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PURIFICATION AND PROPERTIES OF FRUCTOSE DIPHOSPHATASE FROM BUMBLEBEE FLIGHT MUSCLE

ROLE OF THE ENZYME IN CONTROL OF SUBSTRATE CYCLING

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Summary

1. A study of the kinetic, electrophoretic, and thermal properties of bumblebee (*Bombus terrestris*) flight muscle fructose diphosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) was undertaken in an effort to elucidate the metabolic controls of fructose-6-*P* substrate cycling in vivo.

2. After exposure to conditions known to produce different rates of fructose-6-*P* cycling in vivo, groups of bumblebees were quick frozen and the properties of fructose diphosphatase from each group examined. In all groups the physical properties of the enzyme remained constant: enzyme activity remained in the soluble portion of the cytoplasm, only one isozymic form of the enzyme was present, and the specific activity and p*I* of the enzyme remained constant.

3. Fructose diphosphatase was purified to homogeneity by affinity chromatography and kinetically characterized. Inhibitory control of the enzyme is vested in Ca^{2+} (K_i' 25 μM). Physiological concentrations of Li^+ potentiate this inhibition and lower the K_i' for Ca^{2+} to 8 μM, well within the range of Ca^{2+} levels in vivo. The enzyme is potently and specifically activated by oleate and to a lesser extent by linoleate and phosphatidic acid.

4. The kinetic constants of the purified enzyme were altered by decreasing temperature: the K_m for fructose-1,6-P_2 and the K_a for oleate decreased, the K_i' for Ca^{2+} increased.

5. The data indicate that the activity of fructose diphosphatase (and hence the rate of fructose-6-*P* cycling) is likely to be controlled via metabolite modulation of the enzyme. Physiological concentrations of Ca^{2+} (the effect amplified by the presence of Li^+) in the muscle during flight completely inhibit the activity of the enzyme. The effects of temperature on the catalytic prop-

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Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

erties of fructose diphosphate may be partially responsible for the observed increase in fructose-6-*P* cycling in vivo as the environmental temperature is lowered.

Introduction

The presence of high activities of fructose diphosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1), but not of other gluconeogenic enzymes, in the flight muscles of various species of bumblebee led Newsholme et al. [1] to propose that a fructose diphosphatase:phospho-fructokinase-mediated substrate cycle operated in this muscle. The futile cycling of fructose-6-*P* between these two enzymes, with its attendant hydrolysis of ATP, is believed to have a role in heat production. Clark et al. [2] have shown that the rate of substrate cycling is inversely proportional to ambient temperature (maximal rate at 5° C and close to zero at 27° C) and is completely shut off during flight.

Unlike fructose diphosphatase from other sources, the bumblebee flight muscle enzyme is not inhibited by AMP and it is this property that is thought to allow the simultaneous activity of fructose diphosphatase and phospho-fructokinase in the muscle at rest [1]. Using crude homogenates of flight muscles, it was shown that fructose diphosphatase is inhibited by Ca^{2+} [2] and that the rate of fructose-6-*P* cycling is likely regulated by the changes in sarcoplasmic Ca^{2+} concentrations associated with the contractile process [3]. Fluctuations in Ca^{2+} concentration within the physiological range (10^{-8} M in resting muscle, 10^{-6} M in contracting muscle) [4—6] have been demonstrated to influence the activities of several of the enzymes involved in energy production including: phosphorylase *b* kinase [7], mitochondrial glycerol-3-*P* oxidase [8], and NAD^+-linked isocitrate dehydrogenase [9]. However, the concentration of Ca^{2+} reported to be necessary to inhibit bumblebee flight muscle fructose diphosphatase (K_i 100 μM) [2] is higher than most estimates of intracellular Ca^{2+} concentrations in vivo. Moreover, unlike other enzymes in insect flight muscle which respond to Ca^{2+} concentration in the range of 1 μM or less [10,11], flight muscle fructose diphosphatase was not inhibited until the level of Ca^{2+} exceeded 30 μM [2]. Thus the physiological significance of Ca^{2+} inhibition of fructose diphosphatase in vivo is still subject to question. In addition, enzyme regulation by Ca^{2+} alone does not explain another major facet of fructose-6-*P* substrate cycling: the rate of cycling is temperature dependent.

