Antagonistic effects of hexose 1,6-bisphosphates and fructose 2,6-bisphosphate on the activity of 6-phosphofructokinase purified from honey-bee flight muscle

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6-Phosphofructokinase purified from honey-bee flight muscle is inhibited by ATP and, unusually, by glucose 1,6-bisphosphate and fructose 1,6-bisphosphate. The inhibition by either of the bisphosphates is not relieved by AMP, but is relieved by fructose 6-phosphate and especially by fructose 2,6-bisphosphate. Lack of effect by AMP is consistent with a low activity of adenylate kinase in this muscle.

INTRODUCTION

The conversion of fructose 6-phosphate into fructose 1,6-bisphosphate is catalysed by the enzyme 6-phosphofructokinase (EC 2.7.1.11), and it has been shown that its activity is regulated by factors other than the concentration of fructose 6-phosphate, which may include AMP, NH_4^+ , phosphate, ATP, AMP, fructose 1,6-bisphosphate and glucose 1,6-bisphosphate (see Ramaiah, 1974; Sugden & Newsholme, 1975; Beitner, 1979; Uyeda, 1979; Newsholme & Leech, 1983). In recent years, 6-phosphofructokinase from a variety of tissues has been shown to be activated by fructose 2,6-bisphosphate, which is perhaps the most potent activator of the mammalian enzyme (Van Schaftingen *et al.*, 1980; Hers & Van Schaftingen, 1982). There is considerable evidence that fructose 2,6-bisphosphate plays an important role in the regulation of the rates of glycolysis and/or gluconeogenesis in liver; thus fructose 2,6-phosphate activates 6-phosphofructokinase but inhibits fructose-1,6-bisphosphatase to provide control over the direction of glucose metabolism in this tissue (see Hers & Hue, 1983). However, in mammalian muscle, evidence has been obtained to suggest that fructose 2,6-bisphosphate, under some conditions, leads to activation of both 6-phosphofructokinase and fructose-1,6-bisphosphatases, so that the rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate would increase; for example, adrenaline increases the concentration of fructose 2,6-bisphosphate in muscle and increases the flux through both of these enzymes (Bosca *et al.*, 1985).

In some insect flight muscles, the rate of glycolysis from glucose increases more than 100-fold upon the initiation of flight (see Beenakkers *et al.*, 1984, for review), and it has been suggested that substrate cycles between glucose and glucose 6-phosphate and fructose 6-phosphate and fructose 1,6-bisphosphate might be required to increase the sensitivity of the hexokinase and 6-phosphofructokinase reactions, respectively, to changes in concentrations of effectors of these enzymes (Surholt & Newsholme, 1981); flight of the moth *Acherontia atropos* increases the rate of the glucose/glucose 6-phosphate cycle more than 60-fold (Surholt & Newsholme, 1983). Somewhat surprisingly, the flight muscle of the honey-bee *Apis mellifica* possesses no

detectable fructose-1,6-bisphosphatase (Newsholme & Crabtree, 1973), although this muscle depends almost exclusively on carbohydrate oxidation for energy generation. Hence no substrate cycle is possible at this reaction, and some other mechanism for improving the sensitivity of the control mechanism at the 6-phosphofructokinase reaction should be present. It was for this reason that the properties of the enzyme from honey-bee flight muscle were investigated.

MATERIALS AND METHODS

Honey-bees (*Apis mellifica carnica*, collecting worker bees) were obtained from hives in the Department of Zoology, University of Mainz, Germany. Chemicals, biochemicals and enzymes were obtained from sources given previously (Bosca *et al.*, 1985).

6-Phosphofructokinase from flight muscle of *Apis mellifica* was purified to a specific activity of about 130 $\mu\text{mol/min}$ per mg of protein by $(\text{NH}_4)_2\text{SO}_4$ fractionation and successive chromatography on spherical hydroxyapatite (obtained from Merck, Darmstadt, Germany) and Bio-Gel HT (from Bio-Rad, Munich, Germany). The columns were eluted by potassium phosphate (forming linear gradients of 5–50 mM and 30–100 mM respectively) plus 5 mM-mercaptoethanol. During the whole purification procedure the pH was kept at 7.5 and 1 mM-fructose 6-phosphate and 3.5 mM-glucose 6-phosphate were included in all media in order to stabilize enzyme activity. The enzyme was concentrated by ultrafiltration and proved stable for several days at 4 °C.

