The Regulation of Pea-Seed Phosphofructokinase by Phosphoenolpyruvate

By G. J. KELLY AND J. F. TURNER

Department of Agricultural Chemistry, University of Sydney, Sydney, N.S.W., Australia

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1. Pea-seed phosphofructokinase was purified 27-fold by a combination of fractionation with ethanol and ammonium sulphate. Under the conditions of assay, the enzyme was strongly inhibited by phosphoenolpyruvate. This inhibition was reversed by increasing the concentration of fructose 6-phosphate or magnesium chloride, or by lowering the ATP concentration. 2. Citrate, ADP and AMP inhibited phosphofructokinase and increased the sensitivity to phosphoenolpyruvate inhibition. Sulphate and inorganic phosphate stimulated the enzyme activity and decreased the sensitivity to phosphoenolpyruvate. 3. In the presence of inorganic phosphate and low concentrations of ATP, inhibition by phosphoenolpyruvate ceased and phosphoenolpyruvate became stimulatory. 4. The possible significance of these results in the control of plant carbohydrate metabolism is discussed.

The glycolytic enzyme PFK* (ATP-D-fructose 6-phosphate 1-phosphotransferase; EC 2.7.1.11) has been studied with preparations from a variety of tissues. A number of metabolic intermediates, including the substrates F6P and ATP, the products FDP and ADP, and citrate, orthophosphate, AMP and K^+ and NH_4^+ ions are known to affect the kinetic properties of the enzyme from mammalian tissues, yeast and *Escherichia coli* (Passonneau & Lowry, 1964; Atkinson, 1966). It is believed that the interactions of these modifiers with PFK may be important in the control of glycolysis.

Less work has been carried out with the enzyme from higher plants. The action of some modifiers has been studied with PFK prepared from parsley and avocado (Lowry & Passonneau, 1964), carrot and Brussels sprout (Dennis & Coultate, 1966, 1967) and corn (Garrard & Humphreys, 1968).

In an earlier paper (Kelly & Turner, 1968) it was reported that PFK from pea seeds was inhibited by low concentrations of PEP. Blangy, Buc & Monod (1968) found PEP inhibition of *E. coli* PFK and examined the effect of F6P and Mg^{2+} on the inhibition.

In the present investigation the observations on the PEP inhibition of pea-seed PFK were extended. The effects of substrates and some other cellular constituents on the activity of the enzyme and the inhibition by PEP were examined. The concentration of PEP required for inhibition was extremely

low; however, under certain conditions PEP acted as a stimulator.

MATERIALS AND METHODS

Materials. Pea seeds (var. Progress no. 9) were purchased from F. Cooper Ltd., Wellington, New Zealand. The following chemicals and enzymes were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.: sodium salts of F6P, FDP, AMP, ADP, ATP, β -NADH and PEP, 3',5'-(cyclic)-AMP, imidazole, and suspensions in $(NH_4)_2SO_4$ of α -glycerophosphate dehydrogenase, aldolase and triose phosphate isomerase. All other reagents were A.R. grade. Concentrations of F6P, FDP, ATP, ADP and PEP in solutions were determined by enzymic assays. Concentrations of AMP and 3',5'-(cyclic)-AMP were determined by measuring the E_{260} . Enzymes used in assays were dialysed against 5 mM-imidazole-HCl buffer, pH 7.6, before use to remove $(NH_4)_2SO_4$.

Partial purification of pea-seed PFK. Pea seeds were finely ground and the powder was extracted with ether as described by Turner (1957). Defatted pea powder (75g.) was suspended in 225 ml. of a solution (pH 8.2) containing 50 mM- $NaHCO_3$, 1 mM-EDTA and 5 mM- $MgCl_2$ and rotated for 2 hr. on a mechanical roller. The mixture was centrifuged at 15000g for 15 min.; particulate matter was removed from the supernatant by filtration through glass wool and the pH adjusted from 7.0 to 7.8. The supernatant, after centrifugation at 30000g for 20 min., was termed the crude enzyme. All operations, unless otherwise stated, were carried out at 2°.