In this study the role of fructose diphosphatase in substrate cycling was further investigated using enzyme purified to homogeneity from the flight muscle of *Bombus terrestris*.

Materials and Methods

Materials and animals. Substrates, reagents, and coupling enzyme were purchased from Boehringer Mannheim Corp.. Ampholines (pH 3—10) were purchased from LKB Products, Stockholm, Sweden. Phosphocellulose was from Sigma Chemical Co. and inorganics were obtained from Fisher Scientific Co..

Bumblebees, *B. terrestris*, were captured in jam jars from bushes in Oxford during September and October.

Whole animal experiments. Groups of bees were subjected to one of three experimental conditions: (1) flying for 1—2 min at 21°C, (2) resting (flight prevented) for 20 min at 21°C, and (3) resting for 20 min at 5°C. After the required interval, bees were killed by freeze-clamping with tongs cooled in liquid N_2. Frozen flight muscle was teased free of contaminating tissues, weighed, and a crude enzyme homogenate prepared and assayed for fructose diphosphatase and phosphofructokinase activity as described below.

In a separate experiment, groups of bees were subjected to experimental conditions 1, 2, and 3, freeze-clamped after the required interval and crude enzyme homogenates prepared. The crude enzyme was applied to electrofocusing columns (LKB, pH 3—10) and electrophoresis was carried out for 24 h at 4°C [12]. The columns were then drained and assayed for fructose diphosphatase activity as described below.

Enzyme preparation and purification. Bumblebees were decapitated, the flight muscle removed, and the haemolymph blotted away. The muscle was weighed and cut up into homogenizing buffer (20 mM Tris · HCl (pH 8.0)/1 mM EDTA/30 mM β-mercaptoethanol). After homogenization using a ground glass homogenizer, the extract was centrifuged at 44 000 × *g* for 30 min and pellet discarded. The supernatant (which contained 99% of the total fructose diphosphatase activity) was used as the source of crude enzyme.

The purification of fructose diphosphatase followed the method of Black et al. [13]. Heat treatment was omitted, however, as the flight muscle enzyme was found to be very sensitive to heat denaturation. Gel filtration (Sephadex G-200) in homogenization buffer was substituted. The peak fractions were collected and brought to pH 5.8 with solid malonic acid. This solution was applied to a phosphocellulose column [14] equilibrated in 0.1 M malonate buffer (pH 5.8)/1 mM EDTA. The column was washed with equilibration buffer until the A_{280nm} of the eluate dropped to zero. The enzyme was then eluted by the addition of 2 mM fructose-1,6-P_2 to the malonate buffer system. This preparation of fructose diphosphatase was homogeneous as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and contained no contaminating enzymes that would interfere with the basic assay or remove or interconvert any of the added metabolites. The final specific activity of the enzyme was 20 μmol fructose-1,6-P_2 utilized/min per mg protein (pH 7.4, 24°C) in comparison to activities of 28.5 and 35 units/min per mg protein (30°C) for the purified enzyme from rabbit muscle [13] and liver [15], respectively.

Tris · HCl (pH 7.5) and $MgCl_2$ were added to the purified fructose diphosphatase preparation to final concentrations of 50 and 20 mM, respectively. This mixture was incubated for 2 h at room temperature to hydrolyze all traces of fructose-1,6-P_2 and then dialyzed against 10 mM Tris · HCl (pH 8.0) for 24 h at 2°C. The enzyme was stored at −20°C until use.

Protein was measured by the method of Waddell [16].

Assay of enzyme activity. Fructose diphosphatase activity was measured spectrophotometrically by following the rate of $NADP^+$ reduction at 340 nm. Optimal assay conditions consisted of the following (final volume 1 ml):

50 mM Tris · HCl (pH 7.4), 6 mM Mg^{2+} (or 0.2 mM Mn^{2+}), 0.04 mM fructose-1,6-P_2, 0.2 mM $NADP^+$, and excess dialyzed phosphohexoisomerase and glucose-6-*P* dehydrogenase (2 μg each). Reactions were started by the addition of the enzyme preparation, unless otherwise stated. In experiments involving inhibition of enzyme activity, the sampling method of Newsholme et al. [17] was employed to ensure that the metabolite effects were specific for fructose diphosphatase. Kinetic constants were determined from direct linear [18] or double reciprocal [19] plots. Inhibition constants (K_i' or I_{50}) for Ca^{2+} and other inhibitory ions were determined from Hill plots. The values reported are reproducible to within ±5%.

