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INTERACTION OF URIDINE DIPHOSPHATE GLUCOSE WITH CALF LIVER URIDINE DIPHOSPHATE GLUCOSE DEHYDROGENASE

SIGNIFICANCE OF HYDROXYL GROUPS AT C-3, C-4 AND C-6 OF HEXOSYL RESIDUE

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Summary

Analogs of uridine diphosphate glucose (UDPGlc) with a modified hexosyl residue which contained a deoxy-unit at C-3 or C-4 were tested as substrates of calf liver UDPGlc dehydrogenase (EC 1.1.1.22). The 3-deoxyglucose derivative was found not to serve as a substrate for the enzyme whereas the 4-deoxyglucose analog was able to participate in the reaction. The apparent K_m of the latter was 5.3 times that of UDPGlc and the relative V was 0.04. The reaction product was identified as uridine diphosphate deoxyhexuronic acid. UDP-deoxyhexoses were non-competitive inhibitors of UDPGlc enzymic oxidation, inhibition increased in the sequence: 2-deoxy- < 3- and 6-deoxy- < 4-deoxyglucose derivative. The significance of different HO-groups in hexosyl residue for interaction of UDPGlc with the enzyme is discussed.

Introduction

Uridine diphosphate glucose (UDPGlcUA) dehydrogenase (EC 1.1.1.22) catalyses oxidation at C-6″ of the sugar nucleotide with formation of uridine diphosphate glucuronic acid (UDPGlc) in accordance with the equation:

$$UDPGlc + 2\ NAD^+ + H_2O \rightarrow UDPGlcUA + 2\ NADH + 2\ H^+$$

The results of a previous study on the specificity of calf liver UDPGlc dehydrogenase with synthetic UDPGlc analogs gave rise to the conclusion that the NH-group of the uracil nucleus was essential for enzyme-substrate interaction [1] and HO-2″ (hydroxyl group at C-2 of the hexosyl residue) may be

substituted with a hydrogen atom without loss of the substrate properties of the analog [2]. A substrate of UDPGlc dehydrogenase must definitely possess HO-6″, i.e. the hydroxyl group at the reaction center, but it is not clear whether HO-3″ and HO-4″ are also necessary.

UDPgalactose, the epimer UDPGlc at C-4″, was not a substrate for calf liver UDPGlc dehydrogenase [3]. The same is true for UDPmannose [4], but UDP2dGlc* was able to participate in the reaction [2]. This result shows that study of UDPGlc analogs which contain residues of deoxysugars is more conclusive than that of UDPGlc epimers.

The present paper describes the ability of UDP3dGlc and UDP4dGlc to serve as substrates for calf liver UDPGlc dehydrogenase and a comparison of their properties as reaction inhibitors with those of UDP2dGlc and UDP6dGlc. Some of these results were reported in preliminary notes [5,6].

Materials and Methods

Nucleotides

UDPGlc from Merck and NAD^+ from Serva were used throughout the work. Preparation and characterization of UDP2dGlc was described by Druzhinina et al. [2], of UDP3dGlc by Shibaev et al. [7], of UDP4dGlc by Kochetkov et al. [8] and of UDP6dGlc by Kochetkov et al. [9]. Chromatographically pure sugar nucleotide preparations were used.

UDPGlc dehydrogenase

The enzyme was purified from calf liver. The first five stages of purification were as described [3,10]. The resulting preparation was subjected to desalting on a Bio-Gel P-2 column and chromatography on a CM-cellulose column (see Fig. 1). Fractions 21—31 were combined, protein was precipitated with ammonium sulfates (60% of saturation). The precipitate was dissolved in 1 mM sodium acetate pH 5.5 (10 ml). Specific activity of the preparation was 0.16 units per mg of protein (expressed as μmol of UDPGlc oxidized per min, assayed in 1.67 mM glycine pH 8.7).

