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Specificity of GDP-Man:dolichyl-phosphate mannosyltransferase for the guanosine diphosphate esters of mannose analogues containing deoxy and deoxyfluoro substituents

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Guanosine diphosphate (GDP) esters of 2-deoxy-D-glucose (2dGlc), 2-deoxy-2-fluoro-D-mannose (2FMan), 3-deoxy-D-mannose (3dMan), 4-deoxy-D-mannose (4dMan) and 6-deoxy-D-mannose (6dMan) have been synthesised and tested for their ability to act as inhibitors of dolichyl phosphate mannose synthesis (enzyme: GDP-mannose:dolichyl-phosphate mannosyltransferase, EC 2.4.1.83) in chick embryo cell microsomal membranes. The following order of efficiency was found with the apparent K_i in parentheses: GDP-6dMan (0.40 μM $\pm$0.15) > GDP-3dMan (1.0 μM $\pm$0.1) = GDP-2dGlc (1.3 μM $\pm$0.2) > GDP-4dMan (3.1 μM $\pm$0.1) GDP-2FMan (15 μM $\pm$0). For comparison the K_m for GDP-Man was 0.52 μM $\pm$0.02 and the K_i for GDP was 56 μM $\pm$2. These results indicate that the 6-hydroxyl group of mannose is not crucial for emzyme-substrate recognition, whereas the 2- and 3-hydroxyls may have some involvement. The 4-hydroxyl appears to be an important determinant for enzyme-substrate recognition in this mannosyltransferase.

Deoxy-D-glucose, 2-; Deoxy-2-fluoro-D-mannose, 2-; Deoxy-D-mannose, 3-; Deoxy-D-mannose, 4-; Deoxy-D-mannose, 6-; Enzyme inhibition; (Chick embryo cell)

1. INTRODUCTION

Sugar analogues have been found to act as inhibitors of protein glycosylation by virtue of their antiviral properties [1,2]. Thus, analogues of glucose and mannose containing fluoro or deoxy substituents on carbons 2 and 4 were shown to interfere with the glycosylation of viral envelope glycoproteins by inhibiting the assembly of the oligosaccharide precursor, $Glc_3Man_9(GlcNAc)_2$ linked to dolichol pyrophosphate (Dol-PP) [1]. Analogues such as 2-deoxy-D-glucose (2dGlc), 2-deoxy-2-fluoro-D-glucose (2FGlc), 2-deoxy-2-fluoro-D-mannose (2FMan), 4-deoxy-D-mannose (4dMan) and 4-deoxy-4-fluoro-D-mannose (4FMan) are metabolised in vivo to their corresponding GDP and/or UDP derivatives [1,3], and it is these metabolites that are the actual inhibitory agents [4–8].

Although the pathways of protein glycosylation are well documented [9], little is known about how the pathway is regulated or how the enzymes function. Through the use of specific inhibitors it should be possible to gain insight into the mechanism of enzyme-substrate recognition for the glycosyltransferases involved in the assembly of dolichyl phosphate (Dol-P) monosaccharides and dolichol-linked oligosaccharides. With the exception of 2FGlc and 4FMan the GDP derivatives of the above sugar analogues are inhibitors of Man-P-Dol formation in vitro and are substrates for the synthesis of their respective Dol-P derivatives [4,7,8]. Thus, the function of the various hydroxyl groups of the mannose moiety of GDP-Man in the recognition of GDP-Man by the mannosyltransferase (GDP-Man:Dol-P man-

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nosyltransferase, EC 2.4.1.83) catalysing the formation of Man-P-Dol can be investigated. The kinetics of the inhibition of Man-P-Dol synthesis by GDP-2dGlc, GDP-2FMan and GDP-4dMan are presented. In addition, the guanosine diphosphate esters of two novel mannose analogues 3-deoxy-D-mannose (3dMan) and 6-deoxy-D-mannose (6dMan) were synthesised, and their effects on Man-P-Dol synthesis evaluated.