Where temperature was a variable, the pH of the Tris buffer was altered to compensate for the effects of temperature on buffer pH. In studying the effects of Ca^{2+} on fructose diphosphatase activity, Ca^{2+} was added either from a 100 mM stock Ca^{2+} solution (made in double distilled, deionized water) or from a defined EGTA-Ca^{2+} buffer prepared by the method of Portzehl et al. [20]. Both methods yielded essentially identical results, the EGTA added to the assay by the latter method not significantly affecting enzyme activity. In order to improve reproducibility in assays involving the addition of fatty acids, cuvettes were soaked in nitric acid for 10 min between assays, followed by through rinsing with pure water. Assays using fatty acids or their derivatives were started by the addition of fructose-1,6-P_2.

Mitochondrial glycerol-3-*P* oxidase was assayed by the method of Zammit and Newsholme [21]. Phosphofructokinase activity was assayed by the method of Storey and Hochachka [22].

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [23].

Molecular weight determination. Molecular weight determinations on Sephadex G-200 were carried out according to the method of Long and Kaplan [24]. All operations were performed at 4°C. Protein samples were applied to the column in descending order of molecular weight in order to prevent elution overlapping and possible association of proteins. Proteins were eluted in 50 mM Tris · HCl (pH 7.5) and fractions assayed for enzyme activity and/or for absorption at 280 nm. A standard plot of log molecular weight (M_r) versus elution volume was made using the following standards: blue dextran ($M_r \cong 400\,000$), aldolase ($M_r = 158\,000$), beef heart lactate dehydrogenase ($M_r = 142\,000$), ovalbumin ($M_r = 45\,000$), chymotrypsinogen ($M_r = 25\,000$), and ribonuclease ($M_r = 13\,700$).

Measurement of Li^+ concentration in bees. To obtain haemolymph, a small hole was scratched in the head of a live, CO_2-anaesthetized bee and the animal wedged head down in a disposable plastic tip from an Eppendorf pipetter and then placed in a small test tube. This was then spun at low speed in a bench top centrifuge. The haemolymph obtained was clear and free of particulate matter. Flight muscle was dissected out, blotted, weighed, homogenized in two volumes of Li^+-free, distilled, deionized water and then centrifuged at 44 000 × *g*. Li^+ in the haemolymph and muscle homogenates was measured on a Pye Unicam SP800 atomic absorption spectrophotometer. Concentrations were determined by comparison to a standard curve constructed for known concentrations of Li^+.

All solutions used in this study (other than Li^+ and Ca^{2+} stock solutions) were determined to be free of Li^+ (<0.02 ppm) and to contain less than 10^{-9} M Ca^{2+}.

Results

Whole animal experiments

In this study, measurements of specific activity, electrophoretic pattern of enzyme, and subcellular localization of enzyme revealed that there appears to be no physical change in the fructose diphosphatase molecule that correlates with the change in fructose-6-*P* cycling rates accompanying the three experimental regimes used: flying, resting at 21°C, and resting at 5°C. Groups of bees subjected to these three conditions showed no differences in the specific activities of fructose diphosphatase (80 μmol/min per g wet wt.) or of phosphofructokinase (76 μmol/min per g wet wt.). Over 95% of fructose diphosphatase activity in all the bees remained in the soluble cytoplasmic fracton of the cell. In all cases a single isozyme of fructose diphosphatase was found after electrofocusing and no change in position of the band (change in p*I* of the enzyme) or change in number of bands (isozymes) were seen for any group.

Enzyme purification

SDS-polyacrylamide gel electrophoresis and isoelectricfocusing of purifed fructose diphosphatase from flight muscle indicated that the enzyme was homogeneous. Several criteria indicate that the purified enzyme is the native "neutral" enzyme and not the "alkaline" form sometimes produced by proteolytic modification during purification [25]. Firstly, the enzyme occurs as a single band on SDS gels while the degraded enzyme is known to run as a doublet [25]. The ratio of activity at pH 7.4: pH 9.3 remained constant (at 2 : 1) during purification and the optimal pH of the purified enzyme was 8.0, similar to the optimum for purified muscle fructose diphosphatase from other sources [13]. Finally, the molecular weight of the enzyme was determined to be 142 000 ± 10 000 whereas the "alkaline" form of the enzyme typically has a much lower molecular weight [25].

