or cytochalasin
119 B. Thus, the developed NMR-kinetic theory allowed estimations of the unitary rate constants
120 for the GLUT1 transport reaction - these were similar to those obtained by using the more
121 direct magnetisation-transfer methodology with ^{19}F-labelled glucose.[14] However, it was not

122 an *a priori* expectation that substituting an -OH group with an -F would not affect the rate of
123 transport, so having the former (albeit more complicated) method provided a standard for
124 comparison.

125 An interesting aside that emerged from this work, is that the ^{13}C NMR resonance of the β-
126 anomer of [1,^{13}C]D-glucose in a suspension of RBCs has two unresolved components, arising
127 due to the split-peak effect.[15] The two components correspond to D-glucose outside and inside
128 the cell; they are separated by ~5 Hz, with the intracellular component located at the higher
129 frequency. The physical/chemical basis of the splitting is now known to be the differential
130 hydrogen bonding (H-bonding) of water to the H atoms (H-bond donor) and -OH groups (H-
131 bond acceptor and donor, respectively) near the ^{13}C1 atom whether outside or inside the
132 cells.[15]

133 **Molecular mechanism of mutarotase**

134 Major insights into the mechanism of mutarotase-catalysed anomerisation emerged from
135 studies of the enzyme's substrate specificity reported in the 1970s.[7, 16] Several authors showed
136 activity with D-glucose, D-galactose, D-xylose, D-fucose (6-deoxy D-galactose) and L-
137 arabinose, and lack of activity with many other sugars.[7, 16-17] More recently, in addition to this
138 list, D-quinovose (6-deoxy-D-glucose) was shown as a high affinity substrate for mutarotase
139 from *L. lactis*.[18] The molecular structures of the six known substrates of mutarotase, drawn as
140 modified Haworth projections, are shown in Fig. 2. The highest specificity constant ($k_{\mathrm{cat}}/K_{\mathrm{m}}$)
141 of the enzyme has been reported for D-galactose.[19]

142 X-ray crystallography studies of mutarotase in complex with the substrates shown in Fig. 2
143 revealed that four amino acid residues are intimately involved in sugar binding and catalysis:
144 His96, His170, Asp243, and Glu304.[18] The general disposition ('constellation') of these four
145 amino acids is highly conserved between different species, and the reaction mechanism is
146 surmised to be fundamentally the same in all organisms, including humans.[19]
147 Monosaccharides bind to the enzyme's active site by substitution of their H-bond interaction
148 with H_2O by more favourable H-bond interactions with the protein, and no change in the three-
149 dimensional structure of mutarotase occurs upon substrate binding.[18, 20-21] However, the
150 importance of the specific H-bonds in substrate binding is not known.