Purification of the enzyme involved fractionation with ethanol, followed by fractionation with $(NH_4)_2SO_4$. The crude enzyme was cooled in a solid CO_2 -acetone bath and stirred continuously while ethanol at -30° was added dropwise. The ethanol concentration was increased to 28% (v/v)

* Abbreviations: PFK, phosphofructokinase; PEP, phosphoenolpyruvate; F6P, D-fructose 6-phosphate; FDP, D-fructose 1,6-diphosphate.

and the precipitate removed by centrifugation at 27000g for 13 min. The ethanol concentration of the clear supernatant was increased to 35% (v/v) and the mixture was centrifuged at 27000g for 13 min. The precipitate was dissolved in 40 ml. of a solution containing 50 mM-imidazole-HCl buffer, pH 7.6, 5 mM-MgCl₂, 1 mM-EDTA, 0.1 mM-FDP and 0.1 mM-ATP. The preparation at this stage showed a sevenfold increase in relative activity over the crude enzyme.

The preparation, after ethanol fractionation, was treated with saturated (NH₄)₂SO₄ (pH 7.6) and centrifuged at 30000g for 20 min. The (NH₄)₂SO₄ solution was saturated at 2°. The fraction precipitated between 28% and 35% saturation was dissolved in 7.5 ml. of the imidazole buffer solution used in the ethanol fractionation and left overnight before dialysis on a rocking dialyser for 2 hr. against 5 mM-imidazole-HCl buffer, pH 7.6. This purified preparation, which showed a 27-fold increase in relative activity over the crude enzyme, was used for the enzyme kinetic studies. During the first 6 hr. after dialysis there was a 20% loss in enzyme activity but no further loss occurred during storage for 20 days at 2°.

The purified preparation was essentially free from interfering enzymes. Phosphatases acting on F6P, FDP and PEP were absent and phosphohexose isomerase and adenosine triphosphatase activities were negligible. Some adenylate kinase was present but was calculated to have no significant effect on measurements of PFK activity.

Assay of PFK activity. PFK activity was assayed by coupling the production of FDP with the oxidation of NADH through aldolase, triose phosphate isomerase and α -glycerophosphate dehydrogenase. Reaction mixtures for the standard assay contained, in a total volume of 3.0 ml., 60 μ moles of imidazole-HCl buffer, pH 7.6, 4.8 μ moles of MgCl₂, 10.5 μ moles of thioglycollate, 1.5 μ moles of F6P, 0.75 μ mole of ATP, 0.42 μ mole of β -NADH, 50 μ g. of aldolase, 5 μ g. of triose phosphate isomerase, 10 μ g. of α -glycerophosphate dehydrogenase, and 50 μ l. (containing approx. 50 μ g. of protein) of a fivefold dilution of the purified enzyme preparation. The reaction was started by the addition of ATP. The final content of sulphate in the standard assay was adjusted to 0.18 μ mole. Sulphate was determined by a modification of the turbidimetric method described by Snell & Snell (1959).

The reaction mixtures were maintained at 28°. The decrease in E_{340} was followed in a Unicam SP.800A spectrophotometer with an SP.850 scale-expansion accessory, connected to a Varian G-2000 recorder. The reaction velocity of the standard assay was constant for at least 10 min. No induction period was observed after starting the reaction. The reaction velocity was directly proportional to the amount of PFK added for PFK activities up to 5 times the highest reported in this paper. No increase in rate was obtained with a fivefold increase in the amounts of coupling enzymes added. No inhibition by PEP (in concentrations up to 50 μ M) was observed when FDP was incubated with the coupling enzymes in the absence of PFK.

RESULTS

Pea-seed PFK was more susceptible to inhibition by PEP when concentrations of stimulators were low and, in initial studies, stimulators were omitted

Fig. 1. Effect of PEP concentration on the inhibition of PFK activity. The reaction mixtures were as described for the standard assay. The concentration of PEP was varied as shown.

as far as possible. Apart from F6P and Mg²⁺, sulphate (final concn. 60 μ M) was the only stimulator present. This concentration of sulphate was necessary for stability of the PFK. Double-reciprocal (Lineweaver-Burk) plots obtained under these conditions were curves either convex or concave to the 1/[substrate] axis. The addition of phosphate (final concn. 0.5 mM), although increasing the amount of PEP required for inhibition, usually produced less complicated kinetics.

