Temperature and the Regulation of Enzyme Activity in Poikilotherms

REGULATORY PROPERTIES OF FRUCTOSE DIPHOSPHATASE FROM MUSCLE OF THE ALASKAN KING-CRAB

By HANS W. BEHRISCH

The Institute of Arctic Biology, University of Alaska, College, Alaska 99701, U.S.A.

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1. The properties of fructose diphosphatase from skeletal muscle of the Alaskan king-crab (*Paralithodes camtschatica*) were examined over the physiological temperature range of the animal. 2. King-crab muscle fructose diphosphatase is first activated by Na^+ and NH_4^+ and is then partially inhibited by these cations at concentrations higher than 10 mM at 0°, 8° and 15°C. Enzyme activity is stimulated by K^+ at 0°C, but is curtailed at 8°C and 15°C, an effect that could render rate independent of temperature. 3. Affinity for substrate increases with decreasing temperature; below the temperature of acclimatization, K_m for fructose 1,6-diphosphate increases, resulting in a complex U-shaped temperature- K_m curve. 4. King-crab muscle fructose diphosphatase is inhibited by low concentrations of AMP. As with enzymes of other poikilotherms, inhibition by AMP is sensitive to temperature; the enzyme is least sensitive to inhibition by AMP near the temperature of acclimatization. 5. The affinity of fructose diphosphatase for fructose 1,6-diphosphate is enhanced by phosphoenolpyruvate, and this activation is temperature-sensitive; 0.5 mM-phosphoenolpyruvate causes a sevenfold decrease in K_m for fructose 1,6-diphosphate at 15°C but a 25-fold decrease at 0°C. 6. Phosphoenolpyruvate appears to decrease the affinity of king-crab muscle fructose diphosphatase for AMP at low temperature, whereas at the higher temperature it appears to enhance inhibition by AMP. Phosphoenolpyruvate was not observed to cause a reversal of inhibition of fructose diphosphatase activity by AMP. The identification of phosphoenolpyruvate as an activator of a rate-limiting step in gluconeogenesis permits the suggestion of a coupling of the controlling mechanisms of several steps in the glycolytic and gluconeogenic chains. 7. These findings suggest mechanisms for the maintenance and regulation of control of fructose diphosphatase activity in king-crab skeletal muscle at low temperature and under conditions that favour concomitant activity of phosphofructokinase.

In poikilothermic systems evolution in thermally unstable environments appears to have involved a selection for greater enzyme-substrate affinity at low temperatures, to counteract the effect of a decrease in thermal energy available (for discussion see Somero, 1969b; Hochachka & Somero, 1968; Behrisch, 1969). The result of this increase in affinity of enzyme for substrate is a stabilization of catalytic rate over a wide range of temperatures at physiological concentrations of substrate. Work on fructose diphosphatases (D-fructose 1,6-diphosphate 1-phosphohydrolases, EC 3.1.3.11) from cold-blooded organisms indicates that such an increase in enzyme-substrate interaction at low temperature is accompanied by an enhanced affinity of the enzyme for its allosteric inhibitor, AMP

(Hochachka, 1967; Behrisch & Hochachka, 1969a,b), a result also seen with the mammalian enzyme (Taketa & Pogell, 1965). Thus a decrease in temperature from 46°C to 2°C causes a 100-fold increase in affinity of the rat liver enzyme for AMP. Given such conditions, control of fructose diphosphatase activity in a poikilotherm would be expected to be unusually sensitive to changes in environmental temperature, and glucose production would be essentially 'turned off' at low temperatures. This creates a requirement for mechanisms that would lower the sensitivity of the fructose diphosphatase-AMP interaction to temperature changes, and a number of mechanisms that would allow the organism to keep constant the efficiency of control of the enzyme activity over a wide range of temperatures

have been proposed (see Behrisch, 1969). Control of fructose diphosphatase activity in a poikilotherm that may experience wide ranges in environmental temperatures is further complicated by numerous observations that concentrations of various cations (including the cationic cofactor of the enzyme) vary with temperature of acclimatization (Hickman, McNabb, Nelson, Van Breemen & Comfort, 1964; Toews & Hickman, 1969; Heinicke & Houston, 1965; Houston, 1959; Houston & Madden, 1968) and that changes in concentrations of some of these ions may alter the kinetic behaviour of regulatory enzymes (Bygrave, 1967; Behrisch & Hochachka, 1969a,b; Marcus, 1970).

