The Functional Significance of Variants of Fructose 1,6-Diphosphatase in the Gill and Hypodermis of a Marine Crustacean

A KINETIC STUDY

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1. In the hypodermis and gill of the Crustacea fructose 1,6-diphosphatase (EC 3.1.3.11) functions at a primary branch point between glycogen and chitin synthesis. In these tissues of the Arctic king-crab, *Paralithodes camtchatica*, fructose diphosphatase occurs in two electrophoretically distinguishable forms. 2. Fructose diphosphatase I (pI 7.2–7.5) accounts for 70 and 10% of total fructose diphosphatase activity in the hypodermis and gill respectively, whereas fructose diphosphatase II (pI 5.3) accounts for 30 and 90% of the total activity in the two tissues. Both forms display a neutral pH optimum, have an absolute requirement for a bivalent cation, and are potently inhibited by high concentrations of AMP and substrate. 3. Fructose 1,6-diphosphate saturation follows Michaelis-Menten kinetics for both fructose diphosphatases; the K_m (fructose diphosphate) for fructose diphosphatase I is somewhat higher than for fructose diphosphatase II. In the presence of 50–200 mM- K^+ , the K_m (fructose diphosphate) increases and at high concentrations of K^+ fructose diphosphate saturation follows sigmoidal kinetics. 4. UDP-*N*-acetylglucosamine and UDP-glucose at high concentrations specifically and potently inhibit fructose diphosphatase II, but do not significantly affect fructose diphosphatase I activity. 5. Low concentrations of UDP-*N*-acetylglucosamine activate fructose diphosphatase II by a decrease in the apparent K_m (fructose diphosphate), but fructose diphosphatase I is again refractory to UDP-*N*-acetylglucosamine under these conditions. 6. In the presence of K^+ and UDP-*N*-acetylglucosamine, fructose diphosphatase II is able to compete for limiting fructose diphosphate about three times more effectively than is fructose diphosphatase I. 7. AMP inhibition of both forms of the enzyme is subject to three independent variables: (a) alkaline pH increases the K_i (AMP), (b) K^+ decreases the K_i , increases the sigmoidicity of inhibition kinetics, increases the maximum inhibition attained, and abolishes the effect of pH on AMP inhibition, and (c) Mg^{2+} strongly de-inhibits AMP-inhibited fructose diphosphatase. 8. It is postulated that the presence of two forms of fructose diphosphatase aids controlled channelling of carbon through the fructose diphosphatase 'bottleneck' either towards glycogen synthesis or chitin synthesis, but not towards both simultaneously.

The primary mechanism for the control of fructose diphosphate hydrolysis in most tissues is that of negative AMP modulation of the enzyme fructose 1,6-diphosphatase (EC 3.1.3.11). Under energy-depleted conditions, when AMP concentrations are high, fructose diphosphatase function is held in an inhibited state, whereas the activity of phosphofructokinase is AMP-activated. Thus the activities of the two enzymes are co-ordinated to avoid cycling of carbon and energy at the fructose diphosphate-fructose 6-phosphate interconversion. This mechanism is widespread in living organisms. It occurs in mammalian liver, kidney and muscle tissues (News-holme & Gevers, 1967), in lower vertebrates (Behrlich & Hochachka, 1969), in yeast (Horecker *et al.*, 1966) and in plants (Preiss *et al.*, 1967). In pilot studies of the Arctic king-crab, I observed high

fructose diphosphatase activities in the gill and the hypodermis. Because of the multiplicity of pathways occurring at this point in metabolism in these tissues, AMP inhibition of fructose diphosphatase as a sole mechanism of control of the reaction did not appear adequate to account for the different regulatory requirements of the gill and the hypodermis.

The major reactions occurring at this point in the metabolism of the crustacean hypodermis are shown diagrammatically in Scheme 1. Two fundamental functions of the hypodermis are the biosyntheses of large quantities of chitin and of glycogen. The first 'committed' metabolite in the pathway to chitin is UDP-*N*-acetylglucosamine; the first 'committed' metabolite in the pathway to glycogen is UDP-glucose. During the moult cycle in these organisms, a reciprocal relationship is maintained in the relative

Scheme 1. Major metabolic pathways occurring at the level of fructose 6-phosphate metabolism in the hypodermis and gill of Crustacea

In the hypodermis the two major pathways lead to the synthesis of chitin and glycogen, but in the gill glycogen synthesis does not occur to any significant extent. Mannose 6-phosphate can be formed from fructose 6-phosphate in both tissues.

activities of these two pathways; during inter-moult and early pre-moult, glycogen synthesis is high and chitin synthesis does not occur, whereas chitin synthesis is high during later pre-moult and early post-moult, when glycogen breakdown is activated (Passano, 1960). Situated strategically at the primary branching point between these major biosynthetic pathways is the fructose diphosphatase reaction (Scheme 1). It is evident that efficient channelling of carbon through this branch point should favour the glycogen pathway (in intermoult) or the chitin pathway (during moult), but not both simultaneously. This regulatory requirement has been met in part by the evolution of at least two kinds of fructose diphosphatases in the hypodermis. One form, fructose diphosphatase I, is sensitive to feedback inhibition by high concentrations of UDP-*N*-acetylglucosamine, but is unaffected by UDP-glucose; the other form,

fructose diphosphatase II, is regulated by UDP-*N*-acetylglucosamine and UDP-glucose. Both are subject to the usual control by AMP. Through an interplay between these various metabolites it is postulated that an effective channelling of carbon is achieved through the fructose diphosphatase 'bottleneck' to either glycogen or chitin, but not to both at the same time.

