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KINETIC CHARACTERIZATION OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM GERMINATED BARLEY (MALT)

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Key Word Index—*Hordeum vulgare*; Gramineae; UDP-glucose pyrophosphorylase; kinetics; nucleotide sugars; UDP-glucose; pyrophosphate.

Abstract—A complete data set of the kinetics and some features referring to the substrate specificity and inhibition of UDP-glucose pyrophosphorylase from germinated barley (malt) are given in order to evaluate the use of this easily available enzyme for the synthesis of nucleotide sugars in enzymic glycoconjugate synthesis. In the synthesis direction the K_m and V_{max} values were 74 μm and 228 U mg^{-1} for glucose-1-phosphate and 93 μM and 255 U mg^{-1} for UTP at pH 8.35. UTP gave a substrate surplus inhibition with a K_{iS} of 7.09 mM. For the pyrophosphorylysis reaction the kinetic constants K_m and V_{max} were 0.191 mM and 350 U mg^{-1} for UDP-glucose and 0.172 mM and 345 U mg^{-1} for inorganic pyrophosphate. Inhibition studies demonstrated that UDP-glucose is a competitive inhibitor (K_i 0.117 mM) for UTP and a non-competitive inhibitor (0.015 mM) for glucose-1-phosphate. In the pyrophosphorylysis reaction, UTP is a competitive inhibitor (0.169 mM) for UDP-glucose. Inorganic pyrophosphate (K_i 0.213 and 0.952 mM) and inorganic phosphate (K_i 12.2 and 10.9 mM) were identified as non-competitive inhibitors for glucose-1-phosphate and UTP in the synthesis reaction. The analysis of these inhibition studies revealed a sequential ordered Bi-Bi mechanism. The enzyme is inhibited by free UTP and the optimum ratio of Mg^{2+}/UTP for synthesis of UDP-glucose is between 5 and 10. The enzyme shows relative activities for CTP (18.5%), GTP (14.3%) and ATP (13.7%) when glucose-1-phosphate is the second substrate.

INTRODUCTION

UDP-glucose pyrophosphorylase (UDP-Glc PP, EC 2.7.7.9) plays a central role in the biosynthesis of primary and secondary activated sugars in plants, animals and microorganisms. The synthesis reaction of UDP-Glc PP provides UDP-α-D-glucose, which is the precursor for important secondary nucelotide sugars such as UDP-α-D-galactose, UDP-α-D-glucuronic acid, UDP-α-D-xylose and UDP-β-L-arabinose [1]. In the reverse reaction it catalyses, by definition, the reversible magnesium-dependent transfer of a uridylyl group from UDP-α-D-glucose to Mg^{2+}-PP_i with the formation of glucose-1-phosphate and Mg^{2+}-UTP [2]. In plants the formation of UDP-Glc by the sucrose synthase and the pyrophosphorylysis reaction of UDP-Glc PP is involved in sucrose degradation [3].

The aim of our work is the application of enzymes from plant sources in the enzymic synthesis of glycoconjugates since they are most often easily available from seeds or germinated seedlings. In this context we followed two approaches:

(i) In combination with mammalian Leloir glycosyltransferases we have utilized sucrose synthase from rice for the synthesis and the *in situ* regeneration of nucelotide sugars [4–7].

(ii) We have recently purified and characterized UDP-Glc PP from germinated barley seedlings (malt) [8]. Malt is an excellent starting material for enzyme isolation and is produced industrially on a larger scale. The enzyme is a 54 kD-monomer and shows a good pH and temperature stability when supplemented with 0.1 mg ml^{-1} bovine serum albumin. These data are already promising for UDP-Glc PP as a valuable catalyst in nucleotide sugar synthesis.

So far, UDP-Glc PP from yeast and bovine liver has been exploited for the synthesis and *in situ* regeneration of the very expensive nucelotide sugars in preparative carbohydrate synthesis [9–11]. However, a detailed characterization and kinetic coordination of these enzymes involved in such partly very complex systems has not yet been carried out and is expected to be very difficult. Enzyme data like stability and the kinetics, which are relevant for conditions of nucelotide sugar synthesis, are not always available. The intention for our work on UDP-Glc PP from a plant source is also based on the comparison of published kinetic data generally revealing that plant enzymes have a higher

substrate affinity and are less inhibited by substrates or products than the enzyme isolated from animal or microbial sources [3, 12–14].

The present paper summarizes the kinetic characterization, inhibition studies and the substrate specificity of UDP-Glc PP from malt, leading to further implications for the synthesis of UDP-α-D-glucose and derivatives thereof.