Inorganic ion effects

In common with fructose diphosphatase from other sources [26,27], the bumblebee enzyme shows an absolute requirement for divalent ions. Mg^{2+} (K_m 0.2 mM) and Mn^{2+} (K_m 0.025 mM) can satisfy this requirement but not Ca^{2+}, Fe^{2+}, Co^{2+}, Ba^{2+}, Zn^{2+}, or Cu^{2+}.

Amongst monovalent ions, Na^+ inhibited the enzyme (K_i 40 mM). K^+ and NH_4^+ did not inhibit the enzyme at concentrations of up to 200 mM. Cs^+ did not inhibit at 100 μM. Li^+ strongly and specifically inhibited the purified enzyme (K_i 0.08 mM). Li^+ inhibition of fructose diphosphatase from other sources [1,28] is much less pronounced (K_i values being 10—100-fold higher). SO_4^{2-} also inhibited the enzyme (K_i 40 mM).

Of divalent ions tested, Zn^{2+}, Ca^{2+}, and Cu^{2+} inhibited the purified enzyme with K_i' values of 6, 30, and 500 μM, respectively. For the crude enzyme the K_i' for Ca^{2+} is 3-fold higher at 100 μM. This value is in good agreement with

that obtained by Clark et al. [2]. Excess Mg^{2+} or Mn^{2+} partially reversed Ca^{2+} inhibition and the addition of excess EGTA reactivated the Ca^{2+}-inhibited enzyme. Fig. 1 shows the effects of Ca^{2+} inhibition of fructose diphosphatase in relation to buffer pH.

K_i' (I_{50}) values reported for metal ion inhibition are expressed in terms of total (bound + unbound) ion added to the cuvette and not in terms of free (unbound) ion present in the cuvette. For this reason Ca^{2+} inhibition plots appear sigmoidal (Figs. 2 and 3) and Hill plots tend to be non-linear at very low Ca^{2+} concentrations. The deviation of these low Ca^{2+} points from an ideal straight line can perhaps be used to estimate the amount of bound Ca^{2+} (unavailable for inhibition) in the cuvette. The mean estimate for this bound Ca^{2+} is 2.5 ± 0.5 μM. As an identical amount of homogeneous enzyme was added to each cuvette, this value should be constant in all experiments involving Ca^{2+} inhibition.

Fig. 2 shows the effect of Li^+ in potentiating Ca^{2+} inhibition of the purified enzyme. 30 μM Li^+, while not inhibitory in itself, acts to lower the K_i' for Ca^{2+} over 3-fold to 8 μM. Higher concentrations of Li^+ inhibit the enzyme but do not further lower the K_i' for Ca^{2+}. In contrast, up to 10 mM Na^+ does not alter the K_i' for Ca^{2+}.

For comparison, the effects of added Ca^{2+} were tested on another enzyme of flight muscle known to be regulated by cellular Ca^{2+} levels, glycerol-3-*P* oxidase [9,21]. The activation constant (K_a) for Ca^{2+} of the enzyme in crude homogenates was determined to be 10 μM. Thus Ca^{2+} effects on purified fructose diphosphatase occur not only within the physiological range of Ca^{2+} levels in flight muscle, but also within the same range as Ca^{2+} effects on other flight muscle enzymes.