In the gill the situation is somewhat less complex. Here biosynthesis is a much more conservative enterprise. In an average adult king-crab, enough chitin must be synthesized at this site to supply a thin chitinous cover for only about 100g wet wt. of gill tissue. The hypodermis, however, must be capable of synthesizing all of the requisite chitin for the vast exoskeleton laid down at each new moult cycle in an animal that frequently attains a weight as great as 10000g. Although less dramatic, the biosynthesis of

chitin must occur at the gill both during the moult and during gill maintenance in the intermoult. In contrast to the hypodermis, however, significant glycogen synthesis does not occur (Hochachka *et al.*, 1970). Thus the control requirements at the fructose diphosphatase branch-point appear to be quite distinct in the two tissues. In the gill, only one major form of fructose diphosphatase is present. It is remarkably sensitive to feedback regulation by UDP-*N*-acetylglucosamine and UDP-glucose, and is apparently identical with the hypodermal fructose diphosphatase II. When concentrations of either glycogen or chitin precursors are high, the activity of this fructose diphosphatase is held in a strongly inhibited state. A minor, second fructose diphosphatase form also occurs in the gill. It accounts for 10% of the total fructose diphosphatase activity in intermoult animals. It is sensitive to high UDP-*N*-acetylglucosamine concentrations, but is entirely refractory to UDP-glucose. Both forms are inhibited by AMP.

Methods and Materials

Experimental animals

The Arctic king-crab, *Paralithodes camtchatica*, was used in these studies because a relatively large amount of information has been accumulated about the biochemical and physiological features of the species (Hochachka *et al.*, 1970; Somero, 1969). Also, because of the large size of the king-crab many of its tissues occur in large enough quantities to allow extensive biochemical work. The king-crabs used in this study were collected by means of baited 'pots' in Katchemak Bay, near Homer Spit, Homer, Alaska, during June and July, 1971, in approx. 30–40 fathoms of water. Moulting occurs in adult members of this species in March (G. Powell, personal communication); hence the animals used in this study were all in their inter-moult period. The average weight was about 5 kg. Most of the experiments in this study were carried out at 20°C. Since temperature has an important influence on many of the kinetic constants of fructose diphosphatases (Behrisch & Hochachka, 1969), it is important to stress that the lethal temperature for the adult king-crab is over 20°C (C. L. Prosser & C. Mangum, personal communication). However, the species in Nature is restricted to temperatures between 4° and 15°C.

Preparation of enzymes

Hypodermis, removed from the exoskeleton around the gill chamber, and gill filaments were excised from freshly captured king-crabs. The tissues were blotted to remove blood and any adhering sea-

water, cut into small pieces, and homogenized in cold 0.05M-tris-HCl buffer, pH7.5. Usually, 1:3 or 1:4 (w/v) homogenates were prepared. After centrifugation at 30 000g for 7min, the clear supernatant either was dialysed, centrifuged again and used directly as a source of fructose diphosphatase, or was adjusted to 30% saturation with solid (NH₄)₂SO₄ and stirred for at least 1 h at 0°C. The protein that precipitated contained no fructose diphosphatase activity. The supernatant was adjusted to 70% saturation with solid (NH₄)₂SO₄ and stirred again, usually overnight. On centrifugation at about 5000g all of the fructose diphosphatase activity sedimented with the pellet. The pellet was suspended in a small volume of buffer, and, after dialysis against 0.05M-tris-HCl buffer of the desired pH, was used as a source of fructose diphosphatase activity.

Assay of fructose diphosphatase activity

Two assays were used to measure the activity of fructose diphosphatase. For certain experiments, fructose diphosphate hydrolysis was assayed by measuring the concentration of released P_i, by the method of Fiske & SubbaRow (1925) adapted for a Technicon AutoAnalyzer. In these tissues, non-specific phosphatases do not occur; in none of the fructose diphosphatase preparations examined was there any hydrolysis of glucose 6-phosphate, fructose 6-phosphate, mannose 6-phosphate, ribose 5-phosphate, ribulose 5-phosphate, AMP or *p*-nitrophenyl phosphate. For most of the kinetic experiments, a coupled-enzyme assay was used as described by Taketa & Pogell (1965). In this assay, fructose 6-phosphate is converted by phosphohexose isomerase into glucose 6-phosphate, which in turn serves as a substrate for glucose 6-phosphate dehydrogenase. The rate of reduction of NADP⁺ was strictly dependent on fructose diphosphate and Mg²⁺ and was proportional to the amount of enzyme added. The reduction of NADP⁺ was followed in a Hitachi Perkin-Elmer spectrophotometer attached to a linear-log Bausch and Lomb recorder. In all fresh high-speed supernatant and salt-fractionated preparations, endogenous glucose 6-phosphate dehydrogenase and isomerase could be used in the assay system. However, on freezing, the dehydrogenase present in these tissues is irreversibly inactivated, and isomerase and glucose 6-phosphate dehydrogenase were added to the reaction mixture. Reaction velocity, measured as the increase in E₃₄₀ caused by the reduction of NADP⁺, was linear with time for at least 5 min, under conditions of saturating fructose diphosphate concentrations; at very low fructose diphosphate concentrations (in the μmolar range), however, the reaction was linear for only 45–60s. The compositions of assay mixtures are given in the figure legends.