Moreover, although a number of mechanisms that would activate fructose diphosphatase or reverse inhibition by AMP or both have been proposed (Horecker, Pontremoli, Rosen & Rosen, 1966; Pogell, Tanaka & Siddons, 1968; Little, Sanner & Pihl, 1969; Underwood & Newsholme, 1965), these do not appear to reflect the energy status of the cell, around which activities of key enzymes and thus the net direction of carbon flow must revolve (Atkinson, 1966, 1968b). High concentrations of ATP, indicating a high-energy charge that would normally stimulate many biosynthetic reactions (Atkinson, 1968a,b), bring about an inhibition of fructose diphosphatase activity (Mukkada & Bell, 1969). Further, numerous observations of cycling of intermediates at the fructose diphosphatase-phosphofructokinase step in carbohydrate metabolism, (Gevers & Krebs, 1966; Gevers, 1967; Underwood & Newsholme, 1967; Newsholme & Crabtree, 1970) suggest that negative modulation of fructose diphosphatase (and concomitant activation of phosphofructokinase by AMP) is not the sole regulatory mechanism at this level.

To examine the effects of temperature on the regulatory and catalytic properties of this key enzyme in gluconeogenesis, a study was initiated on the roles of various cations in addition to those of substrate, various intermediates and AMP in the regulation of fructose diphosphatase from skeletal muscle of the Alaskan king-crab *Paralithodes camtschatica*.

METHODS AND MATERIALS

Experimental animals. The Alaskan king-crab (*Paralithodes camtschatica*) inhabits the colder waters of the northern Pacific Ocean; temperatures in the range of distribution vary from about 12°C in the summer to about 1.5°C in winter (Johnson & Hartman, 1969). King-crabs have been observed migrating considerably in depth (Mr Guy Powell, Alaska Department of Fish and Game, personal communication) and it is possible that the animal may encounter changes within 1.5–12°C throughout the year.

King-crabs were captured in crabpots in the vicinity of

Kodiak Island, Alaska, U.S.A., in waters at about 6–7°C and were held at 6–7°C on board ship for approx. 12 h after capture. After the animals had been killed tissues were quickly frozen at –55°C and stored at –30°C until use.

Experimental methods. Muscle was taken from the proximal segment of the walking legs, since it was found that skeletal muscle from different regions contained different specific activities of fructose diphosphatase. Homogenate of skeletal muscle was prepared in 10 mM-tris-HCl buffer, pH 7.5, in a VirTis model 23 homogenizer at top speed at 1–2°C. The homogenate was spun at 30000g for 60 min at 2°C and the pellet discarded. The supernatant was brought to 10% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and stirred for at least 4 h at 4°C. The suspension was then centrifuged at 2°C, the pellet discarded and the supernatant slowly brought to 40% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and stirred once more for 4 h at 4°C. The precipitate was collected by centrifugation at 2°C and redissolved in a minimal volume of 10 mM-tris-HCl buffer, pH 7.5, at 4°C. The enzyme solution was then dialysed at 4°C against 10 mM-tris-HCl buffer, pH 7.5, with three changes of buffer at 8 h intervals, before use in the assays. This procedure effectively separated fructose diphosphatase from non-specific alkaline phosphatase, which was assayed with *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) as substrate. The reaction medium contained, in 2.0 ml of 50 mM-tris-HCl buffer of different pH values, various concentrations of fructose 1,6-diphosphate (Sigma Chemical Co.) and MnCl_2 . The reaction was initiated by the addition of the fructose diphosphatase preparation to the medium and was assayed by measuring release of P_i , determined by the method of Fiske & Subbarow (1925) with a Technicon AutoAnalyzer equipped with a 50 mm flow cell. Unless otherwise specified all kinetic experiments were carried out at pH 7.5. Enzyme activity was linear with time for at least 45 min and was proportional to concentration of enzyme. The enzyme activities reported in this paper are the means of at least two separate determinations with variations of less than 10% between individual determinations.