Paper chromatography and electrophoresis

Paper chromatography was performed on Filtrak FN-15 paper with solvent system *n*-butyl alcohol/pyridine/water (6 : 4 : 3). Electrophoresis was performed at 30 V/cm in 0.2 M triethylammonium bicarbonate pH 7.5. Nucleotides were visualized under ultraviolet light, uronic acid derivatives were detected after spraying with aniline/xylose reagent [11].

Interaction of UDP-deoxyhexoses with UDPGlc dehydrogenase and treatment of experimental data

Incubation mixtures contained 180 mM glycine pH 8.7, 1 mM NAD^+ and the enzyme (0.03 units/ml), unless otherwise stated. These components and sugar nucleotides were mixed in a spectrophotometric cell at 25° C and change

* UDP2dGlc, UDP3dGlc, UDP4dGlc and UDP6dGlc are UDPGlc analog with deoxy-unit at C-2, C-3, C-4 or C-6 of the hexosyl residue, respectively.

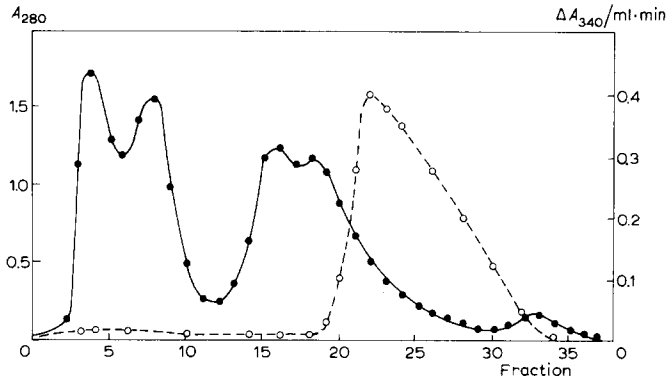

Fig. 1. Chromatography of calf liver UDPGlc dehydrogenase preparation on a column (3 × 9 cm) of CM-cellulose. The column was equilibrated with 0.01 M sodium acetate pH 5.5—0.001 M EDTA. The enzyme preparation (100 mg of protein, 5 mg/ml) was applied at a rate of 1 ml/min. The column was eluted with a linear gradient of NaCl concentration between the equilibrating buffer (500 ml) and 0.4 M NaCl in the buffer (500 ml). Fractions (20 ml) were assayed for A_{280} (———) and UDPGlc dehydrogenase activity (- - - - -).

of absorbance at 340 nm was measured with Unicam SP 8000 spectrophotometer. In inhibition experiments the rate of oxidation of UDP2dGlc or UDP4dGlc was negligible in comparison with that of UDPGlc under assay conditions.

The Michaelis constant and maximal velocity values were determined by treatment of data according to the procedure of Wilkinson [12] with the use of BESM-6 computer. Values of K_i (slope) and K_i (intercept) were calculated as ratio intercept/slope of secondary plots which show dependence of K_m/V or I/V on inhibitor concentration.

Results

UDP4dGlc as a substrate for the reaction

In a preliminary experiment incubation mixture (total volume of 3.6 ml) contained 0.44 mM UDPGlc, 1.67 mM NAD^+, 180 mM glycine pH 8.7 and UDPGlc dehydrogenase (0.03 units/ml). After 20 min incubation an increase of A_{340} was found to be 0.09*. An additional portion of the enzyme (0.08 units) was added and incubation was continued for 24 h. Under these conditions an increase of A_{340} was equal to 7.45, this value corresponded to the formation of 0.62 μmol of UDPhexuronic acid.

The incubation mixture was applied to a column (2 × 90 cm) of Sephadex G-10, the column was washed with water and nucleotide-containing fractions of eluate were concentrated and subjected to preparative paper electrophoresis. The zone with electrophoretic mobility close to that of UDPGlcA was eluted. The product was homogeneous by paper electrophoresis (Table I).

The product was treated with dilute acid (0.1 M HCl, 15 min, 100°C), and the hydrolysate was analyzed with paper chromatography. UDPGlcA, subjected

* In the first experiments described in the preliminary note [6] the reaction was not detected due to lower sensitivity of the assay procedure.

