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A SOURCE OF APPARENT PYROPHOSPHATE:FRUCTOSE 6-PHOSPHATE PHOSPHOTRANSFERASE ACTIVITY IN RABBIT MUSCLE PHOSPHOFRUCTOKINASE

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Summary. In the presence of UDPglucose, rabbit muscle phosphofructokinase appeared to use PP_i as a phosphoryl donor, as reported previously (Biochem. Biophys. Res. Commun. 121, 842-847). This apparent activity was due to conversion of UDPglucose and PP_i to glucose 1-phosphate and UTP, the latter being metabolized by phosphofructokinase. Auxiliary enzymes used in the assays were contaminated by UDPglucose pyrophosphorylase. This contamination was sufficient to account for, and had similar properties to, the apparent PP_i-dependent activity. Without auxiliary enzymes phosphofructokinase could not use PP_i. These findings indicate that the apparent interconversion of phosphofructokinase and PP_i:fructose 6-phosphate phosphotransferase must be re-assessed. © 1985 Academic Press, Inc.

Two enzyme activities, phosphofructokinase (eq. 1) and PP_i: fructose 6-phosphate phosphotransferase (eq. 2), can catalyze the conversion of Fru-6-P to Fru-1,6-P_2.

[1] Fru-6-P + ATP $\xrightarrow{\text{PFK}}$ Fru-1,6-P_2 + ADP

[2] Fru-6-P + PP_i $\xrightarrow{\text{PFP}}$ Fru-1,6-P_2 + P_i

Many higher plant tissues contain both activities (1,2), but their relative importance in glycolysis is unknown. Recently Buchanan and co-workers have proposed that these two activities are reversibly interconverted when incubated with appropriate metabolites (3). They suggest that these interconversions occur in extracts from a range of tissues (4,5), and that such changes contribute to the regulation of glycolysis in plants by adjusting the proportions of PFK and PFP in vivo (4). These workers have also described

Abbreviations: Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; UDPG, UDPglucose; PFK, phosphofructokinase (EC 2.7.1.11); PFP, PP_i:fructose 6-phosphate phosphotransferase (EC 2.7.1.90)

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a similar conversion of PFK from rabbit muscle (6). Incubation of this enzyme, a 320 kDa tetramer, with UDPG produced a 80 kDa form apparently capable of using either ATP or PP_i (6).

Some of the evidence for the above conversions has been criticized. The kinetic data obtained with spinach leaf extracts (3) have been re-interpreted, and indicate that PFK and PFP are two distinct enzymes that are differentially activated by several metabolites (7). Since PFP is restricted to plants and microorganisms, this explanation is unlikely to apply to the PFK-PFP interconversion reported for the purified rabbit muscle PFK (6). Therefore we have investigated the apparent PFP activity from the latter source in more detail. Here we report that the auxiliary enzymes commonly used in the assay for PFK and PFP are contaminated with UDPG pyrophosphorylase (EC 2.7.7.9). Such contamination can account for the apparent PFP activity in preparations of PFK.

MATERIALS AND METHODS

Purified rabbit muscle PFK (type III) was purchased from Sigma (St. Louis, MO) together with all cofactors and auxiliary enzymes, except for glucose 6-phosphate dehydrogenase which was from Boehringer Mannheim (Dorval, QUE). The auxiliary enzymes were dialyzed before use.

PFK and PFP were measured spectrophotometrically as described previously (1). The PFK assay contained, in 1 ml; 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$, 5 mM Fru-6-P, 1 mM ATP, 0.1 mM NADH, 1 U aldolase, 10 U triosephosphate isomerase, 1.2 U glycerol 3-phosphate dehydrogenase. Assay conditions for PFP were identical to those of PFK, except that 0.2 mM PP_i replaced ATP. The reactions were started with ATP and PP_i, respectively, unless stated otherwise. PFK and PFP were also measured in discontinuous assays containing, in 0.5 ml; 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$, 5 mM Fru-6-P and either 1 mM ATP or 0.2 mM PP_i. Following a 5 min pre-incubation, each assay was started by adding Fru-6-P. After 10 min at 25°C each reaction was stopped by adding 0.5 ml 1.41 M $HClO_4$ at 0°C. The samples were then kept at 0°C for 15 min, neutralized with 5 M K_2CO_3 and centrifuged (Beckman microfuge, 3 min). The Fru-1,6-P_2 content of the resulting supernatant was then measured (8).