RESULTS

King-crab muscle fructose diphosphatase, unlike most other muscle fructose diphosphatases examined (Krebs & Woodford, 1965; Fernando, Enser, Pontremoli & Horecker, 1968), has a low affinity for substrate (K_m for fructose 1,6-diphosphate is 5×10^{-5} M at 5°C). Affinity of the king-crab enzyme appears to be quite sensitive to temperature; thus K_m for fructose 1,6-diphosphate (calculated from Hill plots) falls about threefold as temperature is decreased from 20°C to 5°C (Table 1) with optimum concentrations of Mn^{2+} in the medium. Hill plots of the saturation kinetics yield n_H values of about 4 at the upper temperature range (15–20°C), indicating a high degree of fructose 1,6-diphosphate site-site interaction. In this connexion, Behrisch & Hochachka (1969a) reported n_H values of about 4 for rainbow-trout liver fructose diphosphatase. It is noteworthy that as tempera-

Table 1. *Effect of temperature on affinity and values of n_H of substrate (fructose 1,6-diphosphate) of king-crab muscle fructose diphosphatase*

Assays were carried out at pH 7.4 and with 0.1 mM-Mn²⁺.

Temp. (°C)	$10^4 \times K_m$ (M)	n_H	Probability of n_H being zero
0	1.5	1.25	0.00
5	0.5	2.5	0.02
10	0.9	2.43	0.00
15	1.2	4.50	0.00
20	1.5	3.75	0.00

Fig. 1. Substrate saturation curves of king-crab muscle fructose diphosphatase at a series of temperatures: ○, 0°C; △, 5°C; ●, 10°C; ▲, 15°C; □, 20°C. Assays were carried out at pH 7.5 and in the presence of 0.1 mM-Mn²⁺. The inset curve shows the effect of temperature on K_m for fructose 1,6-diphosphate.

ture is lowered from 5°C to 0°C there is a threefold increase in K_m for fructose 1,6-diphosphate and an attendant decrease in the value of n_H (Table 1), indicating an accompanying decrease in fructose 1,6-diphosphate site-site interaction at this temperature. This increase in K_m for substrate is in marked contrast with other poikilothermic fructose diphosphatases, in which K_m for substrate continues to fall as temperature is decreased (Behrisch, 1969; Behrisch & Hochachka, 1969*a,b*). The relationship between temperature and K_m for substrate in the king-crab enzyme yields a K_m -temperature curve (Fig. 1) similar to those reported for lactate dehydrogenases, pyruvate kinases (Hochachka & Somero, 1968; Somero, 1969*a*), isocitrate dehydrogenase (Moon, 1970) and acetylcholinesterase (Baldwin & Hochachka, 1970) from various poikilotherms. In these enzymes, as well as the king-crab fructose diphosphatase, minimum K_m for substrate occurs approximately at the temperature of acclimatization (see Hochachka & Somero, 1968).

Fig. 2. Arrhenius plot for activity of king-crab muscle fructose diphosphatase at pH 7.5 in the presence of optimum concentrations of substrate and Mn²⁺. Each point was made in duplicate with negligible variation between the duplicates.