Inhibition by PEP. The inhibition of pea-seed PFK by increasing concentrations of PEP is shown in Fig. 1. At the higher concentrations of PEP (4 μ M) complete inhibition of the enzyme was approached. An inhibition of 50% was obtained with 1.3 μ M-PEP. In the assay system this was equivalent to 1.0 μ M-free PEP, after calculation from a stability constant of 180 for the MgPEP-complex (Wold & Ballou, 1957). In a previous study (Kelly & Turner, 1968) pea-seed PFK was inhibited 50% by 14 μ M-PEP. The lower sensitivity of the enzyme to PEP in this earlier work was probably due to a higher sulphate content in the reaction mixtures.

The sigmoid curve in Fig. 1 is typical of allosteric inhibition, in which more than one molecule of inhibitor co-operatively binds to the enzyme molecule (Stadtman, 1966). Co-operativity occurs when the binding of one molecule to the enzyme in some manner facilitates the binding of the next. An indication of the degree of co-operativity may be obtained from the Hill plot (Monod, Changeux & Jacob, 1963) shown in Fig. 2. The slope of the line is 2.5.

The PEP preparation used in these experiments was hydrolysed by heating with M-hydrochloric

Fig. 2. Hill plot of the results shown in Fig. 1. v_0 is the rate in the absence of PEP; v is the rate with the different concentrations of PEP. The slope of the line is 2.5.

Fig. 3. Effect of pH on PFK activity in absence and presence of PEP. The reaction mixtures were as described for the standard assay, with the pH of the imidazole buffer varied as shown. $\circ$, No PEP; $\bullet$, $1.4\mu\text{M}$ -PEP.

acid at 100° for 40 min. and neutralized. No inhibition of PFK activity was given by hydrolysed PEP.

Effect of pH. Change in pH affected PFK activity, as shown in the upper curve of Fig. 3. Activity decreased from either direction as pH 7.5

Fig. 4. Effect of F6P concentration on the inhibition of PFK by PEP. The reaction mixtures were as described for the standard assay with the concentration of F6P varied as shown. The concentration of PEP was $1.4\mu\text{M}$.

was approached. This biphasic pH curve is essentially similar to that reported for pea-seed PFK by Axelrod, Saltman, Bandurski & Baker (1952). The lower curve of Fig. 3 shows the change with pH in activity of the PEP-inhibited enzyme. The difference between the two sets of values gave the inhibition due to PEP as a function of pH. With $1.4\mu\text{M}$ -PEP, the maximum inhibition (62%) was at pH 7.5; this fell sharply to 18% at pH 7.85 and increased again as pH 9 was approached.

In separate experiments it was found that varying the PEP concentration at different pH values gave results in agreement with those presented in Fig. 3. For example, $1.1\mu\text{M}$ -PEP was required for 50% inhibition of PFK at pH 8.4, but $3.6\mu\text{M}$ -PEP was necessary for the same inhibition at pH 6.6.

Effect of concentration of F6P. Increasing the concentration of F6P decreased the inhibition of PFK by PEP (Fig. 4). This is supported by the results of the experiments (Table 1) in which the concentration of PEP necessary for 50% inhibition of PFK was increased by increasing the F6P concentration in the reaction mixtures.

Double-reciprocal plots of F6P concentration versus reaction velocity gave a curve convex to the $1/[\text{F6P}]$ axis in the absence of PEP and a curve concave to the $1/[\text{F6P}]$ axis in the presence of PEP. With the addition of phosphate (final concn. 0.5mM) to the reaction mixtures, the convex curve became a straight line but the concave curve remained. Hill plots from assays containing phosphate gave a slope of 1.0 for the uninhibited enzyme and a slope of 1.7 with PEP inhibition. This indicated that, with added phosphate, co-operative interaction between F6P-binding sites was absent, but was introduced by PEP inhibition. Blangy *et*

Table 1. *Effect of concentration of substrates and magnesium chloride on the inhibition of pea-seed PFK by PEP*

The reaction mixtures were as described for the standard assay, with the substrates or MgCl_2 varied as shown. The inhibitions produced by at least seven different concentrations of PEP were determined and the values given below were obtained from plots of the types shown in Figs. 1 and 2.