RESULTS

Synthesis reaction

Table 1 summarizes the kinetic data for the UDP-Glc synthesis reaction using assay A. The K_m and V_{max} values at pH 8.35 are 74 μM and 228 U mg^{-1} for glucose-1-P and 93 μM and 255 U mg^{-1} for UTP. UTP shows a substrate surplus inhibition with an inhibition constant K_{iS} of 7.09 mM. The product PP_i is a non-competitive inhibitor for glucose-1-P and UTP as analysed by Lineweaver–Burk plots. The inhibition constants are 0.213 and 0.952 mM, respectively. The calculation of the inhibition constants by secondary plots of K_m/V_{max} and $1/V_{max}$, respectively, versus the inhibitor concentration gave $K_i < K_{ii}$ (Tables 1 and 2) and confirms a non-competitive inhibition type [15, 16]. Figure 1 demonstrates that PP_i has a much more pronounced inhibitory effect on the enzyme activity and substrate affinity with glucose-1-P as varied substrate.

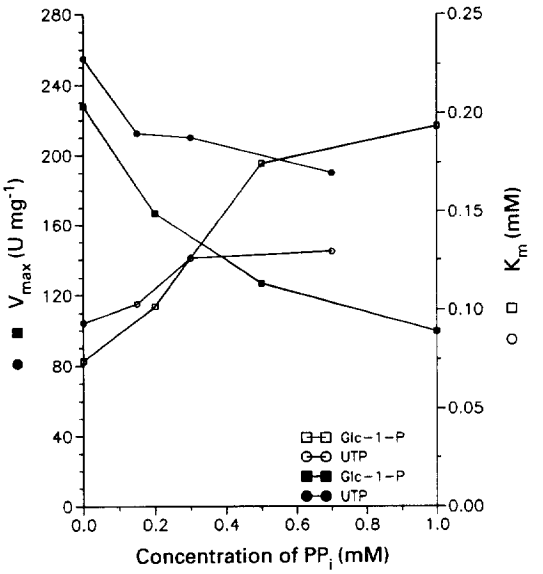

Fig. 1. Effect of different PP_i concentrations on V_{max} and K_m for UTP and glucose-1-phosphate in the synthesis reaction of UDP-Glc PP from germinated barley.

Important for further kinetic studies following product inhibition by UDP-Glc was the development of assay B, which monitors PP_i by a PP_i-dependent phosphofructokinase from potato in a coupled continuous assay [17]. Table 2 shows that UDP-Glc is a

Table 1. Kinetic data and inhibition studies (assay A) of UDP-Glc PP from germinated barley (malt) for the synthesis reaction: Glc-1-P + UTP⇄UDP-Glc + PP_i

Varied substrate (mM)	Constant substrate (mM)	V_{max} (U mg^{-1})	K_m (mM)	Inhibitor (mM)	K_i (mM)	Inhibition type
Glc-1-P (0.02–5.0)	UTP (2.0)	227.8	0.074	—	—	—
Glc-1-P (0.02–5.0)	UTP (2.0)	—	—	PP_i (0.2–1.0)	$K_i = 0.213$ $K_{ii} = 0.851$	Non-competitive
Glc-1-P (0.02–5.0)	UTP (2.0)	—	—	NaH_2PO_4 (2–10)	$K_i = 12.2$ $K_{ii} = 53.2$	Non-competitive
UTP (0.02–2.0)	Glc-1-P (5.0)	255.0	0.093	—	$K_{iS} = 7.09$	Substrate inhibition
UTP (0.02–2.0)	Glc-1-P (5.0)	—	—	PP_i (0.15–0.7)	$K_i = 0.952$ $K_{ii} = 2.492$	Non-competitive
UTP (0.02–2.0)	Glc-1-P (5.0)	—	—	NaH_2PO_4 (2–8)	$K_i = 10.9$ $K_{ii} = 29.8$	Non-competitive

Table 2. Kinetic data and inhibition studies (assay B) of UDP-Glc PP from germinated barley (malt) for the synthesis reaction: Glc-1-P + UTP⇄UDP-Glc + PP_i