Table 2. *Activation energies of king-crab muscle fructose diphosphatase for several temperature ranges in the presence of 0.1 mM-Mn²⁺ and optimum concentration of fructose 1,6-diphosphate*

E_a was calculated for $V_{(opt.)}$.	
Temp. range (°C)	E_a (kcal/mol)
0-5	0
5-10	0
10-15	0.61
15-20	0.52

As in trout and salmon fructose diphosphatases, the king-crab enzyme is partially inhibited by higher concentrations of substrate (Fig. 1) and this inhibition appears to be independent of temperature. This substrate inhibition is consistent with the results of Underwood & Newsholme (1965) and Taketa & Pogell (1965), who suggested a competitive binding of more than one identically charged

substrate molecule to the active site, resulting in a partially inactivated complex. Arrhenius plots (Fig. 2) of these saturation kinetics show that the catalytic rate $V_{(opt.)}$, i.e. at optimum concentrations of substrate, appears to be relatively independent of temperature between 0°C and 10°C and corresponding values of activation energy (E_a) approach zero (Table 2). Above 10°C slopes of the Arrhenius plot rise and values of E_a increase correspondingly.

Inhibition by AMP. King-crab muscle fructose diphosphatase, like other muscle fructose diphosphatases examined (Krebs & Woodford, 1965; Opie & Newsholme, 1967), has a high affinity for its allosteric inhibitor, AMP (K_i is 4×10^{-8} M at 0°C). As observed in other systems (Taketa & Pogell, 1965; Behrisch & Hochachka, 1969a), inhibition of the king-crab enzyme by AMP is sensitive to temperature (Fig. 3). Thus as temperature is increased from 0°C to 8°C K_i for AMP rises (Table 3); as temperature is further increased from 8°C to 15°C K_i for AMP falls to a value close to that at 0°C. It is perhaps significant that conditions of acclimatization roughly correspond to the temperature at which the king-crab enzyme is least sensitive to inhibition by AMP.

Search for a metabolic activator. A search was made for a metabolic intermediate that would activate king-crab muscle fructose diphosphatase or reverse the inhibition by AMP or both. The enzyme

Fig. 3. Inhibition of king-crab muscle fructose diphosphatase by AMP at a series of temperatures: ○, 0°C; ▲, 8°C; △, 15°C. Assays were carried out at pH 7.5 in the presence of 1 mM-fructose 1,6-diphosphate and 0.1 mM- Mn^{2+} .

was not affected by any of the following metabolites tested: glucose 1-phosphate, fructose 6-phosphate, dihydroxyacetone phosphate, 2-phosphoglycerate, pyruvate, lactate, isocitrate and NADH. Two other metabolites that were considered logical choices as possible activators of the enzymes were ATP and phosphoenolpyruvate, since ATP is a negative modulator of fish phosphofructokinase (J. Freed, personal communication) and since the substrate of fructose diphosphatase, fructose 1,6-diphosphate, is a positive modulator of pyruvate kinase (Hess, 1968; Somero, 1969a; Somero & Hochachka, 1968). However, ATP as well as citrate appeared to cause some inhibition of fructose diphosphatase activity, a finding also reported with the pig kidney enzyme (Mendicino, Beaudreau & Bhattacharyan, 1966) and the bacterium *Acinetobacter* (Mukkada & Bell, 1969). In addition, phosphoenolpyruvate at high ($V_{(opt.)}$ and above) concentrations of substrate caused some inhibition of fructose diphosphatase activity. However, at low (approx. K_m) concentrations of fructose 1,6-diphosphate, phosphoenolpyruvate effected a marked increase in the catalytic activity of fructose diphosphatase (Fig. 4). It thus appears that phosphoenolpyruvate enhances the affinity of king-crab muscle fructose diphosphatase for substrate, as shown in the substrate saturation curves at various concentrations of phosphoenolpyruvate and at different temperatures (Fig. 4). The phosphoenolpyruvate-induced activation of the king-crab enzyme is sensitive to temperature, so that 0.5 mM-phosphoenolpyruvate causes about a seven-fold decrease in K_m for fructose 1,6-diphosphate at 15°C but a 25-fold decrease at 0°C (Table 4). Kirtley & Dix (1969) reported hydrolysis of phosphoenolpyruvate by rabbit liver fructose diphosphatase and presented several lines of evidence that phosphoenolpyruvate was bound by the substrate-binding site; however, no hydrolysis of phosphoenolpyruvate by the king-crab enzyme was observed at any of the concentrations tested. It is therefore likely that, although phosphoenolpyruvate is bound at the substrate-binding site of the king-crab enzyme, it cannot satisfy the steric requirements of the catalytic site for hydrolysis of the natural substrate. As is probably the case at low concentrations of fructose 1,6-diphosphate, the binding of