2. MATERIALS AND METHODS

2.1. *Sugar nucleotides*

The GDP derivatives of 2dGlc, 2FMan, 3dMan, 4dMan and 6dMan were synthesised from the parent sugars via the sugar 1-phosphates as in [7]. GDP-[U-^{14}C]Man (300 Ci/mol) was obtained from Amersham Buchler (Braunschweig). GDP-Man, GDP and UDP were from Boehringer Mannheim (Mannheim, FRG). 3dMan and 6dMan were generously provided by Dr S. Kučár and Dr P. Biely of the Slovak Academy of Sciences, Bratislava, Czechoslovakia.

2.2. *Preparation of a microsomal membrane fraction from chick embryo cells*

A crude preparation of microsomal membranes was isolated from chick embryo cells as described [10]. This preparation containing 15–20 mg protein/ml was treated to complete the synthesis of partially assembled lipid-linked oligosaccharides by incubation with 1% by vol. of 0.2 mM GDP-Man and 0.2 mM UDP-Glc for 10 min at 37°C. The membranes were then depleted of Man-P-Dol and Glc-P-Dol by incubation with 1% by vol. of 2 mM GDP and 2 mM UDP for a further 10 min at 37°C. The treated membranes were then diluted with 50 vols of ice-cold buffer containing 20 mM Tris-HCl, pH 7.5 and 150 mM NaCl, and recovered by centrifugation at 100000 × g (60 min at 4°C). The pelleted treated membranes were suspended in 20 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl, 0.4 mM $MgCl_2$ and 0.4 mM $MnCl_2$ at the same protein concentration as above.

2.3. *Assay of GDP-Man:Dol-P mannosyltransferase activity*

Treated microsomal membranes (1.2–1.6 mg protein) and GDP-[^{14}C]Man (1.35 or 6.74 μM) were incubated in 13 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.27 mM $MgCl_2$ and 0.27 mM $MnCl_2$ in a final volume of 120 μl at 37°C for 10 min. The reaction was stopped by the addition of 3 ml ice-cold chloroform-methanol (2:1 by vol., CM) and the incorporation of radioactive label into the CM extract was determined [10].

TLC on silica gel G-60 (Merck) with chloroform-methanol-ammonia-water (65:35:4:4, by vol.) indicated that Man-P-Dol was the sole product soluble in CM. Further extraction with chloroform-methanol-water (10:10:3 by vol., CMW) indicated that no products soluble in CMW (lipid-linked oligosaccharides) were formed. Therefore, the incorporation of label into the CM extract could be used as a criterion for the measurement of GDP-Man:Dol-P mannosyltransferase activity.

3. RESULTS AND DISCUSSION

3.1. *Inhibition of Man-P-Dol synthesis by GDP derivatives of mannose analogues*

The GDP derivatives of 2dGlc, 2FMan and 4dMan have been previously shown to be inhibitors of Man-P-Dol formation in vitro [4,7,8]. In each case, the inhibition was due to the formation of the corresponding sugar analogue-P-Dol derivative. The formation of Dol-P derivatives was inferred from the fact that addition of exogenous Dol-P could overcome the inhibition.

Incubation of radiolabelled GDP-sugar analogues with chick embryo cell microsomes confirmed the formation of the dolichol phosphate sugar analogues [4,7,8]. 3dMan and 6dMan are not taken up into cells in tissue culture. However, it was interesting to synthesise their GDP derivatives and test their efficiency in vitro as inhibitors of the mannosyltransferases involved in the dolichol pathway, since they contain modifications of the hydroxyl groups of carbons 3 and 6. As shown in fig.1A both are strong inhibitors of Man-P-Dol formation. Addition of exogenous Dol-P overcomes the inhibition in the case of GDP-3Man (fig.1B), whereas with GDP-6dMan only a partial recovery is observed (fig.1B). In addition, both nucleotide sugar analogues inhibited the formation of Glc-P-Dol and GlcNAc-PP-Dol (not shown), a property exhibited by the previously characterised sugar analogue guanosine diphosphate esters [4–8]. These results all point to the fact that GDP-3dMan and GDP-6dMan are substrates for the formation of their respective dolichol phosphate derivatives.