TABLE I

PAPER CHROMATOGRAPHY AND ELECTROPHORESIS OF UDP-HEXURONIC ACIDS AND THEIR HYDROLYSIS PRODUCTS

Substrate	UDPhexuronic acids R_{UMP} in paper electrophoresis*	Hexuronic acids and their lactones R_{Glc} in paper chromatography
UDPGlc	1.13	0.35; 1.83
UDP4dGlc	1.10	0.87; 2.20

* For UDPhexoses R_{UMP} = 0.85—0.90.

to the same treatment produced glucuronic acid and glucuronolactone as only spots visible after spraying of the chromatogram with aniline/xylose reagent. Two spots with higher chromatographic mobilities, presumably 4-deoxyglucuronic acid and its lactone were detected in the UDP4dGlc hydrolysate (Table I).

Dependence of the enzymic reaction rate on the concentration of UDP4dGlc is shown in Table II in comparison with similar data for UDPGlc. It may be seen that K_m for UDP4dGlc is 5.3 times higher than that for UDPGlc and maximum velocity corresponds only to 4% of that for the natural substrate.

Study of UDP3dGlc as a substrate of the reaction

UDP3dGlc (1 mM) was incubated with NAD^+ (1 mM) and UDPGlc dehydrogenase (0.03 units/ml) in 83 mM glycine, pH 8.7. No significant change was detected in absorbance at 340 nm after incubation for 1 h. This result shows the inefficiency of UDP3dGlc as a substrate for the enzyme. After incubation for 5 h a very small increase in A_{340} (0.040) was measured. Identification of the reaction products was not attempted due to their negligible yields.

TABLE II

DEPENDENCE OF REACTION RATE ON SUBSTRATES CONCENTRATION

The incubation mixture (a total volume of 1.6 ml) was 94 mM in glycine pH 8.7, 1.85 mM in NAD^+ and contained the enzyme (0.08 units/ml) and sugar nucleotide in the concentrations shown.

Sugar nucleotide concn (mM)	$10^3 \times v$ (ΔA_{340} per min)		Parameters
	Found	Calculated*	
1. UDP4dGlc			
0.188	1.1	1.1 ± 0.1	
0.375	1.5	1.5 ± 0.1	
0.468	1.7	1.6 ± 0.1	K_m (mM) = 0.169 ± 0.031
0.750	1.7	1.8 ± 0.1	
1.310	2.0	1.9 ± 0.1	$10^3 \times V$ = 2.17 ± 0.09 (ΔA_{340}/min)
1.580	1.9	2.0 ± 0.1	
2. UDPGlc			
0.016	16	17 ± 2	
0.035	28	26 ± 3	K_m (mM) = 0.032 ± 0.005
0.058	33	32 ± 3	$10^3 \times V$ = 49.8 ± 3.0 (ΔA_{340}/min)
0.087	36	36 ± 3	
0.116	38	39 ± 3	

* Calculated from the parameters obtained and substrate concentration.

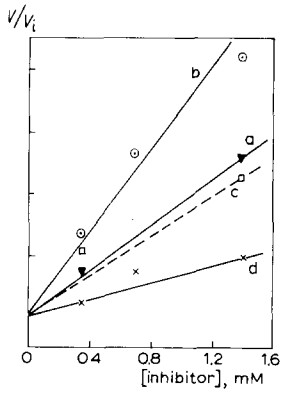

Fig. 2. Inhibition of UDPGlc oxidation with UDPdeoxyhexoses. For experimental details see text. Concentration of UDPGlc was 0.16 mM. a, UDP6dGlc; b, UDP4dGlc; UDP3dGlc; d, UDP2dGlc.

