Biochimica et Biophysica Acta, 403 (1975) 9—16

BBA 67574

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

INFLUENCE OF SUBSTITUENTS AT C-5 OF PYRIMIDINE NUCLEUS

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(Received March 11th, 1975)

Summary

The interaction of α-D-glucopyranosyl pyrophosphates of 5-X-uridines (X = CH_3, NH_2, CH_3O, I, Br, Cl, OH) with uridine diphosphate glucose (UDPGlc) dehydrogenase (EC 1.1.1.22) from calf liver has been studied. All the derivatives investigated were able to serve as substrates for the enzyme. The apparent Michaelis constants for UDPGlc-analogs were dependent both on electronic and steric factors. Increase of substituent negative inductive effect lead to decrease of pK_a for ionization of the NH-group in the uracil nucleus and, consequently, to a diminishing of the proportion of the active analog species under the conditions of assay. After correction for the ionization effect, the K_m values were found to depend on the van der Waals radius of the substituent. The value of 1.95 Å seems to be critical, as the analogs with bulkier substituents at C-5 showed a decreased affinity to the enzyme. The maximal velocity values of the analogs were also dependent on nature of the substituent. Good linear correlation between log V and substituent hydrophobic π-constant was observed for a number of the analogs, although V values for the nucleotides with X = H, OH or NH_2 were higher than would be expected on the basis of the correlation. The significance of the results for understanding of the topography of UDPGlc dehydrogenase active site is discussed.

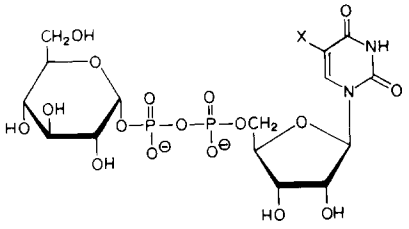
Introduction

Uridine diphosphate glucose (UDPGlc) dehydrogenase (EC 1.1.1.22) from calf liver catalyses oxidation at C-6″ of the sugar nucleotide resulting in formation of UDP-glucuronic acid [1]:

$$UDPGlc + 2NAD^+ + H_2O \rightarrow UDPglucuronic\ acid + 2NADH + 2H^+$$

Among the different structural elements of UDPGlc, the most important substrate-specifying groups for interaction with UDPGlc dehydrogenase were found to be the NH-group of the heterocyclic nucleus and HO-3'' in the hexosyl residue (ref. 1 and references cited therein). Modification of the UDPGlc molecule at other sites lead to the analogs which were able to serve as substrates of UDPGlc dehydrogenase. Particularly, the derivatives of 5-fluorouridine [2], 5-methyluridine [3] and 5-hydroxyuridine [4] were substrates of the reaction, although their efficiency was different.

The aim of this investigation was an attempt to find factors which determine the change of substrate efficiency of UDPGlc analogs modified at C-5 of the uracil nucleus. For this purpose UDPGlc analogs 2—8 with different substituents at this site were assayed as substrates of UDPGlc dehydrogenase under standard conditions.



X = H(1), CH_3 (2), NH_2 (3), CH_3O (4), I(5), Br(6), Cl(7), OH(8).

Materials and Methods

Materials

UDPGlc from Merck and NAD^+ from Reanal were used throughout this work. UDPGlc analogs were prepared from UMP analogs and characterized as described by Kochetkov et al. [5] and Shibaev et al. [6]. The preparations were homogeneous by paper chromatography and electrophoresis. No contamination with UDPGlc as well as from modified uridine 5'-phosphates with UMP was noted. UDPGlc dehydrogenase was purified from calf liver as described by Druzhinina et al. [1].

Assay of UDPGlc analogs as substrates of UDPGlc dehydrogenase

Incubation mixtures contained (in a total volume of 3.0 ml) 30 mM glycine pH 8.75 and 1 mM NAD^+ in addition to the enzyme and the sugar nucleotide. Cells were thermostated at 25°C. The reaction was followed by change in A_{340}, which was registered with Unicam SP 8000 spectrophotometer. Results of experiments were treated for K_m and V determination according to procedure of Wilkinson [7] with the use of a BESM-6 computer. Concentration of the enzyme in the incubation mixture was checked periodically by determination of V for UDPGlc.

Determination of pK_a values

Measurement of pK_a values was performed spectrophotometrically with the following buffer solutions: 0.1 M glycine/NaOH between pH 8.6 and 10.0, 0.1 M Tris · HCl between pH 7.5 and 8.6 and 0.1 M glycine/HCl between pH 2.8 and 3.6.

Results and Discussion

Influence of substituents on pK_a in 5-X-uridine derivatives

Uridine derivatives and their analogues are capable of ionizing in weakly alkaline media as shown:

Only form (a) of UDPGlc was found to participate in interaction with UDPGlc dehydrogenase [2]. In this investigation the reaction was assayed near its pH optimum at pH 8.75. Under these conditions the proportion of species (a) is strongly dependent on the pK_a value of the derivative and information on these values is essential for analysis of the results.