3.2. *Kinetics of inhibition of Man-P-Dol formation by GDP derivatives of mannose analogues*

Since the guanosine diphosphate esters of mannose analogues containing modifications of the hydroxyl groups on carbons 2, 3, 4 and 6 are substrates for the formation of their corresponding dolichol phosphate derivatives, there is the possibility of examining the roles played by each hydroxyl group in the enzyme-substrate interaction between GDP-Man:Dol-P mannosyltransferase and GDP-Man. This was done by determining the apparent inhibition constant (K_i) for each inhibitor and comparing it with the apparent Michaelis con-

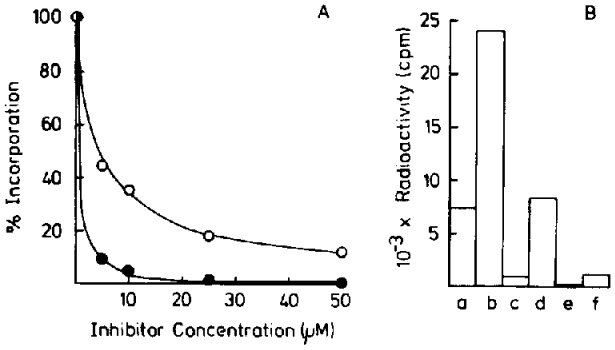

Fig.1. Inhibition of Man-P-Dol formation by GDP-3dMan and GDP-6dMan in vitro (A) and the effect of exogenous Dol-P (B). (A) The formation of Man-P-Dol was determined in the presence of GDP-3dMan (○) or GDP-6dMan (●) as described in section 2. 100% is 11664 cpm incorporated into Man-P-Dol in 10 min. (B) Man-P-Dol formation was determined as described in the text in the absence or presence of exogenous Dol-P (0.3%) in Triton X-100 (0.017%) and GDP-3dMan or GDP-6dMan. (a) Control, (b) Dol-P (0.3%) alone, (c) GDP-3dMan (100 μM) alone, (d) GDP-3dMan + Dol-P, (e) GDP-6dMan (100 μM) alone, (f) GDP-6dMan and Dol-P.

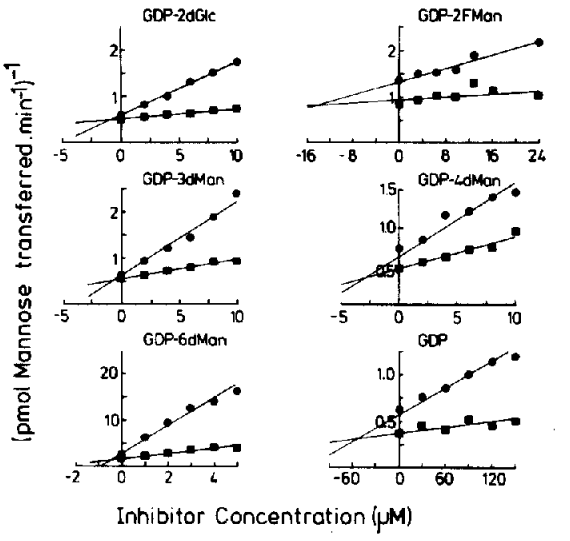

Fig.2. Inhibition of GDP-Man:Dol-P mannosyltransferase by the guanosine diphosphate esters of mannose analogues. The formation of Man-P-Dol was assayed as described in section 2 at two constant GDP-Man concentrations, 1.35 μM (●) and 6.74 μM (■), in the presence of GDP-2dGlc, GDP-2FMan, GDP-3dMan, GDP-4dMan, GDP-6dMan and GDP as indicated. The reciprocal of the reaction velocity (pmol mannose transferred·min^{-1}) was plotted vs inhibitor concentration for each inhibitor at the two specified GDP-Man concentrations. Typical examples for each inhibitor are shown.

stant (K_m) for GDP-Man. A relative order of efficiency could then be constructed.