UDPG pyrophosphorylase was measured by two methods. First, the production of UTP was coupled to NADH oxidation using 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase. The reaction mixture contained, in 1 ml; 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM UDPG, 0.2 mM PP_i, 2.5 mM 3-phosphoglycerate, 0.1 mM NADH, 4 U 3-phosphoglycerate kinase, 1 U glyceraldehyde 3-phosphate dehydrogenase. The reaction was started by adding PP_i. Second, the production of glucose 1-phosphate was coupled to $NADP^+$ reduction using phosphoglucomutase and glucose 6-phosphate dehydrogenase. The reaction mixture contained, in 1 ml; 50 mM Hepes-NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM UDPG, 0.2 mM PP_i, 1 mM $NADP^+$, 1 U phosphoglucomutase, 1 U glucose 6-phosphate dehydrogenase. The reaction was started by adding the preparation containing UDPG pyrophosphorylase.

RESULTS

Under conditions similar to those previously reported to stimulate the conversion of PFK to PFP (6), rabbit muscle PFK activity was stimulated about 50% by dithiothreitol, but was unaffected by UDPG (table 1). However, in the presence of UDPG, the enzyme preparation was also able to use PP_i as the phosphoryl donor. This PP_i-dependent activity was stimulated more than 15-fold by dithiothreitol (table 1). Unlike PFP from plants (9), the activity from rabbit muscle was not stimulated by Fru-2,6-P_2. However, at 1 μM, this compound did enhance activity by 30% in the presence of 5 mM citrate and 10 mM dithiothreitol. PFK showed the expected inhibition by citrate (10), which was relieved by 1 μM Fru-2,6-P_2 (results not shown).

The apparent PFP activity described above was completely dependent on the presence of Fru-6-P, PP_i, UDPG and PFK. The assay for PFP was linear only after 4 to 7 min if the reaction was started by adding PP_i. The initial period of low apparent PFP activity could not be shortened by pre-incubating the remainder of the assay components for up to 5 min before adding PP_i. In contrast, no such lag-phase occurred if either Fru-6-P or PFK was used to start the reaction after a similar 5 min pre-treatment. The lag-phase could

Table 1. Effect of dithiothreitol and UDPglucose on rabbit muscle PFK

Addition	Enzyme activity (μmol.min^{-1}.mg protein^{-1})	
	PFK	PFP
None	26.61 ± 1.47 (100)	0 (0)
Dithiothreitol (10 mM)	40.21 ± 1.00 (100)	0 (0)
UDPglucose (1 mM)	27.66 ± 1.05 (98)	0.57 ± 0.02 (2)
Dithiothreitol (10 mM) + UDPglucose (1 mM)	40.04 ± 0.26 (81)	9.45 ± 0.15 (19)

PFK and PFP were measured spectrophotometrically as described in Materials and Methods. Each assay contained 0.475 μg rabbit muscle PFK. Enzyme activity is expressed per mg protein in the Sigma purified PFK. For each treatment, the figures in parentheses are mean PFK or PFP activity expressed as a percentage of the total phosphorylating activity of both enzymes under those conditions (3). Each value represents the mean ± SE of 4 separate measurements.