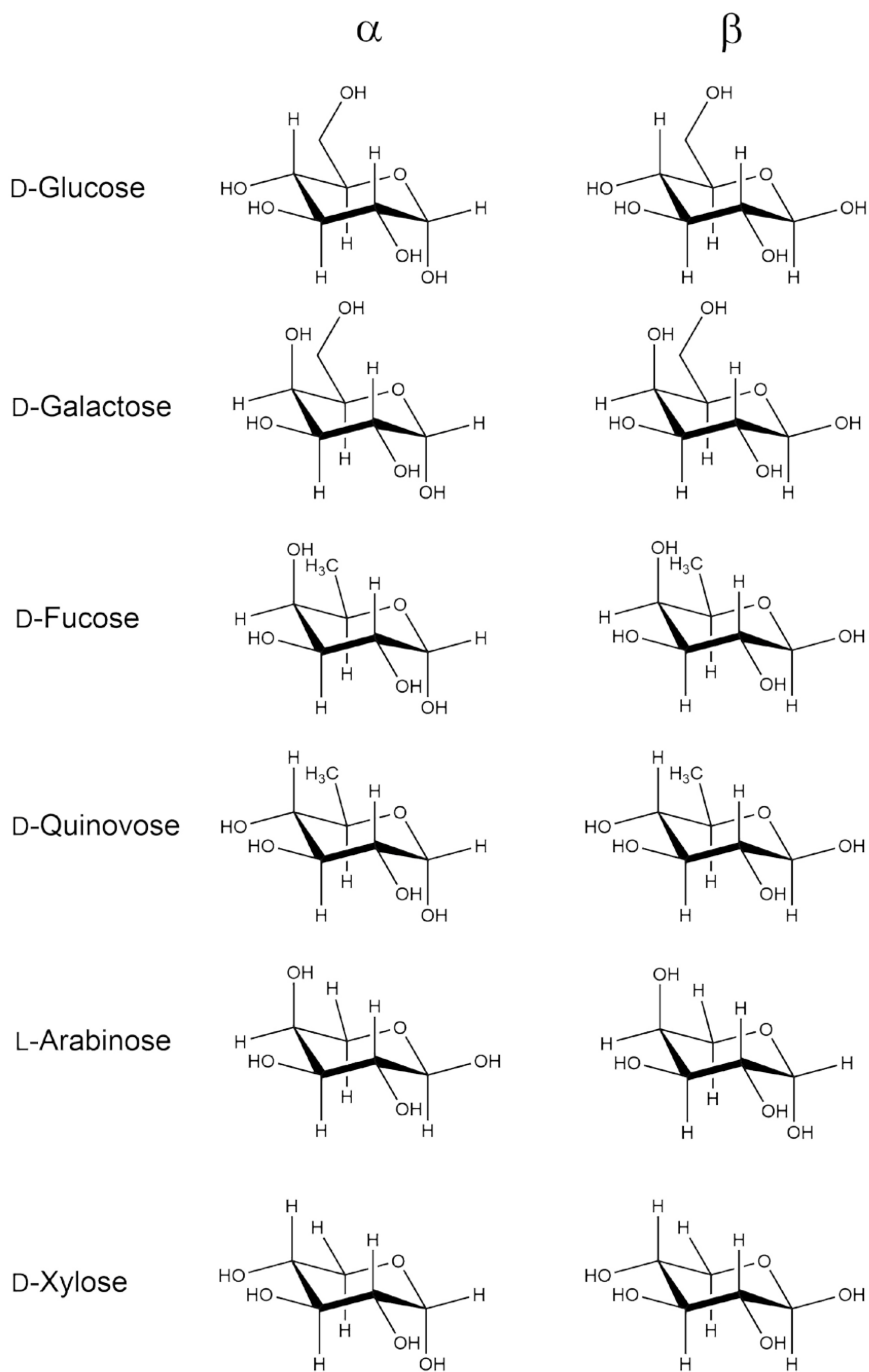

Fig. 2 Monosaccharides that have been reported as good substrates of mutarotase. The α- and β-anomers are shown, with the anomeric carbon C1 located on the right of each chair structure which in all cases is a pyranose ring.

155 **Potential effects of substrate (deoxy)fluorination on substrate binding**

156 (Deoxy)fluorination of sugars is an effective strategy for probing the importance of specific
157 molecular interactions, such as hydrogen bonds, in binding by proteins.[22] In the following, we
158 speculate on how fluorination of D-glucose might affect the catalytic efficiency of mutarotase
159 by considering functional groups in the sugar molecule in terms of their involvement in any
160 crucial molecular interactions with the enzyme's binding site. As the substrates of mutarotase
161 are electrically neutral, there is no need to consider (at least in the absence of other information)
162 electrostatic/salt-bridge interactions, so the primary effect of (deoxy)fluorination will be in the
163 change in free energy of the H-bond interactions. A fluorine atom can act as a hydrogen-bond
164 acceptor; but, unlike the -OH group, it cannot serve as a hydrogen-bond donor. Therefore, it is
165 important to consider whether the -OH groups (to be substituted by -F atoms) participate in any
166 hydrogen-bond interactions with the protein's active site. In the following, we do this analysis
167 in turn for each of the -OH groups attached to the carbon atoms C1-C6.