Table 3. Inhibition by AMP of king-crab muscle fructose diphosphatase at a series of temperatures

Assays were carried out in 50 mM-tris-HCl buffer, pH 7.5, in the presence of 1 mM-fructose 1,6-diphosphate and 0.1 mM- Mn^{2+} .

Temp. (°C)	$10^8 \times K_i$ of AMP (M)	n_H	Probability of n_H being zero
0	4.0	1.05	0.01
8	18.0	0.92	0.04
15	5.05	0.88	0.00

phosphoenolpyruvate effects a conformational change in the enzyme molecule that enhances fructose 1,6-diphosphate site-site interaction and thus increases affinity for substrate. Thus at 0°C an increase in phosphoenolpyruvate concentration from 0 to 0.5 mM in the medium causes a 25-fold decrease in K_m for fructose 1,6-diphosphate with a corresponding increase in values of n_H from 1.2 to 2.2 for the fructose 1,6-diphosphate saturation curves (Table 4), indicating enhanced fructose 1,6-diphosphate site-site interaction.

Phosphoenolpyruvate did not cause a reversal of inhibition of fructose diphosphatase activity by AMP. However, increasing concentrations of phosphoenolpyruvate appear to protect against inhibition of the king-crab enzyme by AMP (Fig. 5) in a manner similar to Mg^{2+} (Behrlich & Hochachka, 1969a). The effect of phosphoenolpyruvate on inhibition by AMP is sensitive to temperature; thus 0.1 mM-phosphoenolpyruvate effects a three- to four-fold increase in K_i for AMP at 0°C whereas it appears to cause an enhancement of inhibition by AMP at 15°C (see Table 5). This result is comparable

with that obtained by Iwatsuki & Okazaki (1967), who observed that a regulatory nucleotide (dCDP) of deoxythymidine kinase from *Escherichia coli* acts as a negative modulator below 21°C and as a positive modulator of the enzyme above 21°C. The protection provided by phosphoenolpyruvate against inhibition by AMP at the lower temperature may be related to the powerful enhancement of enzyme-substrate interaction by phosphoenolpyruvate at this temperature (see Table 4). Also, the nature of the enzyme-substrate or the enzyme-modulator complex, or both, may change with temperature with the result that catalytic activity

Fig. 4. Substrate saturation curves of king-crab muscle fructose diphosphatase at 0°C (a) and 15°C (b) with various concentrations of phosphoenolpyruvate: ○, none; △, 0.05 mM; ●, 0.10 mM; ●, 0.50 mM. Assays were carried out at pH 7.5 and in the presence of 0.1 mM- Mn^{2+} .

Fig. 5. Inhibition of king-crab muscle fructose diphosphatase by AMP at 0°C (a) and 15°C (b) with various concentrations of phosphoenolpyruvate: ○, none; △, 0.05 mM; ●, 0.10 mM. Assays were carried out at pH 7.5 and in the presence of 0.1 mM-fructose 1,6-diphosphate and 0.1 mM- Mn^{2+} .

Table 4. Effect of phosphoenolpyruvate on affinity of king-crab muscle fructose diphosphatase for substrate (fructose 1,6-diphosphate) at different temperatures

Assays were carried out in 50 mM-tris-HCl buffer, pH 7.5, in the presence of 0.1 mM-fructose 1,6-diphosphate and 0.1 mM- Mn^{2+} .