Isoelectrofocusing

The technique of isoelectrofocusing was used to determine the presence or absence of electrophoretic variants of fructose diphosphatase in gill and hypodermis. Freshly prepared high-speed supernatant solutions, dialysed for a period of 4 h at 0°C against 1 mM-tris-HCl buffer, were used as sources of the enzyme for these experiments. An LKB 110 ml electrofocusing column was arranged with the cathode solution at the bottom; a linear sucrose gradient (5–50%, w/v) containing the enzyme preparation was layered on the cathode electrolyte solution by using an LKB gradient former (Haglund, 1967). The temperature of the column was kept at 5°C by circulating fluid around the water jacket of the column from a Forma constant-temperature bath. After completion of the run, as judged by the development of a constant low current, the column was drained at a slow rate (initially at about 0.2 ml/min; finally at about 1 ml/min). Approx. 50 samples were collected per column by using an LKB fraction collector. The various fractions obtained were tested for activity by using the coupled-enzyme assay in the presence of activating (100 mM) concentrations of K^+ . The pH of each fraction was measured with a Coleman pH-meter.

Reagents

All organic reagents and coupling enzymes were from Sigma Chemical Co., St. Louis, Mo., U.S.A. All inorganic compounds used were reagent grade.

Results

Tissue distribution of fructose diphosphatase isoenzymes

Fructose diphosphatase occurs in at least three forms in tissues of the Arctic king-crab. One form is predominant in the hypodermis, a second form constitutes most of the fructose diphosphatase activity in the gill, and a third form is specific to skeletal muscle. Isoelectrofocusing experiments indicate two electrophoretic species of the enzyme in both the gill and the hypodermis (Fig. 1). These are termed fructose diphosphatase I (predominant in the hypodermis) and fructose diphosphatase II (predominant in the gill), and are tentatively considered to be isoenzymes. In the hypodermis of inter-moult king-crabs, fructose diphosphatase I (pI value 7.2–7.5) accounts for about 70% of the total enzyme activity recoverable after isoelectrofocusing. In the gill, fructose diphosphatase I, presumably identical with the hypodermal isoenzyme I, accounts for only about 10% of the total activity. Here fructose diphosphatase II (pI value 5.4) accounts for about 90% of total recoverable activity. In both tissues, both fructose diphosphatases

Fig. 1. Isoelectrofocusing of (a) gill and (b) hypodermal fructose diphosphatases in a pH 3–10 gradient at 5°C

For isoelectrofocusing, initial conditions were 300 V, 6 mA, and final conditions were 400 V, less than 0.5 mA. On completion of each run, the column was drained at about 0.5 ml/min. Each tube was checked for fructose diphosphatase activity by using the coupled-enzyme assay (see the text). v is the relative activity of fructose diphosphatase in each tube. ○, pH; ●, enzyme activity.

I and II display an absolute requirement for a bivalent cation and both are potently inhibited by AMP.

The third type of fructose diphosphatase occurs only in muscle. This form, first identified by Behrlich (1971), displays a remarkably low K_i for AMP, a property shared with muscle fructose diphosphatases from vertebrate sources.

At pH 7.5, 20°C, and optimal assay conditions, the specific activity (in μ mol of fructose diphosphate hydrolysed/min per g wet wt.) is highest in the hypodermis (220.8), intermediate in the gill (153.6) and lowest in skeletal muscle (39.0). Because of the relatively specific tissue distribution of these different forms, the hypodermis was used as a source of isoenzyme I, and the gill was usually used as a source of isoenzyme II. It should be stressed, however, that the

two components in each tissue preparation were not separated for these comparative kinetic studies, and it is only a first approximation that the hypodermal preparations largely display isoenzyme I properties, whereas the gill preparations display isoenzyme II properties. The properties of muscle fructose diphosphatase are not considered in this study.

Effect of pH on gill and hypodermal fructose diphosphatases

In agreement with recent studies of mammalian liver fructose diphosphatases (Marcus *et al.*, 1971), both gill and hypodermal enzymes in the presence of non-inhibitory substrate concentrations show a fairly distinct pH optimum at about pH 7–7.5. At high substrate concentrations, the pH profile is flattened out and the reaction is fairly independent of pH between pH 7.0 and pH 10.0 (Fig. 2).