Treated chick embryo microsomes were incubated with GDP-[^{14}C]Man (1.35 and 6.74 μM) in the presence of GDP-2dGlc, GDP-2FMan, GDP-3dMan, GDP-4dMan, GDP-6dMan and GDP and Man-P-Dol formation was measured. The reciprocal of the reaction velocity (pmol mannose transferred·min^{-1}) was plotted vs the inhibitor concentration (μM) at two constant concentrations of GDP-Man according to Dixon [11] (fig.2). Typical examples for each inhibitor are shown in fig.2, and it is clear that in each case the inhibition is competitive. The apparent K_i is the inhibitor concentration extrapolated from the intersection of the two lines. Table 2 shows the K_i obtained for each inhibitor. The following order of efficiency for the inhibitors can be inferred: GDP-6dMan > GDP-3dMan/GDP-2dGlc > GDP-4dMan > GDP-2FMan ⋙ GDP. There was no significant difference between the K_i for GDP-3dMan and that for GDP-2dGlc ($p > 0.1$ Student's t-test). The K_i for GDP is much greater than those of the GDP-sugar analogues, indicating that the observed effects are due to the intact sugar nucleotides. From the gradients of the lines on the Dixon plots a value for the apparent K_m for GDP-Man was calculated to be 0.52 ± 0.02 μM. This is close to that (0.69 μM) obtained for a partially purified rat liver Man-P-Dol synthetase preparation [12]. Comparison of the apparent K_m for

Table 1

Inhibition constants for the GDP-mannose analogues and GDP calculated from Dixon plots

Inhibitor	K_i (± SE) (μM)
GDP-6dMan	0.40 ± 0.15
GDP-3dMan	1.0 ± 0.1
GDP-2dGlc	1.3 ± 0.2
GDP-4dMan	3.1 ± 0.1
GDP-2FMan	15 ± 0
GDP	56 ± 2

The inhibition constants (K_i) for each inhibitor were obtained from Dixon plots of the reciprocal of enzyme activity (pmol mannose transferred·min^{-1}) vs the inhibitor concentration at two concentrations of GDP-Man (1.35 and 6.74 μM), as shown in fig.2. The K_i is the inhibitor concentration extrapolated from where the two lines intersect. The K_m for GDP-Man was calculated to be 0.52 ± 0.02 μM from the slopes of the lines

GDP-Man with the apparent K_i for each inhibitor indicated that GDP-6dMan and GDP-Man are identical ($p > 0.1$, Student's t-test) in their substrate properties for GDP-Man:Dol-P mannosyltransferase. The other inhibitors are poorer substrates by factors of 2-, 2.5-, 6-, 29- and 108-fold, respectively. It is clear from these results that hydrogen bonding involving the hydroxyl group on carbon 6 does not play a role in enzyme-substrate recognition. The hydroxyls at carbons 2 and 3 may have some involvement in substrate recognition, whereas the hydroxyl on carbon 4 must be more critical. Results obtained with the mannosyltransferases involved in the assembly of the lipid-linked intermediates of *Salmonella* O-antigenic polysaccharides [13] showed that interactions involving the C-2, C-3 and C-6 hydroxyl groups of mannose were not critical for the recognition of GDP-Man by the *Salmonella* mannosyltransferases. Thus, there are similarities with respect to the recognition of the sugar nucleotide donor between mannosyltransferases of bacterial and avian origin utilising lipid-linked acceptors.

Fluorine, when present, seems to have an adverse effect on the enzyme-substrate interaction. Thus, the K_i for GDP-2FMan is over 10-fold higher than that for GDP-2dGlc, and GDP-4dMan is a much better inhibitor of Man-P-Dol formation than GDP-4FMan, which is non-inhibitory up to 200 μM [8]. Presumably the high electronegativity of the fluorine atom promotes stronger hydrogen bonding to the active site of the enzyme than that normally formed by the hydroxyl group. As a result release of the products may be slowed down or bonds may be less susceptible to cleavage.

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