Compound	Final concn. (mm)	PEP concn. required for 50% inhibition (μM)	Slope of Hill plot for PEP
F6P	0.2	0.5	1.9
	0.5	1.3	2.5
	1.5	2.1	1.9
ATP	0.02	1.3	2.5
	0.25	1.3	2.5
	1.0	0.9	2.5
MgCl_2	0.32	0.2	1.9
	1.6	1.3	2.5
	8.0	12.2	2.8

al. (1968) found that PFK from *E. coli* showed co-operativity for F6P in the absence of PEP, and this decreased as the PEP concentration was increased. Phosphate was not added in the experiments of Blangy *et al.* (1968).

The slopes of Hill plots obtained when the PEP concentration was varied with different concentrations of F6P did not show any simple effect of F6P on the co-operativity of the PEP inhibition (Table 1).

Effect of concentration of ATP and Mg^{2+} . In experiments with reaction mixtures with magnesium chloride concentrations between 0.2 mm and 1.6 mm and a range of ATP concentrations, pea-seed PFK was inhibited when the concentration of ATP exceeded that of magnesium chloride. In another series of experiments in which the ratio of the concentration of magnesium chloride to that of ATP was 10:1, no inhibition was obtained even when the concentration of ATP was as high as 5 mm. As the stability constant for the MgATP^{2-} complex is very high (O'Sullivan & Perrin, 1964), these results suggested that free ATP was the inhibitor of PFK. High concentrations of ATP inhibit PFK from several plant tissues (Lowry & Passonneau, 1964; Dennis & Coultate, 1966, 1967; Garrard & Humphreys, 1968).

The effect of ATP concentration on the extent of PEP inhibition of pea-seed PFK is shown in Fig. 5. Increasing the ATP concentration towards 0.02 mm increased the degree of PEP inhibition, but no further effect was observed until the concentration of ATP exceeded 1 mm. The increased inhibition at higher concentrations of ATP may have been at

Fig. 5. *Effect of ATP concentration on the inhibition of PFK by PEP.* The reaction mixtures were as described for the standard assay, with the concentration of ATP varied as shown. The concentration of PEP was $1.4 \mu\text{M}$.

least partly due to an increase in the concentration of free PEP (as opposed to MgPEP^-) after the removal of Mg^{2+} by ATP.

Kinetic analyses of the results obtained from reaction mixtures containing a range of ATP concentrations up to 1.0 mm (magnesium chloride concentration 1.6 mm) gave non-linear double-reciprocal plots convex to the $1/[\text{ATP}]$ axis at high ATP concentrations with and without PEP. These became straight lines in the presence of 0.5 mm-phosphate, and Hill plots with slopes of approximately 1.0 were obtained whether PEP was present or not. In the experiments shown in Table 1, Hill plots for PEP had slopes of 2.5 at each of the three ATP concentrations tested. Thus ATP did not affect the degree of co-operativity shown in PEP inhibition.

Increasing the concentration of Mg^{2+} reversed the PEP inhibition of pea-seed PFK (Fig. 6). Calculation, from a stability constant for the MgPEP^- complex of 180 (Wold & Ballou, 1957), showed that this reversal of the inhibition could not be due solely to complex-formation of PEP with Mg^{2+} . An additional effect at the enzyme level presumably operated. Increasing the concentration of magnesium chloride increased the PEP concentration required for 50% inhibition of PFK activity (Table 1). Blangy *et al.* (1968) reported that Mg^{2+} was unable to relieve the PEP inhibition of *E. coli* PFK.

Double-reciprocal plots obtained from varying the magnesium chloride concentration were curves concave to the $1/[\text{MgCl}_2]$ axis in both the presence and the absence of PEP. When Hill plots for magnesium chloride were obtained from assays containing 0.5 mm-phosphate, the slope was 2.3 whether

Fig. 6. Effect of MgCl₂ concentration on the inhibition of PFK by PEP. The reaction mixtures were as described for the standard assay, with the concentration of MgCl₂ varied as shown. The concentration of PEP was 1.4 μM.

Fig. 8. Effect of AMP concentration on PFK activity in absence and presence of PEP. The reaction mixtures were as described for the standard assay, with the addition of AMP as shown. ○, No PEP; ●, 1.4 μM-PEP.

Fig. 7. Effect of ADP concentration on PFK activity in absence and presence of PEP. The reaction mixtures were as described for the standard assay, with the addition of ADP as shown. ○, No PEP; ●, 1.4 μM-PEP.