Fructose diphosphate saturation kinetics

Fructose diphosphate saturation curves for the two enzymes are very similar. The apparent K_m values, determined by Lineweaver–Burk plots of the results in Fig. 3, are $3\mu\text{M}$ -fructose diphosphate for the gill enzyme and $5.5\mu\text{M}$ for hypodermal fructose diphosphatase, at 5mM-Mg^{2+} and pH 7.5. These values are substantially lower than the estimated tissue fructose diphosphate concentrations for this species *in vivo* (Freed, 1971). Inhibition by fructose diphosphate occurs with both enzymes, but the hypodermal enzyme appears to be somewhat more sensitive; at 1mM -fructose diphosphate, the activity is decreased

Fig. 2. pH profile for fructose diphosphatase of gill and hypodermal isoenzymes

○ and □ are for the hypodermal enzyme in the presence of 0.05 and 1.0mM-fructose diphosphate respectively. △ is for the gill enzyme in the presence of 0.5mM-fructose diphosphate. Activity (v) is expressed as $\Delta E_{340}/\text{min}$ and was assayed at 20°C , 5mM-Mg^{2+} and 0.05M-tris-HCl buffer.

to about 25% of the rate with the optimum concentration of fructose diphosphate (Fig. 3). The gill enzyme at the same fructose diphosphate concentration retains nearly 50% of the control activity.

The activities of both enzymes are profoundly affected by K^+ and other univalent cations (Fig. 4). The effect of K^+ is complex and depends in part on the fructose diphosphate concentration. At low fructose diphosphate concentrations, $[\text{K}^+]$ up to 100mM activates fructose diphosphatase catalytic activity, but high concentrations are inhibitory. At higher fructose diphosphate concentrations, $[\text{K}^+]$ up to 300mM activates the enzyme. Preliminary experiments indicate that NH_4^+ and tris^+ produce comparable activation, an observation made also by Hubert *et al.* (1970) in studies of vertebrate fructose diphosphatases. In contrast, Na^+ inhibits at all concentrations tested.

The complex effect of K^+ on catalysis stems from a strong action of the cation on the apparent K_m for fructose diphosphate (Fig. 5). At 100mM-K^+ and 5mM-Mg^{2+} , the K_m (fructose diphosphate) increases to about $12\mu\text{M}$; at 200mM-K^+ , the apparent K_m increases to $22\mu\text{M}$ -fructose diphosphate. These values fall within measured estimates of the fructose diphosphate concentrations in this species *in vivo* (Freed, 1971). In addition, fructose diphosphate saturation curves for gill fructose diphosphatase in the presence of 200mM-K^+ are distinctly sigmoidal. They are hyperbolic at lower K^+ concentrations (Fig. 5). Physiological concentrations of K^+ in king crab tissues are in the 100mM range (McKay & Prosser, 1970).

Effects of bivalent cations on gill fructose diphosphatase

In common with the homologous enzyme in vertebrates, the crustacean fructose diphosphatases

Fig. 3. Substrate saturation curves for fructose diphosphatases from the gill (●) and the hypodermis (▲)

The assay was carried out at 22°C , pH 7.5, 0.05M-tris-HCl buffer and 5mM-Mg^{2+} . Reaction velocity (v) is expressed as $\Delta E_{340}/\text{min}$.

Fig. 4. Effect of univalent cations on the gill isoenzyme

The assay was carried out at 20°C, pH 7.5, 0.05M-tris-HCl buffer, 5mM-Mg²⁺. The effect of K⁺ was measured in the presence of 0.5mM- (●) and 0.05mM- (○) fructose diphosphate, and that of Na⁺ at 0.5mM-fructose diphosphate (▲). Activity is expressed as a percentage of control rates in the absence of added cations.

have an absolute requirement for a bivalent cation, which can be satisfied by Mg²⁺ (Fig. 6). The Michaelis constant for Mg²⁺ in the absence of K⁺ is about 0.18mM for the gill enzyme and increases to about 0.3mM in the presence of 100mM-K⁺ (Fig. 6). Hubert *et al.* (1970) have reported similar effects of K⁺ on Mg²⁺ saturation of vertebrate fructose diphosphatases. Mg²⁺ saturation curves for the gill enzyme appear to be slightly sigmoidal. Detailed studies of the cation cofactor for these crustacean enzymes are not included in the present paper. Preliminary studies indicate that Mn²⁺ activates these fructose diphosphatases more strongly than does Mg²⁺.

AMP inhibition

As with vertebrate fructose diphosphatases, AMP inhibition is undoubtedly an important control feature of the crustacean fructose diphosphatases. AMP inhibition is subject to at least three independent variables: (1) pH, (2) K⁺ concentration and (3) Mg²⁺ concentration (Figs. 7 and 8, and Table

Fig. 5. Effect of K⁺ on substrate saturation kinetics for the gill enzyme

The assay was carried out at 22°C, pH 7.5, 0.05M-tris-HCl buffer, 5.0mM-Mg²⁺. ●, Control; ▲, 50mM-K⁺; ■, 100mM-K⁺; ▼, 200mM-K⁺. Activity (*v*) is expressed as $\Delta E_{340}/\text{min}$.

Fig. 6. Effect of K⁺ on Mg²⁺ saturation kinetics for the gill enzyme

The assay was carried out at 22°C, pH 7.5, 0.05M-tris-HCl buffer. ○, No K⁺; ▲, plus 100mM-K⁺. Activity (*v*) is expressed as $\Delta E_{340}/\text{min}$.