Unfortunately, reported data on pK_a values of 5-substituted uridine derivatives [8] were not comprehensive enough and not all values were directly comparable due to different conditions of determination. For this reason their measurement was performed under standard conditions which were close to those of the enzymic reaction. The results are presented in Table I.

Values of pK_a for UMP and UDPGlc were found to be indistinguishable within experimental error and further experiments were performed with more accessible UMP derivatives. In most cases spectral shifts between pH 2.0 and 11.0 corresponded to the dissociation of only one proton.

The only exception found was 5-aminouridine 5'-phosphate where two dissociation constants (pK_a 3.37 and 9.62) were determined. The second disso-

TABLE I

IONIZATION CONSTANTS FOR UMP ANALOGS AND UDPGlc

Compound	Useful pH-range	N*	pK_a**	z***
UMP	9.05—10.00	12	9.71 ± 0.02	0.91
UDPGlc	9.05—10.00	5	9.72 ± 0.02	0.91
5-Methyl-UMP	9.60—10.60	11	10.11 ± 0.03	0.96
5-Amino-UMP	2.80— 3.60	4	3.37 ± 0.02	†
	9.40—10.00	4	9.62 ± 0.02	0.88
5-Methoxy-UMP	8.60— 9.20	5	8.96 ± 0.02	0.62
5-Iodo-UMP	8.32— 8.62	6	8.45 ± 0.04	0.39
5-Bromo-UMP	7.77— 8.62	4	8.14 ± 0.02	0.20
5-Chloro-UMP	7.77— 8.62	5	8.12 ± 0.03	0.18
5-Hydroxy-UMP	7.54— 8.42	5	8.33 ± 0.16	††

* A number of experimental points obtained in useful pH-range.

** 95% confidence limits are shown.

*** Proportion of species (a) at pH 8.75 calculated by the equation:

$z = 1/(1 + 10^{pH - pK_a})$

† pK_a for NH_2-group protonation (see the text).

†† pK_a for ionization of HO-group (see the text).

ciation is in the region characteristic for NH-group ionization whereas the first one is probably connected with exocyclic NH_2-group protonation.

The single dissociation in the titration curve of 5-hydroxyuridine 5'-phosphate (pK_a 8.3) is dependent on ionization of HO-, but not the NH-group. This conclusion is based on the appearance of longwavelength absorption maximum in the ultraviolet spectrum of ionized species. In the case of 5-hydroxyuridine such a maximum was shown to be characteristic for HO-group ionization [9].

In all other cases pK_a values obtained are connected with transitions between species (a) and (b), i.e. with ionization of the NH-group in the heterocyclic nucleus. As may be expected, the presence of electron-withdrawing substituents at C-5 resulted in a decrease of pK_a values and diminishing proportion of species (a) of the analog under condition of assay of the enzymic reaction. Linear correlation between pK_a values and induction substituent constants σ_I has been observed (Fig. 1):

$$pK_a = 9.90 - 3.45\,\sigma_I \quad (N = 7,\ r = -0.989)$$

This result suggests that a C-5 substituent influences the pK_a value of the NH-group in a heterocyclic nucleus mainly through its inductive effect.

UDPGlc analogs as substrates for UDPGlc dehydrogenase

Substrate properties of derivatives 2 and 8 were reported previously [3,4]. Our experiments have shown that analogs 3, 4, 5, 6, and 7 also participated in the enzymic reaction.

Table II shows results of experiments on determination of K_m and V values for UDPGlc analogs 2—7. For further analysis we have used values of K_m^r and V^r which represent ratio of the corresponding parameter values for an analog and UDPGlc measured in parallel experiments.

Substituents at C-5 of the uracil nucleus may be arranged in the following sequence on the basis of their influence on the K_m^r value for the analog:

$$H < CH_3 < CH_3O \approx HO < NH_2 < Cl < Br < I.$$

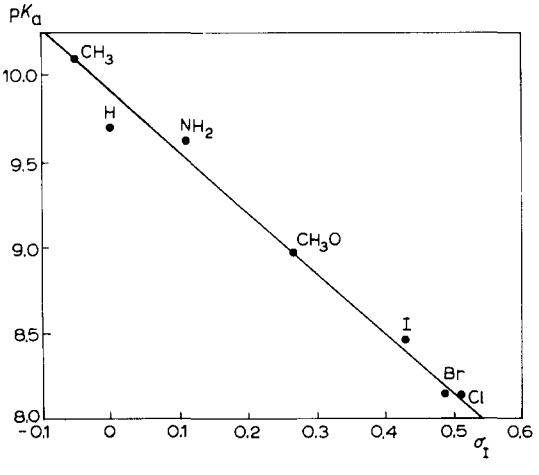


Fig. 1. Correlation of pK_a with σ_I substituent constants for 5-substituted uridine 5'-phosphates. The constants are as cited by Hammett [10].