Varied substrate (mM)	Constant substrate (mM)	V_{max} (U mg^{-1})	K_m (mM)	Inhibitor (mM)	K_i (mM)	Inhibition type
Glc-1-P (0.02–5.0)	UTP (2.0)	290.2	0.243	—	—	—
Glc-1-P (0.02–5.0)	UTP (2.0)	—	—	UDP-Glc (0.025–0.1)	$K_i = 0.015$ $K_{ii} = 0.041$	Non-competitive
UTP (0.02–2.0)	Glc-1-P (5.0)	248.2	0.317	—	—	—
UTP (0.02–2.0)	Glc-1-P (5.0)	—	—	UDP-Glc (0.025–0.1)	$K_i = 0.117$	Competitive

Table 3. Kinetic data and inhibition studies of UDP-Glc PP from germinated barley (malt) for the pyrophosphorylysis reaction: UDP-Glc + PP_i⇌Glc-1-P + UTP

Varied substrate (mM)	Constant substrate (mM)	V_{max} (U mg^{-1})	K_m (mM)	Inhibitor (mM)	K_i (mM)	Inhibition type
UDP-Glc (0.01–1.0)	Na_2PP_i (2.0)	350.0	0.191	—	—	—
UDP-Glc (0.01–1.0)	Na_2PP_i (2.0)	—	—	UTP (0.1–1.0)	K_i = 0.169	Competitive product inhibition
Na_2PP_i (0.02–4.0)	UDP-Glc (0.8)	345.0	0.172	—	—	—

competitive inhibitor for UTP (K_i 0.117 mM) and a non-competitive inhibitor for glucose-1-P (K_i 0.015 mM). However, assay B (Table 2) gave about three-fold higher K_m values for UTP and glucose-1-P when compared with assay A (Table 1). A substrate surplus inhibition by UTP was not detected with assay B. This difference is due to different pH conditions used in assay A (pH 8.35) and assay B (pH 7.4). Although the pH optimum of UDP-Glc PP from malt is between pH 6.5 and 7.5 [8], pH 8.35 was chosen for assay A due to optimized conditions for the auxiliary enzyme UDP-glucose dehydrogenase [18]. At pH 8.35 UDP-Glc PP from malt has 90% residual activity [8].

With respect to the synthesis of nucelotide sugars it is also important to note that inorganic phosphate is a non-competitive inhibitor for glucose-1-P and UTP, but with much higher inhibition constants than PP_i (Table 1). Inorganic phosphate is formed by the combination of pyrophosphorylases with pyrophosphatase in preparative synthesis of nucleotide sugars in order to drive the equilibrium towards the activated sugar.

Pyrophosphorylysis

Table 3 summarizes the kinetic data for the pyrophosphorylysis reaction. The K_m and V_{max} values are 0.191 mM and 350 U mg^{-1} for UDP-Glc and 0.172 mM and 345 U mg^{-1} for PP_i. UTP shows a competitive product inhibition with UDP-Glc as variable substrate. The value for the inhibition constant was 0.169 mM as calculated from a secondary plot.

Enzyme reaction mechanism

The analysis of the bi-substrate reaction via product inhibition studies [16] reveals a sequential ordered Bi-Bi mechanism for UDP-glucose pyrophosphorylase from germinated barley (Table 4 and Fig. 2). In the synthesis direction the free enzyme binds first UTP and subsequently glucose-1-P to form a ternary enzyme–substrate complex. After product formation by the ternary enzyme complex PP_i is first released and subsequently UDP-Glc.

Table 4. Analysis of the bi-substrate synthesis reaction of UDP-Glc PP from germinated barley (malt) via product inhibition studies (Tables 1, 2 and 3). Synthesis reaction: UTP + Glc-1-P⇌PP_i + UDP-Glc

	Variable substrate	
Product inhibition by	UTP	Glc-1-P
PP_i	Non-competitive	Non-competitive
UDP-Glc	Competitive	Non-competitive

Substrate specificity

Screening the activity of UDP-Glc PP with different sugar-1-phosphates and UTP with assay B reveals a high specificity of the enzyme for glucose-1-P. Gal-1-P, $GalNH_2$-1-P, $GlcNH_2$-1-P, GlcNAc-1-P and GalNAc-1-P gave no initial reaction rates with UTP. However, with glucose-1-P and different nucleoside triphosphates the following relative activities were obtained: UTP (100%), CTP (18.5%), GTP (14.3%), ATP (13.7%), ITP (0%) and dTTP (0%).