Table 2. Effect of modifiers on the inhibition of pea-seed PFK by PEP

The reaction mixtures were as described for the standard assay. Modifiers were added as shown. The inhibitions produced by at least seven different concentrations of PEP were determined and the values given below were obtained from plots of the types shown in Figs. 1 and 2.

Modifier	Final concn. (mM)	PEP concn. required for 50% inhibition (μM)	Slope of Hill plot for PEP
None	—	1.3	2.5
ADP	0.39	1.1	2.1
	0.54	0.6	1.5
AMP	0.6	1.0	1.9
	1.6	0.5	1.6
Citrate	0.4	0.7	1.4
	1.0	0.5	1.3
Phosphate	0.08	3.7	4.0
	0.5	17	4.1

PEP was present or not. This indicated that PEP had no effect on the co-operativity of the activation of the enzyme by Mg²⁺ ions.

When concentrations of ATP and magnesium chloride were increased simultaneously (keeping the concentration ratio 1:10) it was found that, as with increasing magnesium chloride concentration alone, the inhibition by PEP was relieved.

Effect of other adenosine phosphates. Pea-seed PFK was inhibited by ADP, and ADP also enhanced the inhibition produced by 1.4 μM-PEP (Fig. 7). AMP at low concentrations had no significant effect on PFK activity but increased the PEP inhibition (Fig. 8). Higher concentrations of AMP inhibited PFK to a small extent. Assays with

3',5'-(cyclic)-AMP showed that it had no effect. These results are in agreement with previous observations on plant PFK preparations: carrots, Brussels sprouts and corn contain PFK susceptible to ADP inhibition and, to a lesser extent, to AMP inhibition (Dennis & Coultate, 1966, 1967; Garrard & Humphreys, 1968). Parsley PFK was unaffected by 3',5'-(cyclic)-AMP (Lowry & Passonneau, 1964).

The results in Table 2 provide further evidence of the enhancement of the PEP inhibition of pea-seed PFK by ADP and AMP. Increasing the concentration of these nucleotides decreased the concentration of PEP necessary for inhibition and decreased the co-operativity of the PEP inhibition.

Fig. 9. Effect of citrate concentration on PFK activity in absence and presence of PEP. The reaction mixtures were as described for the standard assay, with the addition of citrate as shown. ○, No PEP; ●, $1.4 \mu\text{M}$ -PEP.

ADP binds Mg^{2+} ions, although the complex is less stable than the MgATP^{2-} complex (O'Sullivan & Perrin, 1964). It is unlikely that the ADP inhibition was due to a lowering of the magnesium chloride concentration to a point where ATP becomes inhibitory: the ADP concentration (0.5 mM) necessary to inhibit the PFK activity of the standard assay by 50% was insufficient for this.

Effect of citrate. The addition of citrate inhibited pea-seed PFK (Fig. 9). The inhibition by citrate was highly co-operative, as shown by the sigmoid form of the curves in Fig. 9. The slopes of the lines obtained from Hill plots of this data were 5.5 without PEP, and 1.9 with PEP. PFK of other plant tissues is inhibited by citrate (Dennis & Coultate, 1966, 1967; Garrard & Humphreys, 1968).

Citrate also increased the inhibition by PEP (Fig. 9). This was confirmed by the results of the experiments shown in Table 2, where the concentration of PEP needed for 50% inhibition of PFK was decreased when citrate was added. As with ADP and AMP, Hill plots showed that citrate decreased the co-operativity of the PEP inhibition.

Effect of anions. The addition of salts containing the anions sulphate or phosphate to the reaction mixtures stimulated the activity of pea-seed PFK and also relieved the inhibition produced by PEP. At a final concentration of 0.5 mM , both sulphate and phosphate (sodium salts) increased the PFK activity by approx. 20%. Phosphate was the more effective in decreasing the inhibition by PEP. A final concentration of 0.05 mM -phosphate almost completely reversed the inhibition produced by $1.4 \mu\text{M}$ -PEP, whereas 0.3 mM -sulphate was required to produce the same effect.