Concn. of phosphoenolpyruvate (mM)	Assays at 0°C			Assays at 15°C		
	$10^4 \times K_m$ for substrate (m)	n_H	Probability of n_H being zero	$10^4 \times K_m$ substrate (m)	n_H	Probability of n_H being zero
0	1.5	1.25	0.00	1.3	2.3	0.00
0.05	1.25	1.25	0.05	0.85	2.16	0.00
0.10				0.52	2.7	0.13
0.50	0.06	2.2	0.07	0.19	2.9	0.03

Table 5. *Effect of phosphoenolpyruvate on affinity of king-crab muscle fructose diphosphatase for AMP*

Conditions of assay were as described in Table 4.

Concn. of phosphoenolpyruvate (mm)	Assays at 0°C $10^3 \times K_i$ for AMP (m)	Assays at 15°C $10^3 \times K_i$ for AMP (m)
0	4.5	7.1
0.05	5.0	6.1
0.10	7.5	2.0

becomes more sensitive to inhibition by AMP at 15°C.

Combined with the finding that fructose 1,6-diphosphate can act as a feedforward activator of pyruvate kinase (Hess, 1968; Somero, 1969a; Somero & Hochachka, 1968), the identification of phosphoenolpyruvate as a feedforward activator of a regulatory step in gluconeogenesis in the Alaskan king-crab makes it possible to suggest a coupling of the controlling mechanisms of several regulatory reactions in the gluconeogenic and glycolytic chains.

Control by bivalent cations. King-crab muscle fructose diphosphatase, like other fructose diphosphatases examined (Gomori, 1943; Behrisc & Hochachka, 1969a), exhibits an absolute requirement for a bivalent cation, which may be fulfilled by either Mn^{2+} or Mg^{2+} (Fig. 6). However, Mn^{2+} appears to be a far more powerful activator of fructose diphosphatase activity than does Mg^{2+} and, as in other poikilothermic systems (Behrisc & Hochachka, 1969a,b; Behrisc, 1969), affinity for Mn^{2+} is approximately tenfold that for Mg^{2+} . As with the fructose diphosphatases from lungfish and salmon (Behrisc & Hochachka, 1969b; Behrisc, 1969) the Mn^{2+} saturation curve is sigmoid and affinity of the king-crab enzyme for Mn^{2+} is sensitive to temperature; thus an increase in temperature from 0°C to 15°C causes an approximately fourfold decrease in K_a for Mn^{2+} (Table 6). At higher temperatures (10–15°C) higher concentrations of Mn^{2+} tend to inhibit activity of the king-crab enzyme, whereas at 0°C and 5°C no inhibition of the activity is observed. This finding is similar to that of Behrisc (1969), who observed a diminished inhibition of salmon fructose diphosphatase by high concentrations of Mn^{2+} at low temperatures. The Mn^{2+} saturation curves at 0°C and 5°C are somewhat unusual in that, in addition to the sigmoid curve at low concentrations of the cofactor, there is a second inflexion, which occurs at about 60 μM (Fig. 6). Similar results have been observed in the Mg^{2+} saturation curves of salmon fructose diphosphatase (Behrisc, 1969) and substrate saturation curves for pyruvate kinase in the Alaskan king-crab by Somero (1969a), who sug-

Fig. 6. Mn^{2+} saturation curves of king-crab muscle fructose diphosphatase at a series of temperatures: $\circ$, 0°C; $\triangle$, 5°C; $\bullet$, 10°C; $\blacktriangle$, 15°C. Assays were carried out at pH 7.5 in the presence of 1 mM-fructose 1,6-diphosphate.

gested a temperature-dependent interconversion of two or more thermal isomers of the enzyme.

An increase in pH results in a rise in affinity of king-crab fructose diphosphatase for Mn^{2+} (Fig. 7), a finding previously reported for the trout, lungfish and salmon enzymes (Behrisc & Hochachka, 1969a,b; Behrisc, 1969). Thus a rise from pH 7.5 to 9.5 causes a fivefold decrease in K_a for the cationic cofactor (Table 7). In addition, the inhibition of activity by higher concentrations of Mn^{2+} becomes more pronounced at pH 9.5 (Fig. 7). It is noteworthy that a rise from pH 7.5 to 8.0 (the pH optimum for the king-crab enzyme; see Table 8) causes a slight decrease in K_a for Mn^{2+} , but effects an approximately 1.5-fold increase in $V_{(opt.)}$.