UDPdeoxyhexoses as inhibitors of UDPGlc oxidation

Fig. 2 shows results of preliminary experiments on evaluation of UDPdeoxyhexoses as inhibitors of the UDPGlc dehydrogenase reaction. In these experiments, assays were performed with a standard concentration of UDPGlc and three different concentrations of the analogs. It is possible to conclude that UDP4dGlc is the most effective inhibitor of the reaction, inhibition in the presence of UDP2dGlc is very weak, whereas UDP6dGlc and UDP3dGlc are in an intermediate position.

The inhibitory properties of UDP6dGlc, UDP4dGlc and UDP3dGlc were studied more thoroughly. Dependence of initial rate of the reaction on concentration of UDPGlc was studied in presence of the inhibitors. Fig. 3 shows "secondary plots" obtained in these experiments, i.e. dependence of apparent K_m/V and I/V on inhibitor concentration. In all cases investigated inhibition is non-competitive (see Cleland, and cf. ref. 13). Values of K_i (slope) and K_i (intercept) are listed in Table III. These results show clearly that UDP4dGlc is the best reaction inhibitor among the UDPdeoxyhexoses studied.

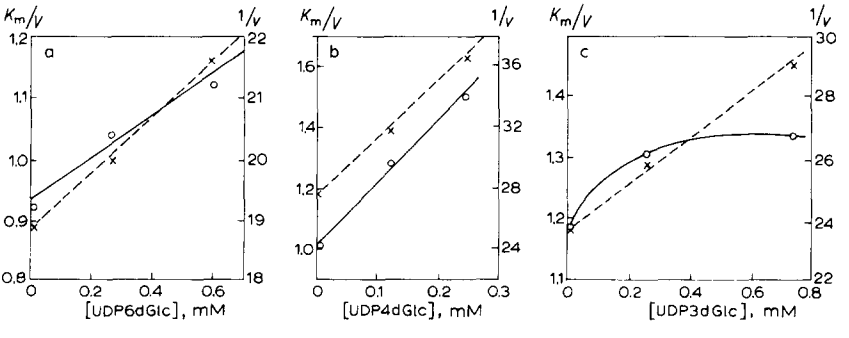

Fig. 3. Secondary plots for inhibition of UDPGlc oxidation with UDP6dGlc (a), UDP4dGlc (b) and UDP3dGlc (c). K_m/V (———) and I/V (- - - - -) dependence on inhibitor concentration. For reaction conditions see the text. Each pair of parameter values was determined in an experiment with at least 8 UDPGlc concentrations between 0.02 mM and 0.35 mM.

TABLE III

INHIBITION OF UDPGlc OXIDATION WITH UDP-DEOXYHEXOSES

Inhibitor	K_i (slope) (mM)	K_i (intercept) (mM)
UDP6dGlc	2.7	4.2
UDP4dGlc	0.51	0.77
UDP3dGlc	2.4*	3.3

* From initial portion of curved secondary plot (Fig. 3c).

Discussion

The results presented demonstrate the different significance of HO-3″ and HO-4″ for the interaction of UDPGlc with calf liver UDPGlc dehydrogenase. The HO-3″ group seems to be significant for the proper enzyme-substrate interaction, as may be concluded from the fact that UDP3dGlc is inefficient as a substrate and a rather weak inhibitor of the dehydrogenase reaction. On the contrary, UDP4dGlc can readily participate in the reaction, consequently the HO-4″ group may be substituted with hydrogen atom without loss of substrate properties for the analog.

UDP3dGlc, UDP6Glc and UDP4dGlc were found to be non-competitive inhibitors in respect to UDPGlc with K_i (slope) not equal to K_i(intercept). Substrate properties of the 4-deoxyglucose derivative suggest that it is bound with the same site of the enzyme as UDPGlc. Competitive inhibition of UDPGlc oxidation in the reaction may be expected. The observed non-competitive inhibition is probably a consequence of multi-subunit structure of the enzyme [14,15]. Equilibrium dialysis experiments showed the presence of three binding sites for UDPGlc with strong negative cooperativity in hexameric UDPGlc dehydrogenase molecule [16]. It is possible that binding of UDPdeoxyhexose on one of the sites may influence the rate of oxidation of UDPGlc which is bound on the other site of the enzyme hexamer. Such effect should lead to apparent non-competitive inhibition of UDPGlc oxidation with UDPdeoxyhexoses.