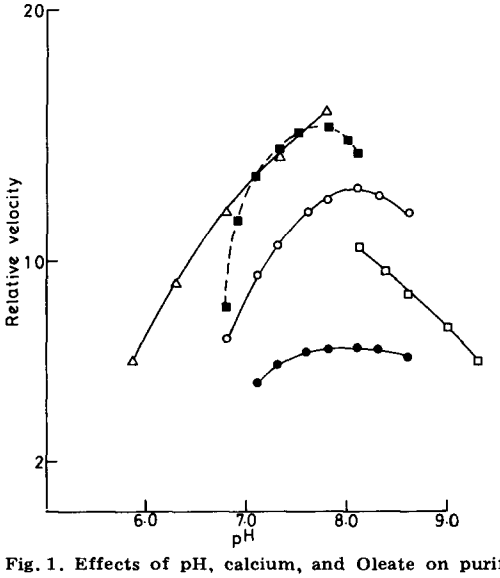

Fig. 1. Effects of pH, calcium, and Oleate on purified bumblebee flight muscle fructose diphophatase. Assay conditions are as in Materials and Methods using Mg^{2+} as cofactor and Ca^{2+} added as a dilution of a 100 mM stock solution. Symbols indicate assay in: ○, 50 mM Tris · HCl; ■, 50 mM Tris · HCl + 30 μM oleate; ●, 50 mM Tris · HCl + 40 μM Ca^{2+}; △, 20 mM Imidazole; □, 20 mM glycine/NaOH.

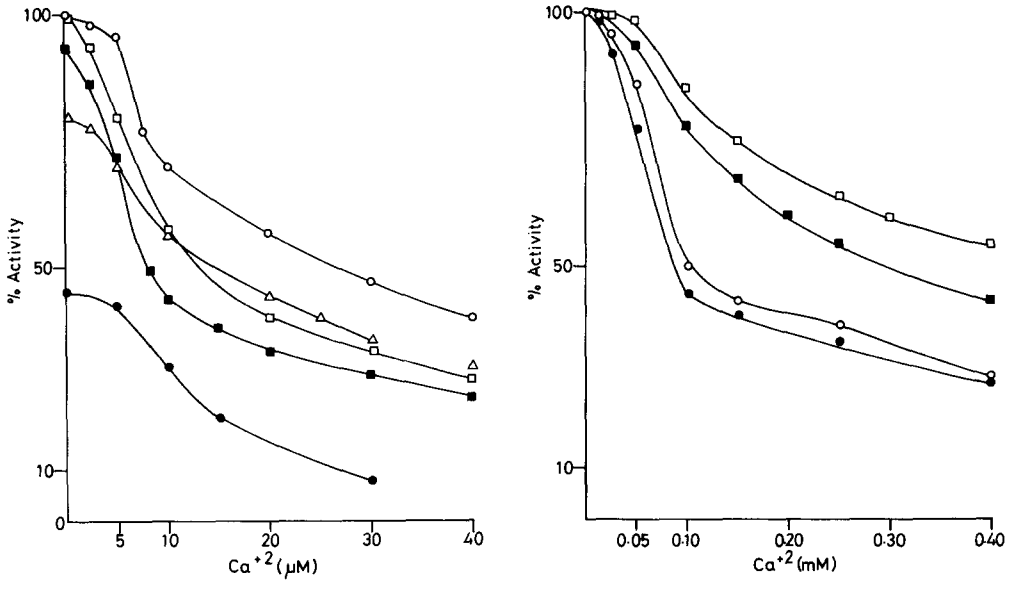

Fig. 2. Effects of Li^+ and Na^+ on Ca^{2+} inhibition of purifed bumblebee flight muscle fructose diphosphatase. Assay conditions are as in Materials and Methods (but at pH 7.1) using Mg^{2+} as cofactor and Ca^{2+} concentrations as indicated. Additions are: ○, none; □, 10 μM LiCl; ■, 30 μM LiCl; ●, 100 μM LiCl; △, 10 mM NaCl. The concentrations of lithium used were checked by atomic absorption.

Fig. 3. Effect of Ca^{2+} and temperature on the activity of crude fructose diphosphatase isolated from bumblebee flight muscle. Assay conditions are as in Materials and Methods (but at pH 7.1) with the pH of the Tris · HCl buffer altered to compensate for temperature effects. ○, ●, Mg^{2+}-activated enzyme at 9 and 35°C, respectively; □, ■, Mn^{2+}-activated enzyme at 9 and 35°C respectively. Similar effects of temperature were measured for the purified enzyme. Activity in the absence of Ca^{2+} is arbitrarily set at 100%.

The effects of Li^+ on Ca^{2+} inhibition of fructose diphosphatase made it necessary to determine Li^+ concentration in flight muscle. Assuming a wet weight to dry weight ratio of 5 : 1 and even distribution of Li^+ in the cells, the concentration of Li^+ in flight muscle was determined to be 25 μM (±20%). Correction for a haemolymph space of 30% in isolated flight muscle [29] (Li^+ concentration was found to be less than 2 μM in haemolymph) raises the calculated intracellular concentration to 30—40 μM, a value well within the range needed to affect Ca^{2+} inhibition of fructose diphosphatase.