Effect of different metal ions

Replacing Mg^{2+} by other divalent cations gave relative activities of 31.8% for Ca^{2+}, 11.2% for Mn^{2+}

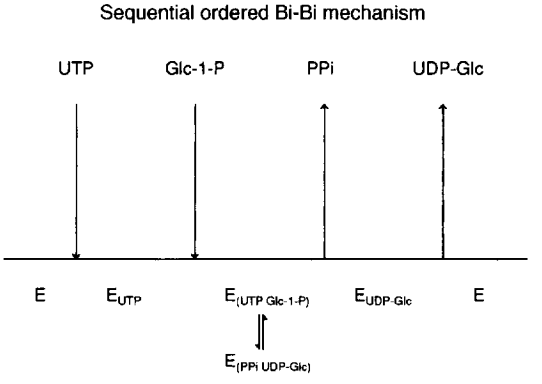

Fig. 2. Reaction mechanism of UDP-glucose pyrophosphorylase from barley malt.

and 3.1% for Ni^{2+} in the synthesis reaction. UDP-Glc PP from malt showed no activity with Cu^{2+} or Zn^{2+}.

Effect of Mg^{2+} and free UTP concentrations on UDP-Glc synthesis

Figure 3 demonstrates that UDP-Glc PP activity is dependent on the Mg^{2+} concentration and the resulting concentration ratio of Mg^{2+} and UTP. All curves point at an optimum ratio of the $MgCl_2$ and UTP concentrations, which was determined to be between 5 and 10 (data not shown). In Fig. 3 the highest activity is obtained with an excess concentration of 10 mM $MgCl_2$ and variation of UTP up to 5 mM. A significant decrease of activity is already obtained at equimolar ratios of $MgCl_2$ and UTP. The decrease of activity is even more pronounced when the concentration of UTP exceeds that of $MgCl_2$ indicating inhibition of UDP-Glc PP by free UTP. Figure 4 illustrates the inhibition of UDP-Glc PP from malt by free UTP. It also indicates that the higher the concentration ratio of Mg^{2+}–UTP and free UTP concentrations the less inhibition of UDP-Glc PP appears; e.g. at 0.8 mM free UTP the activity increases by increasing the ratio of Mg^{2+}–UTP/free UTP from 0.5 to 1.5 and 2.75. This means that free UTP competes with Mg^{2+}–UTP for the same binding site and can be displaced by high Mg^{2+}–UTP concentrations.

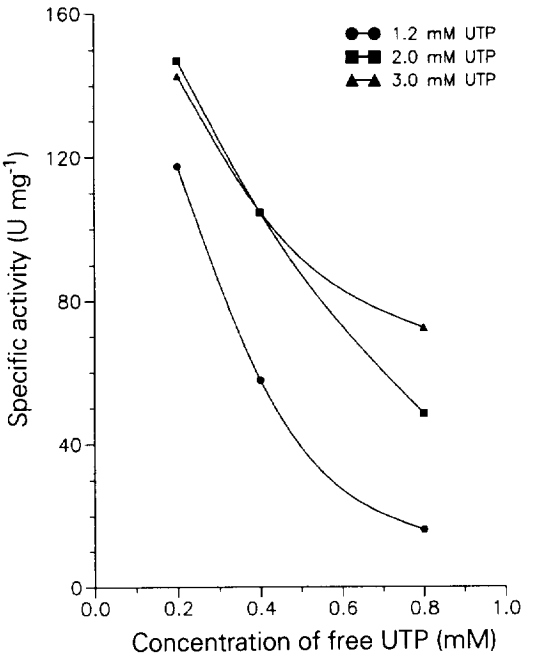

Fig. 4. Effect of free UTP on the specific activity of UDP-Glc PP in the synthesis reaction (assay A). The concentration of free UTP was achieved by varying the concentration of $MgCl_2$ at the indicated constant concentrations of UTP.

DISCUSSION

As far as we know, we here present for the first time the complete kinetic characterization of UDP-Glc PP from barley malt. Since barley malt is produced on an industrial scale the enzyme is easily available for use in nucleotide sugar synthesis. These data are therefore important to evaluate the potential synthetic use of this enzyme compared with other UDP-Glc PP from animal and microbial sources.