1). In general, AMP appears to be less effective at high pH and at low K⁺ concentrations.

The effect of Mg²⁺ is opposite to that of K⁺. Thus an increase in Mg²⁺ concentrations strongly reverses AMP inhibition. With the gill enzyme an increase in Mg²⁺ concentration to 10mM completely reverses the inhibition imposed by 0.1mM-AMP. With hypodermal fructose diphosphatase, only an 80% reversal of the reaction rate can be achieved by Mg²⁺ (Fig. 9).

Fig. 7. Interacting effects of pH and K^+ on AMP inhibition of hypodermal fructose diphosphatase

The conditions were 0.05 mM-fructose diphosphate, 2.5 mM- Mg^{2+} ; (a) pH 7.5; (b) pH 9.0; Δ , plus 100 mM- K^+ ; $\circ$, no K^+ . AMP inhibition of fructose diphosphatase I, isolated from a crude gill preparation by isoelectrofocusing, is shown in (a) ($\square$). The AMP concentration ranges in (a) and (b) are different. Activity is plotted as a percentage of control rates in the absence of AMP.

Effects of UDP-N-acetylglucosamine on gill and hypodermal fructose diphosphatases

Gill fructose diphosphatase activity is remarkably sensitive to UDP-N-acetylglucosamine (Fig. 10). In the absence of K^+ , the major effect at saturating fructose diphosphate concentrations is inhibition. In the presence of 100 mM- K^+ , the effect of UDP-N-acetylglucosamine is altered: under these conditions, low concentrations of UDP-N-acetylglucosamine activate the gill enzyme, apparently by decreasing the K_m for fructose diphosphate, whereas inhibition attained by higher UDP-N-acetylglucosamine concentrations is somewhat accentuated. Thus the

Fig. 8. Interacting effects of pH and K^+ on AMP inhibition of gill fructose diphosphatase

The conditions were 0.05 mM-fructose diphosphate, 2.5 mM- Mg^{2+} ; (a) pH 7.5; (b) pH 9.0; Δ , plus 100 mM- K^+ ; $\circ$, no K^+ . The AMP concentration ranges in (a) and (b) are different. Activity is plotted as a percentage of control rates in the absence of AMP.

presence of K^+ appears to increase the steepness of the UDP-N-acetylglucosamine inhibition curve (Fig. 10). Although UDP-N-acetylglucosamine inhibition follows sigmoidal kinetics in both the presence and the absence of K^+ , the strength of site-site interactions is apparently increased in the presence of K^+ , since the value of the Hill coefficient increases from about 2.5 to 4.0. Preincubation of the enzyme with UDP-N-acetylglucosamine before addition of fructose diphosphate to the reaction mixture leads to an even greater inhibition (Table 2); this preincubation phenomenon was not examined in any detail.

The response of hypodermal fructose diphosphatase to UDP-N-acetylglucosamine markedly contrasts with the behaviour of the gill enzyme. In

Table 1. Values of the apparent K_i and the Hill coefficient (n) for AMP inhibition of gill and hypodermal fructose diphosphatases

The values are calculated from the Hill treatment of AMP inhibition curves (Figs. 7 and 8). For experimental details see the text.

Conditions	n		K_i (μM)	
	Gill	Hypodermis	Gill	Hypodermis
pH 7.5, no K^+	2.3	1.7	55	60
plus K^+	3.1	2.7	32	35
pH 9.0, no K^+	3.1	4.2	80	90
plus K^+	1.9	3.1	25	25

Fig. 9. Mg^{2+} reversal of gill and hypodermal fructose diphosphatases inhibited by 0.1 mM-AMP at 0.05 mM-fructose diphosphate

Other conditions were 2.5 mM- Mg^{2+} , pH 9.0, 20°C. Activity is plotted as a percentage of control rates at 0.05 mM-fructose diphosphate, 2.5 mM- Mg^{2+} , and no AMP. ●, Gill enzymes; ▲, hypodermis enzymes.

Fig. 10. Effect of UDP-N-acetylglucosamine on gill (○, △) and hypodermal (●, ▲) fructose diphosphatases

The conditions were 2.5 mM- Mg^{2+} , 0.05 mM-fructose diphosphate, pH 7.5, 0.05 M-tris-HCl buffer, 20°C. Activity is plotted as a percentage of control rates in the absence of UDP-N-acetylglucosamine. ●, ○, No K^+ ; ▲, △, plus 100 mM- K^+ .

this case, the activity shows dramatically decreased UDP-N-acetylglucosamine sensitivity, and the inhibition is not strongly altered by K^+ (Fig. 10). UDP-N-acetylglucosamine inhibition of isolated gill fructose diphosphatase I follows the curves for hypodermal fructose diphosphatase shown in Fig. 10, indicating that the UDP-N-acetylglucosamine insensitivity resides in this fructose diphosphatase variant.