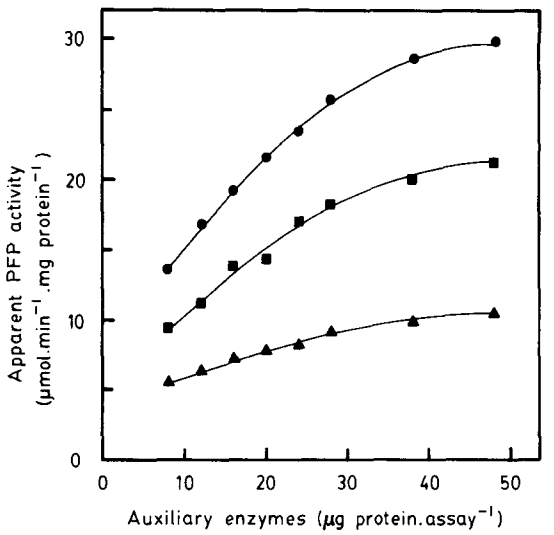

Fig. 1. Effect of auxiliary enzymes on apparent PFP activity of rabbit muscle PFK. PFP activity was measured spectrophotometrically as described in Materials and Methods. Each assay contained 10 mM dithiothreitol, 1 mM UDPG, 1 U aldolase and either 0.238 (●), 0.475 (■) or 0.950 (▲) μg rabbit muscle PFK. The auxiliary enzymes, triosephosphate isomerase (1250 U.mg protein^{-1}) plus glycerol 3-phosphate dehydrogenase (150 U.mg protein^{-1}), were varied as shown.

be abolished only if the pre-incubation mixture contained UDPG, PP_i and triosephosphate isomerase plus glycerol 3-phosphate dehydrogenase (supplied as a mixture).

We investigated the role of triosephosphate isomerase plus glycerol 3-phosphate dehydrogenase by varying the level of these auxiliary enzymes in the assay. Apparent PFP activity more than doubled as the amount of auxiliary enzymes increased from 8 to 48 μg (fig. 1). This is unlikely to be due to either triosephosphate isomerase or glycerol 3-phosphate dehydrogenase limiting the assay, since under the same range of conditions, PFK activity remained constant at 40.16 ± 0.19 μmol.min^{-1}.mg protein^{-1} (greater than the maximum PFP activity). These results suggest that some component in the preparation of auxiliary enzymes is responsible for the apparent PFP activity.

Discontinous assays, which allowed PFK and PFP to be measured in the absence of auxiliary enzymes, confirmed that apparent PFP activity was dependent on the triosephosphate isomerase plus glycerol 3-phosphate dehydrogenase preparation. Under each set of conditions described in table 1, PFK activity measured in the discontinuous assay was between 92 and 101% of

that obtained with the spectrophotometric assay. However, no PFP activity was observed using the discontinuous assay unless the reaction mixture also contained the triosephosphate isomerase plus glycerol 3-phosphate dehydrogenase preparation. In the presence of 8 μg auxiliary enzymes (the same amount as used in the standard spectrophotometric assay) the apparent PFP activity was 9.15 ± 0.39 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ (mean ± SE of 4 separate measurements).

We tested the possibility that UTP formed from UDPG might contribute to the apparent PFP activity. First, we confirmed a previous report (11) that UTP was a suitable phosphoryl donor for rabbit muscle PFK. PFK activity measured with 1 mM UTP was between 94 and 108% of that obtained with 1 mM ATP. Second, the triosephosphate isomerase plus glycerol 3-phosphate dehydrogenase preparation (Sigma, type III, lot no. 123F-9530) used throughout this study contained measurable UDPG pyrophosphorylase activity. The production of UTP was dependent on both UDPG and PP_i, and was stimulated about 15-fold by dithiothreitol (table 2). Under similar conditions, glucose 1-phosphate

Table 2. Contamination of auxiliary enzymes by UDPglucose pyrophosphorylase

Addition	UDPglucose pyrophosphorylase activity	
	$nmol \cdot min^{-1} \cdot mg\ protein^{-1}$	activity in standard PFK assay as % of apparent PFP
Control (- UDPglucose)	0	-
Dithiothreitol	0	-
UDPglucose	32.6 ± 2.4	96.0 ± 6.8
Dithiothreitol + UDPglucose	561.1 ± 9.6	99.0 ± 1.7