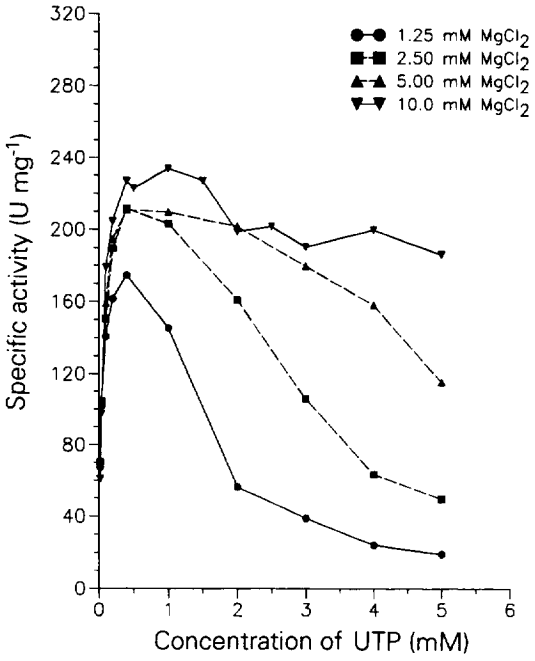

Fig. 3. Specific activity of UDP-Glc PP at different UTP and $MgCl_2$ concentrations in the synthesis reaction (assay A).

The K_m values for all substrates are consistent with those recently reviewed for UDP-Glc pyrophosphorylases from plants [3]. Among these the enzyme from barley malt has higher affinities towards glucose-1-P and UTP than towards UDP-Glc and PP_i (Tables 1 and 3).

The data for UDP-Glc PP from barley malt also confirm that enzymes from plant sources have a higher substrate affinity than those from animal and microbial sources [14]. In the direction of UDP-Glc synthesis the K_m values for the enzyme from calf liver [19] and human erythrocytes [12] are 0.055 mM (Glc-1-P), 0.20 mM (UTP) and 0.17 mM (Glc-1-P), 0.33 mM (UTP), respectively. UDP-Glc PP from barley malt shows also a less pronounced product inhibition by UDP-Glc than the enzymes from animal sources [20]. In conclusion, UDP-Glc PP from barley malt can be now offered as an alternative enzyme to the commercially available enzymes from yeast or bovine liver, which are mostly used in enzymic carbohydrate synthesis [10]. So far, complete kinetic data for the synthesis direction of the enzyme from yeast are not available [20].

The inhibition studies and the deduced enzyme mechanism for UDP-Glc PP from barley malt fit well

with the features currently summarized for known plant enzymes [3]. It has been suggested that the sequential ordered Bi-Bi mechanism is a typical feature of the nucleoside diphosphoglucose family including also the enzymes from animal sources and yeast [20, 21]. However, pyrophosphorylases from bacterial sources follow a different reaction mechanism. dTDP-Glc PP and CDP-Glc PP from Salmonella have been characterized to exhibit a ping-pong Bi-Bi mechanism [22, 23]. It is suggested by this reaction mechanism that in the synthesis reaction the nucleoside monophosphate moiety from the nucleoside triphosphate substrate is covalently bound to the free enzyme under the release of PP_i before glucose-1-P binds next and UDP-Glc is finally released.

UDP-Glc PP from barley malt is specific for glucose-1-P as substrate in the synthesis reaction with UTP. A relative broad specificity has been obtained with different nucleoside triphosphates (CTP, ATP and GTP) and glucose-1-P. The former is consistent with data from other plant enzymes [3]; the latter has been partly demonstrated for enzymes from animal sources [20].

In summary, our data indicate that the use of UDP-Glc PP from barley malt for the synthesis and *in situ* regeneration of nucleotide sugars should be favourable over the enzyme currently used. Work is in progress in our laboratory to exploit these features in combination with mammalian glycosyltransferases [24].

EXPERIMENTAL

All sugar-1-phosphates, nuceotides and nucleotide sugars used were purchased from (Sigma). NAD^+, NADH and NADP were from Biomol (Hamburg). The enzymes used in the assays were all purchased from Boehringer (Mannheim). All other chemicals were obtained from E. Merck (Darmstadt) and were of analyt. grade. A UV-160 spectrophotometer, equipped with a CPS controller and a CPS cell positioner, was used for all enzyme assays. For all assays one enzyme unit is defined as the amount of enzyme producing 1 μmol product min^{-1} under standard conditions. One enzyme unit corresponds to 16.67 nkat (10^{-9} mol sec^{-1}).

Enzyme activity assays

Assay A: formation of UDP-Glc in the synthesis direction. UDP-Glc PP activity was measured in a coupled assay by monitoring 2 mol NADH mol^{-1} UDP-Glc at 340 nm [18]. The assay mixt. (1 ml) contained 5 mM Glc-1-P, 2 mM UTP, 10 mM $MgCl_2$, 2.11 mM NAD^+ and 3.2 mU UDP-Glc dehydrogenase in 50 mM Tris–HCl (pH 8.35) at 25°.