The activity of 6-phosphofructokinase was measured spectrophotometrically at 25 °C and at pH 7.2 by monitoring either the rate of formation of fructose 1,6-bisphosphate (assay system I) or the rate of formation of ADP (assay system II). All enzyme activities are given in units, with 1 unit corresponding to 1 μmol of substrate transformed/min at 25 °C. Both assay systems contained, in a final volume of 500 μl , 50 mM-triethanolamine/HCl, 50 mM-KCl, 2 mM- MgCl_2 , 0.1–0.2 mM-NADH, 5 μg (1.7 units) of phosphoglucose isomerase, and fructose 6-phosphate, ATP and AMP, the concentrations of which are given in the Figures. ATP was always added as Mg-ATP, which had been

Fig. 1. Effect of the concentration of Mg-ATP on the activity of purified 6-phosphofructokinase from the flight muscle of the honey-bee

The activity of 6-phosphofructokinase was measured in assay system I. The assay medium contained 0.1 mM-fructose 6-phosphate, 0.1 mM-AMP and 4 mM-phosphate. Addition of 2 mM-fructose 6-phosphate yielded a $V_{\max}$ of 0.8 $\mu\text{mol/min per ml}$.

neutralized with NaHCO_3 ; fructose 6-phosphate was given together with a 3.5-fold surplus of glucose 6-phosphate to buffer the substrate concentration. Assay system I contained 100 μg (0.9 unit) of aldolase, 40 μg (6.8 units) of glycerol 3-phosphate dehydrogenase and 1 μg (5 units) of triosephosphate isomerase; in addition an ATP-regenerating system was often used, which comprised 10 mM-phosphocreatine and 20 μg (5 units) of creatine kinase per assay. Assay system II contained 1 mM-phosphoenolpyruvate, 10 μg (2 units) of pyruvate kinase and 10 μg (5.5 units) of lactate dehydrogenase. All auxiliary enzymes were extensively dialysed before use. Adenylate kinase was assayed as described by Leech & Newsholme (1978).

RESULTS AND DISCUSSION

In general, ATP is known to be a potent inhibitor of animal 6-phosphofructokinase and, for the enzyme from many muscles, the ATP inhibition is enhanced by phosphocreatine (or phosphoarginine) and by citrate, both of which probably play a major role in decreasing the activity of this enzyme in resting muscle (Sugden & Newsholme, 1975). The enzyme from honey-bee flight muscle was shown to be inhibited by ATP (Fig. 1), but the flight muscle of the honey-bee contains very little phosphoarginine (Beis & Newsholme, 1975) and citrate does not inhibit 6-phosphofructokinase from flight muscles of various insects (Newsholme *et al.*, 1977), including that of the honey-bee (G. Wegener, unpublished work). In the search for other effectors of the honey-bee enzyme, both glucose 1,6-bisphosphate and fructose 1,6-bisphosphate were found to be potent inhibitors. At approximate physiological concentrations of ATP, AMP, phosphate and fructose 6-phosphate, about 80 μM -glucose 1,6-bisphosphate or about 20 μM -fructose

1,6-bisphosphate causes 50% inhibition of the enzyme (Fig. 2).

This is the first report of a purified animal 6-phosphofructokinase that is strongly inhibited by either fructose 1,6-bisphosphate or glucose 1,6-bisphosphate; the enzyme from many other sources is considered to be activated by one or both of these compounds (see the Introduction). In mammalian phosphofructokinases, e.g. from erythrocytes (Heylen *et al.*, 1982) or muscle (Tornheim, 1985), and in the yeast enzyme (Bartrons *et al.*, 1982), the activity can be lowered by fructose 1,6-bisphosphate, under certain conditions. However, in these cases, fructose 1,6-bisphosphate is itself not an inhibitor, but counteracts the stimulatory effect of added fructose 2,6-bisphosphate. Phosphofructokinase from tumour cells has been found not to be affected by fructose 1,6-bisphosphate (Bosca *et al.*, 1982).