TABLE II

INTERACTION OF 5-SUBSTITUTED UDPGlc ANALOGS WITH CALF LIVER UDPGlc DEHYDROGENASE

Compound	C-5 substituent	Concentration range (mM)	N^*	K_m, mM	$V \times 10^3$ (ΔA_{340}/min)	K_m^{r**}	$K_m^r(a)^{***}$	V^{r**}
1	H	0.013—0.132	11	0.039 ± 0.003	58.1 ± 1.8	1.0	1.0	1.0
2	CH_3	0.027—0.190	12	0.104 ± 0.013	23.6 ± 1.5	2.7	2.8	0.37
3	NH_2	0.094—0.56	5	0.220 ± 0.009	69.2 ± 1.3	4.4	4.2	0.77
4	CH_3O	0.042—0.423	12	0.155 ± 0.009	17.9 ± 0.5	4.0	2.7	0.28
5	I	0.046—0.446	10	1.29 ± 0.15	47.1 ± 4.4	33.1	14.2	0.81
6	Br	0.036—0.249	9	0.274 ± 0.006	37.9 ± 0.5	7.0	1.5	0.65
7	Cl	0.057—0.402	14	0.253 ± 0.018	27.7 ± 1.0	6.5	1.3	0.50
8	OH	0.008—0.043	8	0.157 ± 0.028	17.8 ± 0.2	4.0	—	0.32

* A number of experimental points in an experiment on K_m and V determination.

** Relative kinetic parameters, ratio of the parameters for an analog and UDPGlc under identical conditions. Different enzyme concentrations were used in experiments with some analogs, V^r values were calculated with V values for UDPGlc with the same enzyme concentration.

*** Relative K_m in terms of species (a) concentration for the sugar nucleotide (see the text) $K_m^r(a) = K_m^r \times z/z_0$ where z and z_0 are proportions of the species (a) at pH 8.75 for the analog and UDPGlc (see Table I).

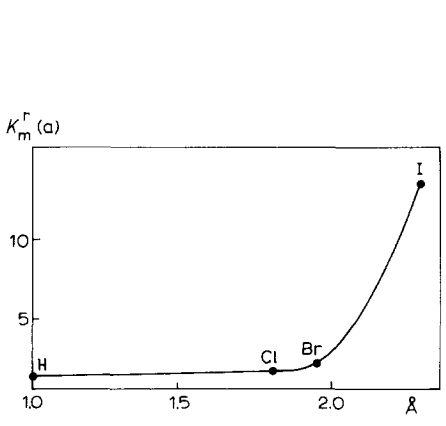
As discussed above, change in K_m values with change of C-5 substituent may depend on a different proportion of the protonated species (a) under conditions of assay for different UDPGlc analogs. Correction for this effect may be readily introduced in all cases except the case of the 5-hydroxyuridine derivative. For this purpose K_m values were calculated in terms of concentration of the form (a) for all UDPGlc analogs, the corresponding relative values K_m^r (a) are included in Table II. It may be seen that these values were not identical and following the series of C-5 substituent influence on K_m^r (a) the following values may be written:

$$H < Cl < Br < CH_3O \approx CH_3 < NH_2 < I.$$

This sequence suggests significant influence of steric factors on interaction of UDPGlc analogs with the enzyme. In Fig. 2 the values of K_m^r (a) are plotted against van der Waals radii for substituent atoms. The value of 1.95 Å for van der Waals radius of C-5 substituent seems to be critical for proper analog-enzyme interaction as a sharp increase in K_m^r (a) value was observed when the bromine atom at C-5 was substituted with iodine atom.

The 5-methyluridine derivative 2 shows an intermediate value of K_m^r (a) in comparison with analogs 5 and 6. This result implies that steric limitation of the enzyme-substrate interaction is not connected with substituent size along the C_5-X axis. Inspection of molecular models clearly shows that in this direction van der Waals radius of the methyl group is less than that for chlorine atom. At the same time van der Waals radius of the CH_3-group in the direction perpendicular to the group axis lies between the values characteristic for bromine and iodine atoms. It is probable that steric interactions of the substituent with enzyme group(s) situated in this direction are responsible for lowering of the substrate analog affinity to the enzyme. This hypothesis is in accordance with close K_m^r (a) values for the 5-methyluridine and 5-methoxyuridine derivatives.

