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An improved assay for UDPglucose pyrophosphorylase and other enzymes that have nucleotide products

R. G. Duggleby[a,*], H.-L. Peng[b] and H.-Y. Chang[c]

[a] *Centre for Protein Structure, Function and Engineering, Department of Biochemistry, University of Queensland, Brisbane 4072 (Australia), Fax +617 3365 4699, e-mail: duggleby@biosci.uq.edu.au*
[b] *Department of Microbiology and Immunology, Chang-Gung Medical College, Kwei-San, Taiwan (Republic of China)*
[c] *Department of Molecular and Cellular Biology, Chang-Gung Medical College, Kwei-San, Taiwan (Republic of China)*
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Abstract. UDPglucose pyrophosphorylase catalyses the interconversion UDPglucose plus pyrophosphate and glucose 1-phosphate plus UTP. Several assay methods for this enzyme have been described but the only one that can be used to investigate the specificity with respect to various UDPsugars is based on coupling to UTP formation. This assay employs phosphoglycerate kinase to catalyse the formation 1,3-*bis*phosphoglycerate which is then used to oxidise NADH in the presence of glyceraldehyde 3-phosphate dehydrogenase. We have found that the activity of phosphoglycerate kinase towards UTP is low which limits the usefulness of the assay to very low rates, in agreement with the published recommendation of Hansen et al.[5]. Here it is shown that the dynamic range of the assay is increased by more than five fold on addition of nucleoside diphosphate kinase and ADP, which convert UTP to the preferred phosphoglycerate kinase substrate, ATP. It is also shown that the improved assay is suitable for enzymes with other nucleotide triphosphate products.
Key words. UDPglucose pyrophosphorylase; coupled assay; nucleotide products; nucleoside diphosphate kinase; uridine triphosphate.

Abbreviations. ADP = adenosine 5′-diphosphate; MgADP = magnesium complex of adenosine 5′-diphosphate; ATP = adenosine 5′-triphosphate; MgATP = magnesium complex of adenosine 5′-triphosphate; dATP = 2′-deoxyadenosine 5′-triphosphate; UDP = uridine 5′-diphosphate; UTP = uridine 5′-triphosphate; MgUTP = magnesium complex of uridine 5′-triphosphate; CTP = cytidine 5′-triphosphate; dCTP = 2′-deoxycytidine 5′-triphosphate; dTTP = 2′-deoxythymidine 5′-triphosphate; TTP = thymidine 5′-triphosphate; GTP = guanosine 5′-triphosphate; MgGTP = magnesium complex of guanosine 5′-triphosphate; dGTP = 2′-deoxyguanosine 5′-triphosphate; NADH = reduced nicotinamide-adenine dinucleotide; cDNA = complementary deoxyribonucleic acid; IPTG = isopropyl β-D-thiogalactopyranoside; DEAE = diethylaminoethyl.

Many enzymes have MgATP as a product and their activities are readily measured in a coupled assay; using hexokinase and glucose 6-phosphate dehydrogenase[1], for example. Enzymes that produce other nucleoside triphosphates are less easily measured due to the paucity of efficient coupling systems that are available. This laboratory has recently isolated a cDNA clone of human liver UDPglucose pyrophosphorylase[2] which catalyses the reaction:

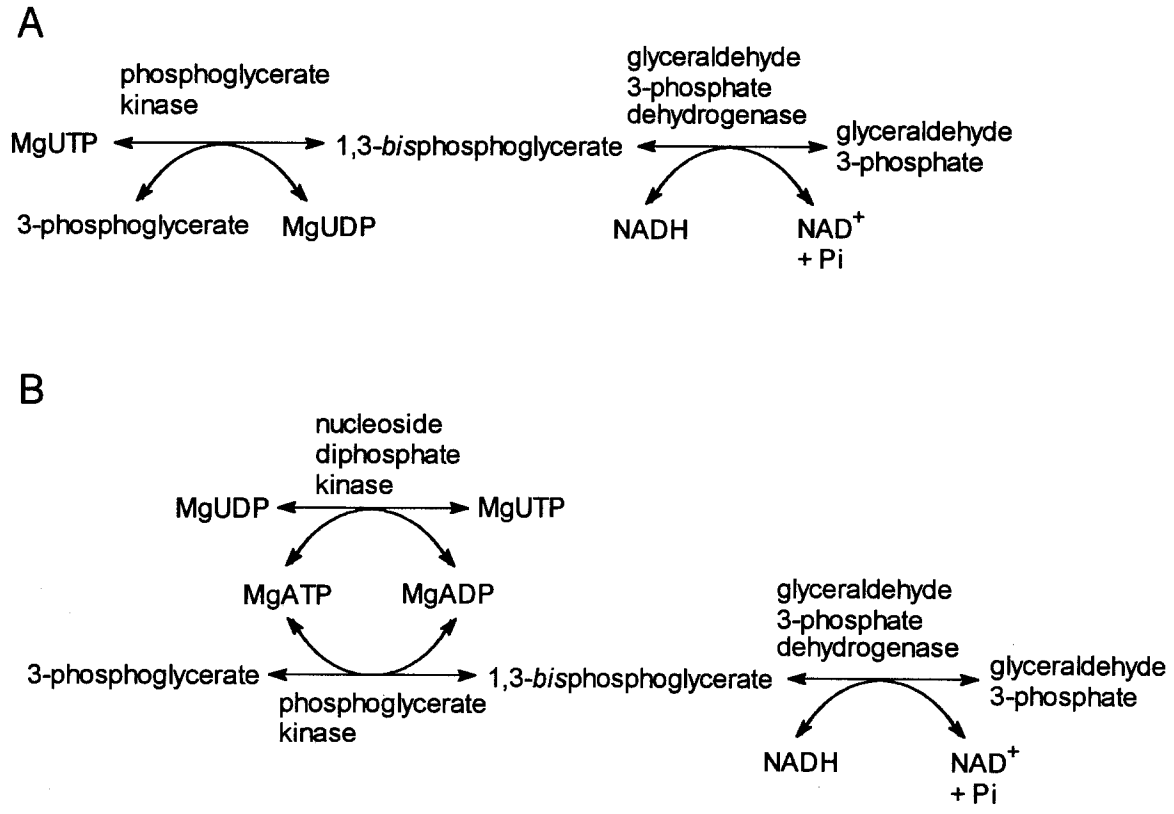
$$\text{UDPglucose} + \text{MgPPi} \rightleftharpoons \text{MgUTP} + \text{glucose 1-phosphate}$$

We are now mutagenising and expressing this cDNA, then purifying and characterising the resultant protein (Chang et al.[3]).

The enzyme can be assayed in several ways (Tsuboi et al.[4]). The most obvious of these is to couple to glucose 1-phosphate formation, using phosphoglucomutase and glucose 6-phosphate dehydrogenase. In the opposite direction, coupling to UDPglucose formation is possible, using UDPglucose dehydrogenase. However, due to the specificity of these coupling systems for glucose derivatives, neither is suitable for assessing other sugar derivatives as potential substrates. We are particularly interested in trying alternative substrates in our mutagenesis studies.

Hansen et al.[5] have described an assay that couples to MgUTP formation (scheme 1A). The assay exploits the ability of phosphoglycerate kinase to utilise MgUTP rather than the preferred substrate, MgATP. The unfavourable equilibrium of the system is overcome by including hydrazine to trap glyceraldehyde 3-phosphate as its hydrazone. We have observed that this assay is of limited usefulness due to long time lags before a constant rate of NADH oxidation is obtained. Presumably, these lags result from the need for MgUTP to accumulate to an extent where it becomes effectively utilised by phosphoglycerate kinase. Moreover, the steady-state rate of NADH oxidation must be kept low if it is to

* Corresponding author.



Scheme 1. Reactions occurring in the conventional (*A*) and improved (*B*) assay of UDPglucose pyrophosphorylase.

accurately reflect the quantity of UDPglucose pyrophosphorylase added.

Here we report a modification of the assay that overcomes the limitations mentioned above. Lags can be reduced to less that 20 seconds and the rate of NADH oxidation can be five to ten times higher without compromising the accuracy of the assay.