AMP is largely ineffective in removing the inhibition by glucose 1,6-bisphosphate or fructose 1,6-bisphosphate; thus, increasing the AMP concentration from 1 to 15 mM had no effect on these inhibitions. This observation suggests that in honey-bee flight muscle, unlike other muscles, changes in the AMP concentration will be of little or no importance in regulating the activity of

Fig. 2. Effect of the concentration of glucose 1,6-bisphosphate or fructose 1,6-bisphosphate on the activity of purified 6-phosphofructokinase from the flight muscle of the honey-bee

The activity of 6-phosphofructokinase was measured by using assay system I, for the effect of glucose 1,6-bisphosphate (○), and assay system II, for the effect of fructose 1,6-bisphosphate (●). The assay systems contained 0.1 mM-fructose 6-phosphate, 5 mM-ATP, 1 mM-AMP, 1 mM-phosphate and an ATP-regenerating system. The activity in the absence of either bisphosphate was about 70% of maximum activity (1.4 $\mu\text{mol/min per ml}$), which could be attained at any concentration of bisphosphate by addition of 10 mM-fructose 6-phosphate.

Table 1. Maximal activities of adenylate kinase and the calculated rate of ATP turnover in muscles from insects and birds

The activity of adenylate kinase and the calculated rate of ATP turnover are expressed as $\mu\text{mol}/\text{min}$ per g fresh wt. of muscle at 25 °C. Results are expressed as means $\pm$ S.E.M. or range of activities, with the numbers of different animals used given in parentheses. Data for calculated maximum rate of ATP turnover are from Newsholme *et al.* (1978).

Animal	Muscle	Adenylate kinase activity	Calculated maximum rate of ATP turnover
Locust (<i>Schistocerca gregaria</i>)	Flight	83 ± 9.4 (7)	352
	Femoral	303 (259–356) (3)	—
Yellow under-wing moth (<i>Noctua pronuba</i>)	Flight	277 ± 51 (4)	637
Giant water-bug (<i>Lethocerus cordofanus</i>)	Flight	591 ± 58 (5)	—
Bumble-bee (<i>Bombus agrorum</i>)	Flight	120 (107–130) (3)	1017
Honey-bee (<i>Apis mellifica</i>)	Flight	49 (41–54) (3)	954
Pheasant (<i>Phasianus colchicus</i>)	Pectoralis major	419 (413–425) (3)	429
Pigeon (<i>Columba livia</i>)	Pectoralis major	316 (277–348)	63

6-phosphofructokinase as part of a mechanism for regulation of glycolytic flux in relation to the demand for energy. Indeed, it is likely that changes in AMP concentration in the flight muscle of the honey-bee will be very small during flight: thus the AMP concentration in the muscle of the resting bee is very high (Beis & Newsholme, 1975), and the activity of adenylate kinase in honey-bee flight muscle is low in comparison with that in other muscles and in comparison with the rate of ATP turnover during flight (see Table 1). The role of adenylate kinase in muscle and other tissues is considered to be to catalyse rapidly the near-equilibrium between ATP, ADP and AMP, such that a small change in the ATP/ADP concentration ratio will produce a much larger change in that of AMP, which in turn would be expected to increase the activity of 6-phosphofructokinase as part of an overall mechanism to increase the rate of ATP formation in response to an increase in demand for ATP (Newsholme & Start, 1973). A high activity of adenylate kinase would be necessary to ensure that the reaction was maintained close to equilibrium and that the reaction could respond rapidly to a change in the ATP/ADP concentration ratio. As shown in Table 1, the maximum activity of adenylate kinase is much lower than the maximum rate of ATP turnover for flight muscles of the honey-bee and bumble-bee. For other muscles, except the locust, the activities are similar to or greater than the maximum calculated rate of ATP turnover. The apparent lack of an effector role of AMP for honey-bee flight muscle 6-phosphofructokinase, at physiological concentrations of substrate and product, led to a search for other activators or de/inhibitors of this enzyme.