The complex effect of UDP-N-acetylglucosamine on gill fructose diphosphatase stems in part from a change in the nature of fructose diphosphate saturation kinetics. Fig. 11 shows that at low fructose diphosphate concentrations UDP-N-acetylglucosamine activates the reaction by decreasing the apparent K_m ; in the presence of UDP-N-acetylglucosamine and K^+ the apparent K_m is decreased to

about 2–3 μM -fructose diphosphate, compared to control values for gill fructose diphosphatase of 5–6 μM . Although in the absence of K^+ the K_m (fructose diphosphate) for both gill and hypodermal fructose diphosphatases are comparable (Fig. 3), in the presence of 100 mM- K^+ the apparent K_m (fructose diphosphate) for hypodermal fructose diphosphatase (8 μM -fructose diphosphate) is substantially greater and the saturation curve is sigmoidal (Fig. 11).

The effects of UDP-N-acetylglucosamine on crude hypodermal enzyme preparations at low fructose diphosphate concentrations are complex because of a disproportionate contribution to activity by fructose diphosphatase II; since the K_m is important in determining the reaction velocity at low substrate

Table 2. *Effect of preincubating gill fructose diphosphatase with 0.5 mM-UDP-N-acetylglucosamine*

Preincubation conditions were pH 7.5, 0.05 M-tris-HCl buffer, 20°C, and assay conditions were 0.05 mM-fructose diphosphate, 2.5 mM-Mg²⁺. Activity is expressed as a percentage of the control rate in the absence of UDP-N-acetylglucosamine.

Conditions	Activity (% of control)
Control (no UDP-N-acetylglucosamine)	100
No preincubation	71
5 min preincubation	20
10 min preincubation	14

Fig. 11. *Effect of UDP-N-acetylglucosamine on fructose diphosphate saturation kinetics of gill fructose diphosphatase*

Activating conditions (0.1 mM-UDP-N-acetylglucosamine; 100 mM-K⁺) are shown by ▲; inhibiting conditions (0.7 mM-UDP-N-acetylglucosamine; no K⁺) are shown by △. Rates in the absence of UDP-N-acetylglucosamine are shown for gill fructose diphosphatase without K⁺ (○) and in the presence of 100 mM-K⁺ (●). The rate for hypodermal enzyme in the presence of 100 mM-K⁺ is shown as (----; ■). Fructose diphosphatase activity was assayed at pH 7.5, 0.05 M-tris-HCl buffer, 2.5 mM-Mg²⁺, 20°C. Reaction velocity (*v*) is plotted as a fraction of *v*_{opt.} (the velocity at optimum fructose diphosphate concentrations).

concentrations and since the *K_m* for fructose diphosphatase II is further decreased by UDP-N-acetylglucosamine, the contribution of this form to activity under these conditions becomes appreciable. This problem was circumvented by examining the effects of UDP-N-acetylglucosamine on isolated fructose diphosphatase I, the dominant form in the hypodermis. In contrast to the marked decrease in the *K_m*

Table 3. *Effect of low (0.1 mM) concentrations of UDP-N-acetylglucosamine on the catalytic activity of fructose diphosphatase I isolated from isoenzyme II by electrofocusing*

After electrofocusing, isoenzyme I was dialysed against 0.05 M-tris-HCl buffer, pH 7.5. Assay conditions were 2.5 mM-Mg²⁺, 100 mM-K⁺, 0.05 M-tris-HCl buffer, pH 7.5, 20°C. Activity in the presence of UDP-N-acetylglucosamine is expressed as a percentage of control rates at different fructose diphosphate concentrations.

Fructose diphosphate concentration (μM)	Activity (% of control)
1.0	93
2.0	87
4.0	84
10.0	96

(fructose diphosphate) for fructose diphosphatase II (Fig. 11), the catalytic rate of fructose diphosphatase I at low fructose diphosphate concentrations is largely unaffected by UDP-N-acetylglucosamine (Table 3). Thus the reaction velocity of the major hypodermal fructose diphosphatase (form I) appears to be insensitive to UDP-N-acetylglucosamine both at low and at saturating fructose diphosphate concentrations.

Effects of UDP-glucose on gill and hypodermal fructose diphosphatases

Another distinction between the two fructose diphosphatases lies in their sensitivity to UDP-glucose. Gill fructose diphosphatase is strongly inhibited, whereas hypodermal fructose diphosphatase is completely insensitive to UDP-glucose at concentrations as high as 5 mM (Fig. 12). The inhibition curve is sigmoidal in nature, with a Hill coefficient of about 3.0.

Other regulatory metabolites

Because of the unusual metabolism characterizing these tissues, a search for other potential regulatory metabolites was made. ATP at high concentrations (10 mM) inhibits both enzymes; the inhibition is reversed by adding high concentrations of Mg²⁺. Indeed, under these conditions, ATP activates the enzyme to about 130% of control values. Because of reported effects on the crustacean muscle enzyme (Behrisch, 1971), phosphoenolpyruvate was tested at both low and high fructose diphosphate concentrations, but had no effect on either gill or hypodermal enzymes. Mannose 6-phosphate, glucose 6-phosphate, fructose 6-phosphate and glutamine were also without effect.