Assay B: formation of PP_i in the synthesis reaction. UDP-Glc PP activity was measured in a coupled assay by monitoring 2 mol NAD mol^{-1} PP_i at 340 nm [17]. The assay mixt. (100 μl) contained 5 mM Glc-1-P, 2 mM UTP, 10 mM $MgCl_2$, 0.15 mM NADH, 5 mM fructose-6-phosphate, 1 μM fructose-2,6-bisphosphate, 100 mU PP_i-dependent phosphofructokinase (potato), 45 mU aldolase, 500 mU triose phosphate isomerase and 170 mU glycerol-3-phosphate dehydrogenase in 50 mM Tris–HCl (pH 7.4) at 30° in a microtitre plate. Nucleotidyltransferase substrate screening assay was also used for screening the substrate specificity of UDP-Glc PP from malt by testing different nucleoside triphosphates and sugar-1-phosphates.

Assay C: formation of UTP by pyrophosphorylysis. UDP-Glc PP activity was measured in a coupled assay following the decrease of NADH concn at 340 nm according to ref. [19]. The assay mixt. (1 ml) contained 2 mM $MgCl_2$, 2 mM Na_2-pyrophosphate, 1.2 mM 3-P-glycerate, 0.24 mM NADH, 0.4 mM UDP-Glc, 1 U 3-phosphoglycerate-kinase and 2.5 U glyceraldehyde-3-phosphate dehydrogenase in 50 mM Tris–HCl (pH 8.0) at 25°.

Assay D: formation of glucose-1-phosphate by pyrophosphorylysis. UDP-Glc PP was measured in a coupled assay following the increase of NADPH concn at 340 nm according to ref. [19]. The assay mixt. (1 ml) contained 2 mM $MgCl_2$, 2 mM Na_2 pyrophosphate, 4 mM NADH, 0.4 mM UDP-Glc, 1 U phosphoglucomutase and 2.5 U glucose-6-phosphate dehydrogenase in 90 mM Tris–HCl (pH 8) at 25°.

Enzyme kinetics. For kinetic measurements one substrate was varied at a constant concn of the second substrate. In the synthesis direction Glc-1-P (0.02–5.0 mM) and UTP (0.02–2.0 mM) were varied at constant concns of UTP (2 mM) and Glc-1-P (5 mM), respectively. In the pyrophorylysis direction UDP-Glc (0.01–1.0 mM) and Na_2PP_i (0.02–4 mM) were varied at constant concns of Na_2PP_i (2 mM) and UDP-Glc (0.8 mM), respectively.

Inhibition studies. To study different inhibition types in the synthesis direction, kinetic measurements were performed as described above in the presence of different concns of Na_2PP_i (0.2–1.0 mM) and NaH_2PO_4 (2–10 mM) in assay A. Studies describing product inhibition by UDP-glucose (variation between 0.025 and 0.1 mM) were performed with assay B. In the pyrophosphorylysis direction, production inhibition by UTP (0.1–1.0 mM) was determined with assay D.

Determination of kinetic parameters. Experimental data from all kinetic measurements were analysed by a non-linear regression analysis (iteration method according to Marquardt) using the graphic and statistic computer program PlotIt® (Scientific Programming Enterprises, Haslett, U.S.A.). The computer program calculated the Michaelis–Menten constants K_m and V_{max} according to the given equation by minimization of least-square deviation values. The equation for substrate surplus inhibition was used for determination of K_m, V_{max} and K_{iS} [15]. In the case of competitive or non-competitive inhibition studies the calculated values for K_m and V_{max} were used for graphical demonstration of the inhibition type by Lineweaver–Burk plots. Secondary plots of K_m/V_{max} or $1/_{Vmax}$ versus the inhibitor concn gave the inhibition constants K_i and K_{ii}, respectively, by linear regression analysis and calculation of the intercept with the x-axis [15].

Effect of Mg^{2+} free UTP concns on UDP-glucose synthesis. The concn of UTP was varied between 0.02 and 5 mM at different constant concns of $MgCl_2$ and 5 mM Glc-1-P in assay A. The enzyme activity was finally plotted as a function of the concn ratio of UTP and $MgCl_2$. To study the effect of free UTP on activity of UDP-Glc PP, concns of UTP and $MgCl_2$ were simultaneously varied to obtain concns of 0.2, 0.4 and 0.8 mM free UTP.

Effect of different metal ions. UDP-Glc PP activity was tested with assay A replacing 10 mM $MgCl_2$ by 10 mM $MnCl_2$, $ZnCl_2$, $NiCl_2$, $CaCl_2$ and $CuCl_2$.

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