In the experiments given in Table 2 the addition

Fig. 10. Effect of low concentrations of ATP on the effect of PEP on PFK in the presence of phosphate. The reaction mixtures were as described for the standard assay, with the concentration of ATP varied as shown and the addition of $1.5 \mu\text{moles}$ of phosphate. The concentration of PEP was $15 \mu\text{M}$.

of 0.08 mM - and 0.5 mM -phosphate increased the concentration of PEP required for 50% inhibition of PFK activity from $1.3 \mu\text{M}$ to $3.7 \mu\text{M}$ and $17 \mu\text{M}$ respectively. The slopes of the Hill plots indicated that phosphate increased the co-operativity of the PEP inhibition.

Stimulation of PFK by PEP. In reaction mixtures containing phosphate, a stimulation of pea-seed PFK by PEP was observed when the concentration of ATP was low (Fig. 10). The effect of PEP showed a gradual transition from inhibition to stimulation as the ATP concentration was decreased below $40 \mu\text{M}$.

This stimulation was not obtained if phosphate was omitted from the reaction mixtures, although the extent of the inhibition by PEP decreased when the ATP concentration was decreased to low values (Fig. 5).

DISCUSSION

The results of the present investigation suggest that PEP is a potentially powerful modifier of pea-seed PFK activity. Previous reports of PEP modifying PFK activity indicated much lower sensitivity of the enzyme to PEP. Uyeda & Racker (1965) reported that rabbit muscle PFK required PEP concentrations of 1 mM and 2 mM for inhibitions of 41% and 66% respectively. Lou & Kinoshita (1967) showed calf lens PFK was inhibited 8% by 2.5 mM -PEP. Several workers found no effect of

PEP on PFK preparations, or assayed the enzyme in the presence of PEP (Passonneau & Lowry, 1962; Lowry & Passonneau, 1964; Viñuela, Salas & Sols, 1963; Underwood & Newsholme, 1967; Baumann & Wright, 1968; Shen, Fall, Walton & Atkinson, 1968).

Blangy *et al.* (1968) described PEP inhibition of *E. coli* PFK and gave results that indicated 50% inhibition with PEP concentrations between 0.2 mM and 0.5 mM, depending on the concentration of F6P. The inhibition was co-operative, and this was accounted for by the concerted-transition theory proposed by Monod, Wyman & Changeux (1965). According to this model, PEP binds to the PFK molecule, effectively inducing a change in the molecular configuration, and reducing the affinity of the catalytic site for the substrates. The PEP inhibition of pea-seed PFK was co-operative, and a similar mechanism may be applicable to the pea-seed enzyme. Other interpretations may be possible.

Preliminary experiments in this laboratory have shown PEP inhibition of PFK preparations from wheat grains and parsley and silver-beet leaves. It is possible that PEP regulation of PFK may be a common property of the plant enzyme, but the relevance of this to the control of carbohydrate metabolism within the cell cannot be fully assessed until more information is available. Barker, Khan & Solomos (1967) found that the PEP content of mature green peas was 8 μ moles/100g. fresh wt. This concentration of PEP could totally inhibit pea-seed PFK. However, modifiers relieving the inhibition would almost certainly be present but these, as well as PEP, may be subject to compartmentation.

Glycolytic control has been studied recently in several plant tissues. Garrard & Humphreys (1968) found no evidence to support a regulatory role for PFK in glycolysis in corn scutellum. Givan (1968) suggested that ATP inhibition of PFK was a controlling factor in glycolysis in cells of *Acer pseudo-platanus* under aerobic conditions. Unfortunately, changes in PEP content were not measured in either of these investigations. Barker *et al.* (1967) followed changes in a comprehensive series of glycolytic intermediates in pea seeds transferred from air to nitrogen and suggested that the enzymes of glycolysis may be confined to an organized structure showing a difference in accessibility for ADP and ATP. The authors observed a rapid fall in the PEP content, which coincided with the development of the Pasteur effect, and shortly after this the FDP content of the pea seeds increased. It is not inconceivable that relief of a PEP inhibition of PFK may have played some part in this system.

Although the significance *in vivo* of the PEP regulation of PFK is not known, the present study

in vitro suggests that the phenomenon could be of significance as a control factor. The concentrations of PEP required to alter the PFK activity are very low and a large number of interactions occur between PEP and other modifiers of the enzyme activity. It may also be significant that the change from inhibition to stimulation by PEP is favoured by low ATP and high phosphate concentrations, conditions that, *in vivo*, would probably be associated with accelerated glycolysis.

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