Fig. 12. Effect of UDP-glucose on fructose diphosphatases from the gill (○) and the hypodermis (Δ)

The conditions were pH 7.5, 0.05M-tris-HCl buffer, 0.05mM-fructose diphosphate, 2.5mM-Mg²⁺, 20°C. Reaction velocity is plotted as a percentage of control rates in the absence of UDP-glucose.

Fig. 13. Effect of ribose 5-phosphate on fructose diphosphatases from the gill (○) and the hypodermis (Δ)

The conditions were pH 7.5, 0.05M-tris-HCl buffer, 0.05mM-fructose diphosphate, 2.5mM-Mg²⁺, 20°C. Reaction velocity is plotted as a percentage of control rates in the absence of ribose 5-phosphate.

Both gill and hypodermal fructose diphosphatases are inhibited by ribose 5-phosphate, but the degree of inhibition differs greatly (Fig. 13). The inhibition

curve for the gill enzyme is sigmoidal, and the Hill coefficient is slightly greater than 3.0.

Discussion

For many unidirectional exergonic reactions such as that catalysed by fructose diphosphatase the relative concentrations of ATP, ADP and AMP are important control factors (Atkinson, 1968), and AMP is an inhibitor of all known fructose diphosphatases. Under energy-saturating conditions (ATP concentrations high, AMP concentrations low), when biosynthetic events such as glucogenesis are favoured, fructose diphosphatases in both the gill and hypodermis of king-crabs are in de-inhibited states. Under energy-depleted conditions, however, AMP accumulation would lead to potent and specific inhibition of both gill and hypodermal isoenzymes. Although in general this regulatory characteristic is also displayed by homologous enzymes in vertebrates, the particular effects of cations on AMP regulation appear to be more specific to the crustacean fructose diphosphatases. Thus, in both the gill and hypodermis, AMP control is itself markedly dependent on the ionic environment. In the presence of K⁺ at concentrations within the physiological range (McKay & Prosser, 1970), the K_i for AMP lies in the estimated concentration range of AMP *in vivo* (Freed, 1971) and is essentially pH-independent. Such a pH-independence of the K_i for AMP is not typically observed for fructose diphosphatases (Horecker *et al.*, 1966), possibly because most previous studies, with the notable exception of those by Hubert *et al.* (1970), have been carried out in the absence of K⁺ ions. Hubert *et al.* (1970) found that AMP inhibition of mammalian liver and kidney fructose diphosphatases is not strongly affected by K⁺, but inhibition of the mammalian muscle enzyme and of certain fish liver fructose diphosphatases is accentuated at high concentrations of K⁺ ions. In their results, as in those reported here, both the maximum inhibition and the sigmoidicity of AMP-inhibition kinetics are increased by K⁺ ions. Hubert *et al.* (1970), however, did not examine the interacting effects of K⁺ and pH on AMP inhibition. Hence it is not possible to decide whether the pH-independence of the K_i (AMP) is unique to these crustacean fructose diphosphatases.

A second critical factor capable of modulating the AMP control of fructose diphosphatase is the Mg²⁺ concentration. This phenomenon has been observed by other workers (Behrisch & Hochachka, 1969; Underwood & Newsholme, 1965), but the degree of Mg²⁺ de-inhibition of AMP-inhibited isoenzymes I and II appears to be especially prominent with the king-crab enzymes. Under my experimental conditions, the maximum Mg²⁺ concentrations involved approach 10mM. For this species, estimated

intracellular concentrations of Mg^{2+} are in this range (McKay & Prosser, 1970). Since the concentrations of Mg^{2+} , Na^+ and probably K^+ (by transport coupling) are regulated by active transport across the epithelium of the gill and of the hypodermis, these effects of the Mg^{2+} cofactor and of K^+ on AMP inhibition of fructose diphosphatase are of possible physiological relevance.

The influence of K^+ on fructose diphosphate saturation kinetics may also be important in this connexion. Concentrations of K^+ between 50 and 200 mM (1) increase the K_m for fructose diphosphate so that it coincides more closely with physiological concentrations for this species (Freed, 1971), and (2) alter the maximum catalytic ability of the enzyme ($V_{max.}$). At K_m concentrations of substrate, the K^+ decreases the reaction velocity, whereas at saturating substrate concentrations it increases the catalytic rate (see Fig. 5). Moreover, at high concentrations of K^+ , substrate-saturation kinetics become distinctly sigmoidal. Positive fructose diphosphate site-site interactions in other fructose diphosphatases have been observed previously (Behrisch & Hochachka, 1969), but evidently the effect of K^+ on this characteristic has been overlooked (Hubert *et al.*, 1970).

It is evident that in the king-crab gill and hypodermis, interactions between fructose diphosphate, AMP, Mg^{2+} and K^+ on the enzyme could lead to highly flexible control of catalysis, since all the relevant apparent affinity constants are within measured concentration ranges of these compounds and ions *in vivo*. It would indeed be surprising if these control parameters were not operative *in vivo*. Yet, given the particular set of metabolic pathways operating in these tissues (see Scheme 1), the above control mechanisms, in the absence of further signals, lack the specificity required for efficient channelling of carbon through the fructose diphosphatase 'bottleneck' to either glycogen or chitin, but not to both simultaneously. UDP-*N*-acetylglucosamine and UDP-glucose, the first 'committed' metabolites in the synthesis of chitin and glycogen respectively, appear to supply such additional signals. The control functions played by these two metabolites appear to be different in the gill and the hypodermis.