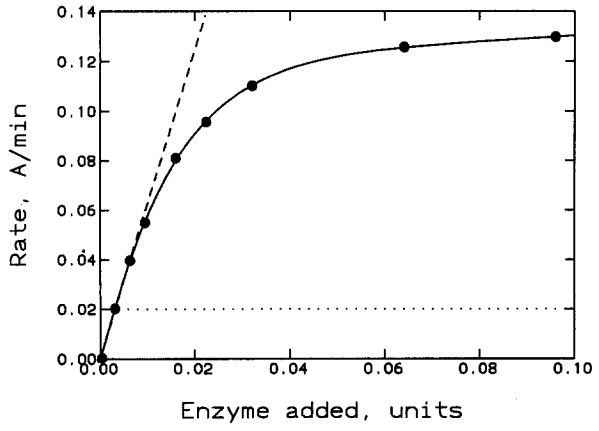
Materials and methods

Tetrasodium pyrophosphate was purchased from Nacalai Tesque Inc. (Kyoto, Japan), triethanolamine from Ajax Chemicals, (Auburn, Australia), ammonium sulfate from Baker (Phillipsburg, NJ, USA), HCl from Merck (Darmstadt, FRG), pBluescript from Stratagene (La Jolla, CA, USA), Centricon-50 ultrafilters from Amicon (Beverley, MA, USA) and DEAE-cellulose (DE52) was obtained from Whatman. Nucleotides were obtained from Pharmacia P-L Biochemicals Inc. (Milwaukee WI, USA) and other chemicals, biochemicals and enzymes were purchased from Sigma (St Louis, MO, USA).

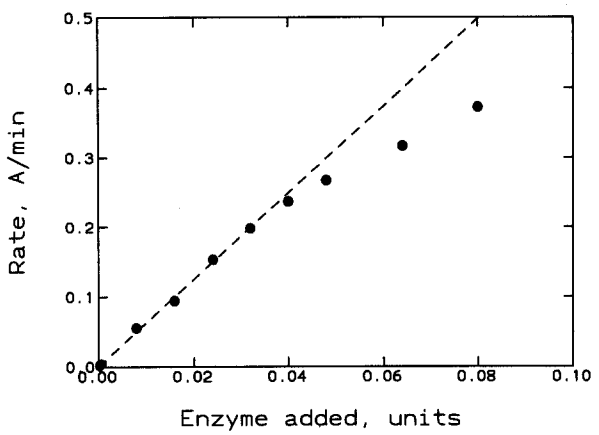
Human UDPglucose pyrophosphorylase was obtained by cloning the cDNA into pBluescript under the control of the *lac* promotor; the enzyme was then expressed in *Escherichia coli* by adding 0.2 mM IPTG. A cell-free extract was obtained by centrifugation after disrupting the cells by sonication, and the enzyme enriched to approximately 10% purity by streptomycin sulfate treatment, ammonium sulfate fractionation, and chromatography on DEAE-cellulose. Details of the procedure are described elsewhere (Duggleby et al.[6]). Enzyme concentrations were standardised using an assay coupled to glucose 1-phosphate formation, similar to that described by Tsuboi et al.[4].

The unmodified assay was essentially as described by Hansen et al.[5] and contained in a final volume of 1 ml the following components (dissolved in 100 mM triethanolamine-HCl buffer, pH 8.0), added in the order given: hydrazine sulfate (1 mM), $MgCl_2$, (2 mM), sodium pyrophosphate (1 mM), 3-phosphoglycerate (1.2 mM), NADH (0.15 mM), UDPglucose (1 mM), dithiothreitol (1 mM), phosphoglycerate kinase (40 U/ml) and glyceraldehyde 3-phosphate dehydrogenase (10 U/ml). The reaction was started by the addition of UDPglucose pyrophosphorylase and followed at 340 nm and 25°C.

In the improved assay, ADP (40 μM) and nucleoside diphosphate kinase (2 U/ml) were added immediately following dithiothreitol, while the concentrations of phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase were each 20 U/ml. More glyceraldehyde 3-phosphate dehydrogenase was required to sustain the higher rates of NADH oxidation that the improved assay will support. However, less phosphoglycerate kinase is needed because the assay is no longer



(*A*)



(*B*)

Figure 1. Effect of enzyme concentration on the assay for UDPglucose pyrophosphorylase. The conventional assay is shown in panel A in which the dotted line represents the rate limit recommended by Hansen et al.[5] while panel B shows the improved assay. For both assays, the broken line shows the expected relationship between units of enzyme added and the rate. No rate was observed when either phosphoglycerate kinase or glyceraldehyde 3-phosphate dehydrogenase was omitted. The data shown are representative of replicate experiments.