168 (1) C1 is the "special" carbon atom in D-glucose – it only has an -OH group substituent upon
169 the formation of the hemiacetal in pyranose-ring closure. Thus, (deoxy)fluorination of C1 in
170 either α- or β-anomer would totally prevent formation of an aldehyde, leading to complete
171 absence of the anomerisation reaction. On the other hand, C1-F derivatives could still
172 potentially bind to mutarotase and inhibit it.

173 (2) In all the known substrates of mutarotase (Fig. 2), the orientation of the -OH groups attached
174 to C2 and C3 are equatorial relative to the plane of the pyranose ring (in the 4C1 conformation).
175 This suggests that these -OH groups are important in binding and correct positioning of the
176 substrate in the enzyme's active site. Indeed, according to the crystal structure, both of
177 these -OH groups are involved as H-bond donors forming an interaction with the two O atoms
178 of the carboxylate group of Asp243.[20] Therefore, we predict that substitution of the -OH
179 groups at either C2 or C3 by an F atom, which cannot serve as an H-bond donor, would
180 compromise binding and reaction of the substrate. Thus, this analysis predicts that sugars such
181 as 2-fluoro-2-deoxy-D-glucose or 3-fluoro-3-deoxy-D-glucose would be poor substrates of
182 mutarotase.

183 (3) The C4 of D-galactose, D-fucose and L-arabinose has its -OH directed axially, while in the
184 case of D-glucose, D-xylose and D-quinovose, it is equatorial (Fig. 2). According to the reported
185 crystal structures, when the -OH group is axial, it is capable of serving as a hydrogen-bond
186 acceptor and thus forms an interaction with the side-chain of Arg71.[20] However, when

the -OH group is equatorial, no amino acid is located in its vicinity for the formation of an H-bond.[18] This is one of the possible explanations for why D-galactose is a better substrate for mutarotase than D-glucose.[18] In any case, substituting the -OH by an -F would not significantly impede substrate binding, since the F atom is capable of serving as an H-bond acceptor, thus maintaining the interactions with the side chains of Arg71. This observation predicts that (deoxy)fluorination at position C4 would not significantly compromise substrate binding by mutarotase. Thus, 4-fluoro-4-deoxy-D-glucose should still be a good substrate of the enzyme.

(4) Among the known substrates of mutarotase (Fig. 2), the C5 atom has -CH_2OH, -CH_3, or -H attached, in addition to the O atom that serves as the bridge when closing the sugar ring (it is present in this position in all six saccharides). This implies that a range of potential binding partners to these groups might interact with the enzyme, but none of these interactions seem crucial for overall substrate binding. Thus, we would not expect that the nature of the substituent on C5 would strongly affect the H-bond interactions.

(5) Since the pentose substrates (L-arabinose and D-xylose) have no C atom at the C6 position and given their ready mutarotation by the enzyme, it can be surmised that substitution of the -OH on C6 of the 6-carbon saccharides would not have a major effect on the reaction rate. In fact, it was previously noted that D-fucose (6-deoxy-D-galactose) binds to mutarotase with the same affinity as D-galactose.[23] Moreover, according to the crystal structure, the -OH group at C6 does not participate in H-bonding to the enzyme. [20] Thus, we would not expect a strong effect of this substitution and fluorination of C6 atom.

In summary, using insights from previous work on substrate specificity and molecular structures of mutarotase, we surmise that, at least some fluorinated analogues of D-glucose would be poor substrates of the enzyme. In the context of using these in *in vivo* studies, they might accumulate in the kidney and be potentially used as probes of intracellular volume in magnetic resonance imaging/spectroscopy experiments of this organ. Alternatively, the inhibitory properties of some of the fluorinated sugars might be used to study the kinetics of the enzyme *in vitro* by using various biochemical assays. Thus, anomerisation of fluorinated sugars by mutarotase was deemed to be ripe for study by ^{19}F NMR spectroscopy, which provides unprecedented access to sub-second kinetics. The results of these novel studies are reported in the following paper.

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220 ## Conflicts of interest

221 The authors declare no conflicts of interest.
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