In the post-moult and inter-moult animals used in this study, glycogen synthesis in the gill does not occur. Concentrations are so low that glycogen is hardly detectable (Hochachka *et al.*, 1970); it is therefore assumed that UDP-glucose occurs in low concentrations and is not involved in the control of fructose diphosphatase activity. The presence of an active fructose diphosphatase in the gill is presumably a specific adaptation for supplying carbon from amino acids and other glucogenic precursors for chitin synthesis. Chitin synthesis necessarily occurs concomitantly with the extensive cell and membrane turnover

which typifies gill tissue. Although concentrations *in vivo* are not known, it appears that UDP-*N*-acetylglucosamine exhibits two important controlling effects on gill fructose diphosphatase (isoenzyme II). First, at low concentrations, UDP-*N*-acetylglucosamine activates isoenzyme II mainly by decreasing the K_m (fructose diphosphate). At 5 μ M-fructose diphosphate and 100 mM- K^+ , for example, 0.1 mM-UDP-*N*-acetylglucosamine increases the reaction velocity to 170% of control rates. At higher UDP-*N*-acetylglucosamine concentrations, which would be expected when chitin synthesis is blocked, UDP-*N*-acetylglucosamine presumably acts as a typical negative feedback modulator; under these conditions, isoenzyme II is potently and specifically inhibited. Any oscillations in UDP-*N*-acetylglucosamine concentrations (possibly as a result of oscillations in the adenylate ratio) can immediately be reflected in the catalytic rate of fructose diphosphatase II. This form of the enzyme, then, appears to be sensitively geared up for controlled channelling of carbon in the direction of chitin (Scheme 2a). In the gill, isoenzyme I (10% of the total activity) is largely insensitive to UDP-*N*-acetylglucosamine and entirely refractory to UDP-glucose; presumably this isoenzyme is responsible for continued fructose diphosphatase function even in the presence of high concentrations of UDP-*N*-acetylglucosamine or UDP-glucose.

The situation in the gill appears comparable with that in the hypodermis during periods of active chitin synthesis. In the late pre-moult and early post-moult animal, the major biosynthetic job in the hypodermis is the synthesis of chitin for the endocuticle of a large exoskeleton. During this time, glycogen synthesis does not occur; instead, extensive glycogenolysis is activated (Passano, 1960). Since the direction of carbon flow at this level in metabolism is reversed (Scheme 2a), UDP-glucose concentrations are probably much decreased. On activation of chitin synthesis, UDP-*N*-acetylglucosamine concentrations also would fall because of activation of chitin synthetase. As we have seen, low concentrations of UDP-*N*-acetylglucosamine activate fructose diphosphatase II by a decrease in the K_m (fructose diphosphate), whereas fructose diphosphatase I is refractory to low concentrations of UDP-*N*-acetylglucosamine. Under physiological K^+ concentrations, the apparent K_m (fructose diphosphate) for fructose diphosphatase II in the presence of UDP-*N*-acetylglucosamine is 2–3 μ M; under comparable conditions, the K_m (fructose diphosphate) for fructose diphosphatase I is at least threefold higher. In king crab tissues, reported fructose diphosphate concentrations *in vivo* are in the 10 μ M range (Freed, 1971). Since at these concentrations of fructose diphosphate the K_m is important in determining the fructose diphosphatase catalytic rate, it is evident that fructose diphosphatase II is able to compete for

Scheme 2. Postulated feedback regulation of fructose diphosphatase II activity in the gill and hypodermis during periods of chitin synthesis (a) and in the hypodermis during periods of glycogen synthesis (b)

In the gill, fructose diphosphatase II accounts for about 90% of total fructose diphosphatase activity; in the hypodermis of the intermoult animal, fructose diphosphatase II accounts for only about 30% of total fructose diphosphatase activity. Potential control interactions are described in detail in the text.

limiting fructose diphosphate about three times more effectively than is fructose diphosphatase I. During the period of most active chitin synthesis for the new exoskeleton, then, these effects would put a premium on a relative increase in fructose diphosphatase II and a relative decrease in fructose diphosphatase I activities in the hypodermis.

The above situation is precisely opposite to that observed during periods of peak glycogen synthesis. At these times (in the inter-moult period), the predominant activity (isoenzyme I) is entirely refractory to UDP-glucose (hence carbon flow towards glycogen will be favoured even if UDP-glucose accumulates), and is only 50% inhibited by high UDP-N-acetylglucosamine concentrations. This appears to be an adequate situation in a tissue (1) which is synthesizing and storing glycogen, (2) which therefore may require high rates of glycogenesis from amino acids and other precursors, and (3) in which UDP-N-acetylglucosamine concentrations may have accumulated owing to inhibition of chitin synthetase. Under these conditions, any isoenzyme II that was present would probably be held in an inactive state by UDP-N-acetylglucosamine and UDP-glucose (Scheme 2b).

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