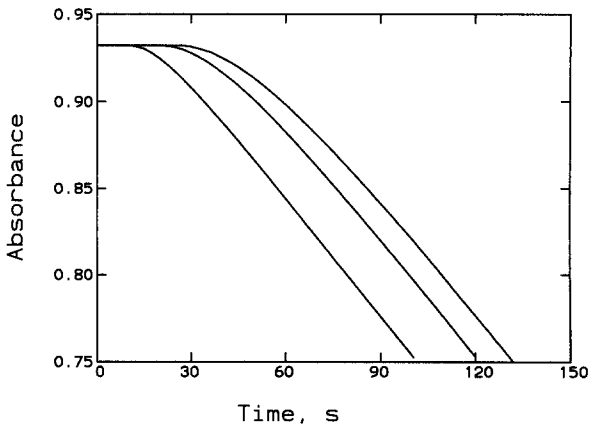


Figure 2. Dependence of the lag on ADP concentration in the assay for UDPglucose pyrophosphorylase. The curves, from left to right, represent 40, 100 and 200 μM ADP. The data shown are representative of replicate experiments.

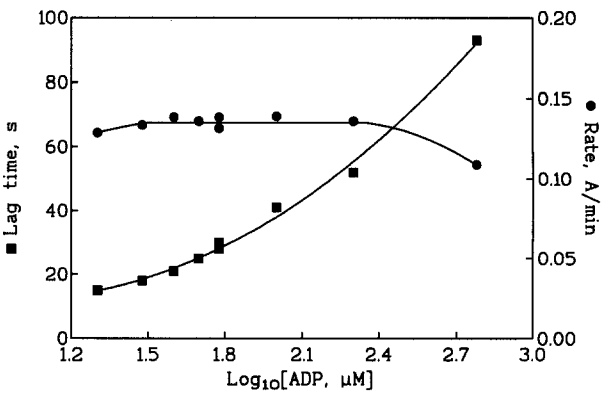


Figure 3. Dependence of the steady-state rate and the lag on ADP concentration in the assay for UDPglucose pyrophosphorylase. The circles represent the steady-state rate plotted on the scale shown on the right ordinate, while the lags are shown as squares using the left ordinate scale.

reliant upon the weak activity of this enzyme with UTP as substrate.

We have observed that human UDPglucose pyrophosphorylase, like the bovine mammary gland enzyme[7] is inhibited by sulfate ion. Thus it was necessary to limit carryover of this ion into the assay from phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase, which were purchased as suspensions in concentrated ammonium sulfate. For the latter enzyme centrifugation, followed by removal of the supernatant, was effective. For the former enzyme, centrifugation did not result in sedimentation; consequently the enzyme was dissolved by adding two volumes of buffer and concentrated to half the original volume by centrifugal ultrafiltration (Centricon-50). This dilution and concentration step was then repeated.

Results and discussion

The measured rate in the unmodified assay[5], as a function of added enzyme, is shown in figure 1A. Clearly, this assay exhibits major departures from the expected linear relationship (broken line); it would only be reliable when the rate is no greater than 0.04 A per minute, somewhat better than the recommendation of the authors who state that rates of less than 0.3 A per 15 minutes are desirable (fig. 1A, dotted line).

Nucleoside diphosphate kinase catalyses the transfer of the terminal phosphate between MgATP and other MgNTPs, thereby maintaining adequate cellular nucleoside triphosphate concentrations for nucleic acid and protein synthesis. In sharp contrast to other kinases, the metabolic role of this enzyme demands that it has a broad nucleotide specificity. We reasoned that

Table 1. Effect of nucleoside diphosphate kinase (NDPK) and ADP on the substrate activity of various nucleoside triphosphates. Assays were as described in 'Materials and methods' for the improved assay, except that UDPglucose, pyrophosphate and UDPglucose pyrophosphorylase were omitted, while the phosphoglycerate kinase activity was reduced to 0.035 U/ml.