Temperature effects

The effect of temperature on the kinetic parameters of fructose diphosphatase was studied in order to determine whether the increase in fructose-6-*P* cycling at low temperatures is due to a direct effect of temperature on enzyme activity. Fig. 3 shows that as reaction temperature is lowered the K_i' for Ca^{2+} of the crude enzyme is increased. The temperature effect is more pronounced with Mn^{2+} as the divalent cation. A slightly more pronounced temperature relationship was found using the purified enzyme (Table I); these data also show that the affinity of the purified enzyme for substrate, fructose-1,6-P_2, is increased with decreasing temperature. Both Mg^{2+}- and Mn^{2+}-stimulated enzymes behave similarly. Since similar changes were seen in affinity param-

TABLE I

THE EFFECT OF TEMPERATURE ON THE KINETIC CONSTANTS OF BUMBLEBEE FLIGHT MUSCLE FRUCTOSE DIPHOSPHATASE

The purified enzyme was assayed as described in Materials and Methods. K_i for Ca^{2+} was determined at pH 7.1.

Kinetic constant (mM)	Divalent cation	Temperature (°C)					
		35	31	27	22	17	9
$K_{m(FDP)}$	Mg^{2+}	0.011	0.011	0.010	0.005	0.004	0.003
$K_{m(FDP)}$	Mn^{2+}	0.008	0.010	0.009	0.008	0.005	0.003
$K_{m(Mg^{2+})}$	—	0.20					0.15
$K_{m(Mn^{2+})}$	—	0.03					0.02
$K_{a(oleate)}$	Mg^{2+} or Mn^{2+}				0.010		0.003
$K_{i(Ca^{2+})}$	Mg^{2+}				0.030		0.017

eters of the crude enzyme, these effects are not likely to be artifacts of purification.

Effect of lipids

The effects of the following substances on purified flight muscle fructose diphosphatase were determined: 10—30 μM palmitic, elaidic, stearic, myristic, arachidonic, linoleic, and phosphatidic acids, oleyl alcohol, deoxycholate, SDS, and palmitoyl carnitine; 1 mM β-hydroxybutyrate, acetoacetate, carnitine, and acetyl carnitine. Only oleic, linoleic, and phosphatidic acids affected enzyme activity; the three compounds activated the enzyme 20—100% in this concentration range and exhibited K_a values of 10, 15, and 0.2 μM, respectively, at 22° C. Oleate was the most effective activator of fructose diphophatase activity, especially at pH 7—8 (Fig. 1). Fructose diphosphatase from other sources also exhibits oleate activation in the neutral pH range [15,20]. The K_a for oleate was decreased 3-fold at 9° C.

The effects of oleate on bumblebee flight muscle phosphofructokinase were also measured. Oleate was inhibitory; the K_i' (25 μM) was in the same range as that determined for phosphofructokinase from other sources [31]. The K_i' for oleate decreased 2-fold at 9° C.

Effects of other metabolites

The following compounds had no effect on the V activity of fructose diphosphatase or on the K_i' for Ca^{2+} at either 17 or 30° C: NTP, NDP, NMP (N = A, U, C, or G) (2 mM), glycolytic intermediates (2 mM) except for fructose-1,6-P_2 (which inhibits at 0.3 mM), tricarboxylic acid cycle intermediates (2 mM), NAD(P)(H) (0.2 mM), arginine, proline, glutamate, alanine, histidine, aspartate, or serine (10 mM), dithiothreitol, glutathione (reduced or oxidized) (2 mM), inorganic phosphate (5 mM), pyrophosphate (1 mM), arginine phosphate (10 mM), acetyl-CoA (0.1 mM), trehalose (5 mM), acetyl phosphate (1 mM), and UDPglucose (0.2 mM).

Discussion

Although modulation of both fructose diphosphatase and phosphofructokinase is likely to be involved in altering the rate of fructose-6-*P* substrate cycling, control at fructose diphosphatase itself appears to be key in regulation under certain conditions, notably flight. Thus, substrate cycling is completely turned off during flight, a result explained by data in this study which shows that the enzyme is inhibited by physiological levels of Ca^{2+} in the presence of Li^{+}. This study also suggests that the temperature dependence of cycling may be regulated by modulation of both component enzymes in concert.