Some other factors may influence the interaction of 5-substituted UDPGlc



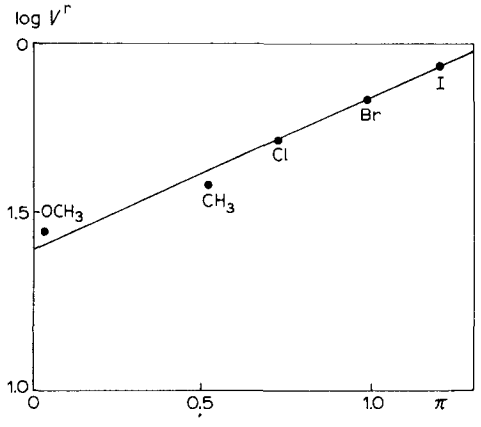


Fig. 2. Dependence of K_m^r(a) on van der Waals radii of C-5 substituents for interaction of UDPGlc analogs with UDPGlc dehydrogenase. The values of the atomic radii were taken from ref. 11. Only substituents with spherical symmetry are included. For a discussion of the situation with other substituents see the text.

Fig. 3. Correlation of V^r with hydrophobic π substituent constants for interaction of UDPGlc analoge with UDPGlc dehydrogenase. The constants used were taken from the book of Alberty [12]. The correlation shown was obtained with a set of constants for phenoxyacetic acids which was recommended for use in systems with weak interaction of substituents. With set of constants characteristic for phenols similar correlation was observed: log $V^r = 0.315\ \pi - 0.564$ ($r = 0.986$). Using a set of "general" aromatic π-constants reported more recently [13] produced slightly worse, although a significant correlation: log $V^r = 0.410\ \pi - 0.573$ ($r = 0.959$).

analogs with UDPGlc dehydrogenase. Particularly, K_m^r (a) value for the 5-aminouridine analog is lower than may be expected from NH_2-group dimensions.

Table II shows that the nature of C-5 substituent also influences the maximal velocity of the reaction. The following sequence of the substituent effects was observed:

$$H > I \approx NH_2 > Br > Cl > CH_3 > HO > CH_3O.$$

The series for V^r change is different from that for K_m^r change. We were unable to find straightforward explanation for such type of substituents influence. It is of interest that for UDPGlc analogs with hydrophobic substituents at C-5 (compounds 2, 4—7) V^r values increased with increase in substituent hydrophobicity and significant correlation between log V^r and hydrophobic π-constant was observed (Fig. 3):

$$\log V^r = 0.402\ \pi - 0.587\ (N = 5,\ r = 0.986)$$

UDPGlc and its analogs with hydrophilic substituents (3 and 8) showed higher V^r values than expected from the correlation observed.

Possible mechanisms of substituents influence

The mechanism of the enzyme-substrate interaction for UDPGlc dehydrogenase discussed in the previous paper [1] suggested binding of nucleoside and hexoxyl portions of the substrate on different subsites of the enzyme with the uracil nucleus acting as "autosteric effector" [14] for the formation step of hexosyl-binding subsite and/or catalytic site of the enzyme. The NH-group of

the heterocyclic nucleus was found to be most important for this interaction.

The results of this investigation are in accordance with such a mechanism. Moreover, these results show that presence of a C-5 substituent with small van der Waals radius does not influence significantly the enzyme-substrate interaction and an apparent change of K_m value is connected with change in pK_a of the substrate due to the inductive effect of C-5 substituent. With increase in size of the substituent its disruptive effect on the normal pattern of interaction becomes evident, steric limitations in a direction perpendicular to the C_5-X axis seem to be more significant.

The presented results show the measurable effect of the C-5 substituent on maximal velocity of the reaction which occur at C-6'' of the substrate. This effect is connected presumably with distortion of proper orientation of catalytic groups of the enzyme in the enzyme-substrate analogs complexes. Three kinds of substituent that influence the mechanism are probably operating here. Firstly, UDPGlc is the best substrate of the enzyme, and consequently the presence of the hydrogen atom at C-5 results in the best geometry of interaction which is partially distorted with bulkier substituent at C-5. Secondly, the increase in the hydrophobicity of the substituent produces the reverse effect, i.e. improving of the induced fit probably as a result of the substituent interaction with a non-polar side chain group of the enzyme. Finally some kind of interaction of protic, hydrophilic substituents at C-5 with the enzyme groups also probably exists, but its mechanism remains unclear.

The results of this investigation suggest that the active site of calf liver UDPGlc dehydrogenase contains amino acid residues located near C-5 of the substrate in the enzyme—UDPGlc complex which are capable of interacting with substituents in this position. UDPGlc analogs modified at C-5 may be considered as probes for the characterization of the topography of the enzyme active site in this region. Their use should be helpful in study of other enzymes which employ UDPGlc as a substrate. A similar approach may be of importance for probing active sites of other enzymes specific for different nucleotide anhydrides.

Acknowledgement

The authors are greatly indebted to Dr T.N. Druzhinina for consultation on the enzyme purification and to Dr M.M. Mazhul who performed several preliminary experiments.

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