UDPG pyrophosphorylase activity was assayed by measuring production of UTP as described in Materials and Methods. In addition, each assay contained 8 μg triosephosphate isomerase plus glycerol 3-phosphate dehydrogenase and, when present, 10 mM dithiothreitol and 1 mM UDPG. Activity is expressed per mg protein in the auxiliary enzyme preparation, and was calculated assuming that glyceraldehyde 3-phosphate produced in the assay was further metabolized through triosephosphate isomerase and glycerol 3-phosphate dehydrogenase. The activity present in the standard PFK assay is expressed as a percentage of the mean apparent PFP activity for each treatment. Each value is the mean ± SE of 4 separate measurements.

production was between 92 and 104% of the rates shown in table 2. Neither PFK nor aldolase (the other auxiliary enzyme used in the PFK assay) contained detectable UDPG pyrophosphorylase activity.

DISCUSSION

The apparent PFP activity described in this paper is not due to conversion of PFK to a form capable of using both ATP and PP_i. The PP_i-dependent activity measured in the present study was not proportional to the amount of PFK, but instead was strongly dependent on the quantity of auxiliary enzymes, triosephosphate isomerase plus glycerol 3-phosphate dehydrogenase, in the assay. Discontinuous assays indicate that apparent PFP activity was completely dependent on the presence of the auxiliary enzymes as well as PFK.

The auxiliary enzyme preparation was contaminated by UDPG pyrophosphorylase and the apparent PFP activity was due to a combination of this UDPG pyrophosphorylase activity and PFK as shown in equations 3 and 4.

[3] UDPglucose + PP_i ⟶ Glucose 1-phosphate + UTP

[4] Fru-6-P + UTP ⟶ Fru-1,6-P_2 + UDP

The production of Fru-1,6-P_2 by such a system is dependent on UDPG, Fru-6-P and PP_i; the same requirements as the UDPG-induced PFP activity proposed by others (6). Even though the contaminating UDPG pyrophosphorylase activity is less than 0.5% of the activity of either triosephosphate isomerase or glycerol 3-phosphate dehydrogenase, there is sufficient to account for the apparent PFP activity (table 2). The UTP produced by this reaction (eq. 3) can be rapidly metabolized by rabbit muscle PFK (eq. 4). Thus, the scheme outlined above can quantitatively account for the apparent PFP activities reported in this study. In addition, both UDPG pyrophosphorylase and the apparent PFP activity are strongly activated by dithiothreitol.

This contamination of auxiliary enzymes probably accounts for the apparent PFP activity of rabbit muscle PFK reported previously (6). As in the present paper, apparent PP_i-dependent activity required UDPG and was stimulated by dithiothreitol. Further, in the presence of dithiothreitol and citrate, both activities are enhanced by Fru-2,6-P_2. In the previous report

(6), the apparent PFP activity in the presence of UDPG was associated with the original 320 kDa enzyme as well as with a 80 kDa monomer of PFK (fig. 1 in 6). The association of apparent PFP activity with both forms of the enzyme is consistent with the explanation proposed in this paper.

It is uncertain to what extent UDPG pyrophosphorylase contamination of auxiliary enzymes may account for the apparent interconversion of PFK and PFP in plants (3-5). Plant PFK is capable of using UTP as a phosphoryl donor (12), and is thus prone to interference by UDPG metabolism. However, plants also contain a separate, specific PFP which is unable to use ATP (9). Therefore both the contamination described above and differential activation of PFK and PFP as suggested earlier (7) probably contribute to the apparent interconversion of the two enzymes. Both of these possibilities need to be excluded before we accept that PFK and PFP are interconverted as described by others (3-6).

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