Substrate	Rate (% of 40 μM ATP)	
	−NDPK/ADP	+NDPK/ADP
40 μM ATP	100	nd
1 mM ATP	358	nd
1 mM GTP	268	213
1 mM UTP	12	107
1 mM CTP	1	108
1 mM dATP	120	163
1 mM dGTP	164	161
1 mM dTTP	3	88
1 mM dCTP	2	105
No substrate	nd	0

nd = not determined.

addition of nucleoside diphosphate kinase, in the presence of MgADP, would catalyse the conversion of MgUTP to MgATP which should be a much better substrate for phosphoglycerate kinase. Preliminary experiments confirmed this hypothesis; in the presence of 1 mM MgUTP and a limiting amount of phosphoglycerate kinase, there was a nine fold stimulation of NADH oxidation upon addition of nucleoside diphosphate kinase (2 U/ml) and MgADP (200 μM).

The improved assay (scheme 1B) was optimised by varying the concentration of each component in the coupling system. Although there were significant lags (fig. 2), these were reduced dramatically by lowering the MgADP concentration (figs 2 and 3). The steady-state rate was independent of the MgADP concentration over the range 30 to 200 μM but was reduced slightly outside this range. We selected an MgADP concentration of 40 μM where the lag was approximately 20 seconds, little more than the mixing time in manual assays. The rate, as a function of added enzyme, is shown in figure 1B from which it is seen that the assay is linear to at least 0.2 A per minute, a five to ten fold improvement over the original assay. At higher enzyme concentrations the rates are less than expected (fig. 1B broken line). Preliminary experiments suggested that this was because glyceraldehyde 3-phosphate dehydrogenase is beginning to become rate-limiting. We have not attempted to extend the range of linearity because the present assay was suitable for our purposes.

We developed this assay with the specific purpose of investigating the properties of UDPglucose pyrophosphorylase, which has MgUTP as a product. However, the same principle could be applied to enzymes with other MgNTP products as is shown in table 1. The other purine nucleotides are reasonably good substrates of phosphoglycerate kinase although none are used as effectively as an equivalent concentration of MgATP. For these compounds, addition of nucleoside diphosphate kinase and MgADP offers no advantage; indeed, there is some disadvantage for MgGTP due, presumably, to inhibition of phosphoglycerate kinase by MgADP. In contrast, none of the pyrimidine nucleotides are good substrates and addition of nucleoside diphosphate kinase and MgADP greatly stimulates their rates of utilisation. For these compounds, the assay offers clear benefits.

Our intention in this study was to improve an existing assay. However, it should be noted that the presence of MgADP and nucleoside diphosphate kinase frees the assay from its dependence on the ability of phosphoglycerate kinase to use alternative nucleoside triphosphate substrates. Thus, the same principle could be applied to other MgATP coupling systems such as the hexokinase/glucose 6-phosphate dehydrogenase couple mentioned in the 'Introduction'. Perhaps such an assay might be preferable to the present one as it would not require a trapping agent to impose irreversibility, and would entail following an increase, rather than a decrease, in A_{340}.

The principle might also be extended to enzymes that have a nucleoside diphosphate product (glycogen synthase, for example). In this case, addition of nucleoside diphosphate kinase and MgATP would produce MgADP, which could then be coupled to NADH oxidation using pyruvate kinase and lactate dehydrogenase[1].

In the present assay, MgADP behaves catalytically (scheme 1B) being converted to MgATP in the nucleoside diphosphate kinase reaction and reformed in the reaction catalysed by phosphoglycerate kinase. Thus, we were initially surprised that lags were accentuated by increasing the MgADP concentration. We suspect that this is due to product inhibition of phosphoglycerate kinase and that the lag represents the time required for MgATP to accumulate to the extent that this inhibition is reversed. Under the conditions employed here, 40 μM MgADP was found to be optimal; however, under different conditions of pH, temperature, buffer and so on, or for different enzymes, the MgADP concentration would have to be optimised individually.

In summary, we have developed an improved assay for UDPglucose pyrophosphorylase that has a five to ten fold higher dynamic range than that described by Hansen et al.[5]. The general strategy has wider implications and could form the basis of assays for any enzyme that has a nucleoside triphosphate or diphosphate product.

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