The HO-6″ group of UDPGlc is significant for UDPGlc dehydrogenase not

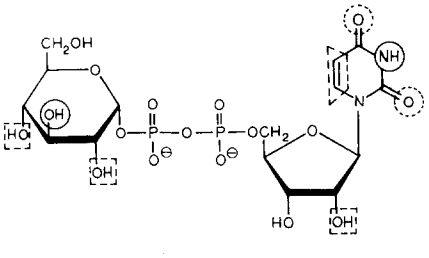

Fig. 4. Significance of different structural elements of UDPGlc for its interaction with calf liver UDPGlc dehydrogenase. The solid line encloses groups which are essential for the enzyme-substrate interaction and cannot be changed without loss of substrate properties of the sugar nucleotide. Structural modification without loss of substrate properties are possible in the areas enclosed in dashed line. For oxygen atoms enclosed in the dotted line only substitution with sulfur atoms is still investigated and shown to be permissive.

only as the reaction center but probably as one of the centers responsible for the enzyme-substrate complex formation. This conclusion is based on the rather weak inhibition efficiency of UDP6dGlc. At the same time UDPxylose which differs from UDPGlc and UDP6dGlc only by a substituent at C-5'' was found to be a very effective inhibitor of calf liver UDPGlc dehydrogenase reaction [17]. Our results suggest this inhibition is dependent on the binding of UDPxylose on a specific allosteric site of the enzyme, which is unable to interact with UDP6dGlc.

Fig. 4 summarizes the results of this and our previous studies [1,2,18] on the significance of UDPGlc functional groups for its specific recognition with calf liver UDPGlc dehydrogenase. Two sites of the sugar nucleotide molecule may be identified as essential, "substrate-specifying" groups. These are HO-3'' and the NH-group of the heterocyclic base. Modification of UDPGlc at these points leads to analogs which are unable to serve as substrates for the enzyme, whereas other sites of the sugar nucleotide molecule may be modified without loss of substrate properties.

Significance of the HO-3'' and NH-groups for recognition of UDPGlc with enzyme was demonstrated in our earlier studies for four other enzymes specific for UDPGlc [1,19,20]. Scarce data reported from other laboratories are also in accordance with this conclusion (for review see ref. 21). The ability of UDP3dGlc to serve as a substrate instead of UDPGlc was noted for yeast glycogen synthetase [22], but even in this case the 3-deoxyderivative is the worst substrate among UDPdeoxyhexoses, i.e. the HO-3'' group seems the most significant of hydroxyl groups in hexosyl residue of UDPGlc. Similarity in requirements to substrate structure for different enzymes suggests a similar mechanism of UDPGlc recognition with these enzymes.

Binding of the nucleotide and hexosyl moieties of UDPGlc takes place probably on different sub-sites of the substrate-binding site of an enzyme and the HO-3'' and NH-groups may be essential for proper binding of the corresponding substrate portions. It seems probable that precise formation of the hexosyl-binding subsite and or the catalytic center of the enzyme is dependent on the interaction of heterocyclic base of UDPGlc with the nucleotide-binding sub-site, which may produce some conformational change of the enzyme.

It is interesting that the ability of calf liver UDPGlc dehydrogenase to discriminate the glucose nucleotide from the mannose and galactose derivatives seems to depend mainly not on specfic interaction of the HO-2'' and HO-4'' groups with some groups of the enzyme but on blockage of proper enzyme-substrate interactions due to axial substituents at C-2'' or C-4'' in UDPGlc epimers. Similar specificity to substrates epimeric at C-2'' was noted for UDPGlc epimerases [2]. Such a principle of recognition of epimeric substrates may operate for other enzymes specific for carbohydrate derivatives.

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