Two factors were discovered that would decrease the extent of inhibition of the honey-bee flight muscle enzyme glucose 1,6-bisphosphate or fructose 1,6-bisphosphate; these were the substrate, fructose 6-phosphate, and particularly fructose 2,6-bisphosphate. For example, the inhibition produced by a high concentration (0.3 mM) of glucose 1,6-bisphosphate was relieved by 50% by about 8 μM -fructose 2,6-bisphosphate (see Fig. 3). The concentration of fructose 2,6-bisphosphate in flight muscle of honey-bees not in flight was found to be 4.4 ± 0.73 nmol/g fresh wt., or about 8 μM in the

Fig. 3. Effect of the concentration of fructose 2,6-bisphosphate on the activity of purified 6-phosphofructokinase from the flight muscle of the honey-bee, inhibited by 0.3 mM-glucose 1,6-bisphosphate

The activity of 6-phosphofructokinase was measured by using assay system I. The assay system contained 0.1 mM-fructose 6-phosphate, 5 mM-ATP, 1 mM-AMP, 1 mM-phosphate, 0.3 mM-glucose 1,6-bisphosphate and an ATP-regenerating system. In the absence of fructose 2,6-bisphosphate, the enzyme exhibited only 10% of its maximal activity. Maximum activity could be achieved by addition of 10 mM-fructose 6-phosphate, and was 1.3 $\mu\text{mol}/\text{min}$ per ml.

intracellular fluid. Storey (1983, 1985) has shown that, for 6-phosphofructokinase from cockroach flight muscle, fructose 2,6-bisphosphate decreases the $s_{0.5}$ and the extent of ATP inhibition and has a very similar effect to that on the mammalian enzyme (see Hers & Van Schaftingen, 1982). It is suggested that, in resting honey-bees, the activity of flight muscle 6-phosphofructokinase is maintained very low by the effects of ATP and/or

fructose 1,6-bisphosphate plus glucose 1,6-bisphosphate, but that, on initiation of flight, these effects are relieved by increased concentrations of both fructose 6-phosphate and fructose 2,6-bisphosphate. Storey (1983) has shown that the concentration of fructose 2,6-bisphosphate in cockroach flight muscle is about 2-fold higher when the insects are forced to fly compared with those maintained at rest. It is not known how the concentration of fructose 2,6-bisphosphate is regulated in honey-bee flight muscle, but an increase in the concentrations of fructose 6-phosphate would be expected to increase not only the activity of 6-phosphofructo-1-kinase, which catalyses the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate, but also that of 6-phosphofructo-2-kinase, which catalyses the conversion of fructose 6-phosphate into fructose 2,6-bisphosphate. The concentration of fructose 6-phosphate, which is low in resting honey-bee flight muscle (Beis & Newsholme, 1975), might be expected to increase dramatically upon the initiation of flight, since the rate of glucose phosphorylation in this muscle may be controlled by a substrate cycle between glucose and glucose 6-phosphate (Surholt & Newsholme, 1981). In addition, it is tempting to speculate that the different mechanism of control of 6-phosphofructokinase in honey-bee flight muscle in comparison with that in other insects [e.g. cockroach (Storey, 1983, 1985) and locust (Wegener *et al.*, 1986)] is related to the social life of this insect.

We are grateful to the late Professor R. R. Porter, F.R.S., for his interest and encouragement. The work was started during a sabbatical leave to Oxford (G.W.), which was facilitated by the support of Professor K. Urich (Mainz) and by financial support from the Deutsche Forschungsgemeinschaft (Bonn; grant We 494/5-1). We are indebted to Professor E. Thomas for providing the honey-bees. The excellent technical assistance from Heike Bender is gratefully acknowledged.

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Received 13 January 1986/14 March 1986; accepted 17 April 1986