Phosphorylation and adenylation of enzymes to produced essentially "on-off" types of control are well known [32,33] but no data for flight muscle fructose diphosphatase was available in this regard. Initially, then there was the possibility that substrate cycling was wholly or partially controlled by a physical modification of the enzyme molecule to produce an enzyme with altered kinetic properties. Clark et al. [2] determined that the rates of cycling of fructose-6-*P* are 0.0, 0.48, and 10.4 μmol/min per g flight muscle for a bumblebee in flight, resting at 21°C, or resting at 5°C, respectively. In this study, when bees were held under these varying environmental conditions, two parameters which would be indicative of a physical modification of the enzyme, a change in specific activity and a change in electrophoretic pattern or mobility, showed no alteration under any of the environmental conditions. As well, the presence of a single isozyme of fructose diphosphatase in flight muscle rules out differential activity of isozymes as a means of controlling cycling activity. Control of the enzyme in vivo is therefore most likely to be invested in metabolite modulation of enzyme activity.

Bumblebee fructose diphophatase is similar to the enzyme from other sources in several respects including: its molecular weight and pH optimum, the occurrence of a single isozyme, activation by fatty acids, and inhibition by divalent and monovalent cations [13,27]. As first reported by Newsholme et al. [1], the bumblebee enzyme differs from other animal fructose diphosphatases [34] in being refractory to inhibition by AMP and other mono-, di-, and trinucleotides. The purified enzyme is also unaffected by inorganic phosphate, citrate, and phosphagen (arginine-*P*), compounds known to activate fructose diphosphatase from other sources [35].

The data in this study indicate that, as was first proposed by Clark et al. [2], the major mechanism controlling the rate of fructose-6-*P* cycling under resting versus flight conditions is the concentration of sarcoplasmic Ca^{2+}. Although the initial report on Ca^{2+} inhibition indicated that high (and likely non-physiological) concentrations of Ca^{2+} were needed to inhibit fructose diphosphatase, this present study demonstrates that the K_i' for Ca^{2+} of the purified enzyme is fully 3-fold lower than that for the crude enzyme (perhaps due to Ca^{2+} chelation by other proteins in the crude preparation) and that the presence of physiological concentrations of Li^{+} further reduces the K_i' for Ca^{2+} bringing it into the range of Ca^{2+} concentrations in vivo. This novel interaction between a monovalent and a divalent cation is unique to the Ca^{2+}-Li^{+} interaction as no other combination of monovalents and divalents tested produced such an effect. The significance of Ca^{2+} inhibition in vivo is further strengthened by the observation that

Ca^{2+} effects on both fructose diphosphatase and glycerol-3-*P* oxidase occur in the same concentration range (approx. 10 μM).

An investigation of the effects of temperature on fructose disphosphatase activity was undertaken in an attempt to correlate enzyme parameters with the rates of fructose-6-*P* cycling as determined in whole animal experiments. The effects of temperature on enzyme kinetics were such that with decreasing temperature the affinity for substrate increased, the affinity for inhibitor (Ca^{2+}) decreased, and the affinity for activator (oleate) increased. Each of these changes would result in an increase in fructose diphosphatase activity, with a subsequent increase in fructose-6-*P* cycling, as temperature is lowered.

The activation of fructose diphosphatase by fatty acids, especially oleate, and its accentuation by low temperature is particularly interesting as oleate in the same range inhibits bumblebee flight muscle phosphofructokinase and this inhibition increases as the temperature is lowered. A "reciprocal" type control at this locus by fatty acid would serve to greatly increase fructose-6-*P* cycling at low temperatures.

Some controversy exists as to the specificity of the effects of fatty acids on enzyme activity [15,31]. Several factors, however, indicate that the activation of fructose diphosphatase by fatty acids is not due to a non-specific detergent effect. Firstly, a free carboxyl group is clearly required for activation since fatty alcohols and fatty carnitines had no effect on enzyme activity. Secondly, the detergents, SDS and deoxycholate, were without effect on the enzyme and thirdly, activation by oleate is much greater than activation by other acids, indicating a specific effect of oleate.

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