In the presence of 1 mM- or 10 mM- Mg^{2+} at 0°C pH appears to have little effect on activity of the king-crab enzyme (Table 8). In contrast, at 10°C and with 1 mM- Mg^{2+} in the medium there is a sharp increase in activity at pH 8.0, and at this temperature an increase in Mg^{2+} concentration to 10 mM results in a sharp peak of activity at pH 7.5, the accepted physiological pH range. These results are similar to those of Preiss, Biggs & Greenberg (1967) and Behrisc & Hochachka (1969a), who observed a shift in the pH optimum towards the neutral

Table 6. *Effect of temperature on affinity of king-crab muscle fructose diphosphatase for Mn^{2+}*

Assays were carried out in 50mm-tris-HCl buffer, pH 7.5, in the presence of 1 mM-fructose 1,6-diphosphate.

Temp. ($^{\circ}C$)	$10^5 \times K_s$ for Mn^{2+} (M)	n_H	Probability of n_H being zero
0	3.1	1.72	0.02
5	1.9	1.68	0.03
10	1.07	2.31	0.00
15	0.67	2.60	0.00

Fig. 7. Effect of pH on affinity of king-crab muscle fructose diphosphatase for Mn^{2+} : ●, pH 7.5; △, pH 8.0; ○, pH 9.5. Assays were carried out at $10^{\circ}C$ in the presence of 1 mM-fructose 1,6-diphosphate.

DISCUSSION

It is generally accepted that the major means of regulation of fructose diphosphatase is by negative modulation by AMP (see Horecker *et al.* 1966; Atkinson, 1966). Thus when concentrations of AMP are high, indicating a low energy charge, the enzyme is inhibited and gluconeogenesis is curtailed. This postulate is supported by studies on liver homogenates (Gevers & Krebs, 1966; Newsholme & Gevers, 1967) and kidney-cortex slices (Weidemann, Hems & Krebs, 1969) insofar as modulation of activity at this point by the adenylates affects the net flow of carbohydrate metabolism.

The remarkable temperature-dependence of the fructose diphosphatase-AMP interaction complicates control at this step in a poikilotherm since a fall in temperature causes a large increase in affinity of the enzyme for AMP. Thus in a cold-blooded organism one would expect gluconeogenesis to be essentially inhibited at low temperatures. Previous studies (Underwood & Newsholme, 1965; Behrisch & Hochachka, 1969a) have shown that inhibition of fructose diphosphatase activity by AMP can be minimized and reversed by increasing concentrations of the cationic cofactor (Mn^{2+} or Mg^{2+}). In addition, an increase in pH will cause an increase in K_i for AMP (Behrisch & Hochachka, 1969a) and a concomitant decrease in K_s for Mn^{2+} or Mg^{2+} (see also Fig. 7). Further, the observation that a decrease in temperature effects an increase in intracellular pH (Reeves & Wilson, 1969) in poikilotherms, and thus lowers the thermal sensitivity of the fructose diphosphatase-AMP interaction, provides a possible mechanism to permit maintenance of fructose diphosphatase activity at all temperatures (for discussion see Behrisch, 1969). In a series of experiments at several temperatures in which pH was varied in accordance with the observations of Reeves & Wilson (1969) the interaction between rainbow-trout liver fructose diphosphatase and AMP was found to be independent of temperature (H. W. Behrisch, unpublished work). Similar results have been obtained for citrate synthase from rainbow-trout liver, in which, under these conditions, the temperature-sensitivity

range with an increase in concentration of the cationic cofactor.

Effects of univalent cations on activity. Numerous publications report activation of enzyme activity by the univalent cations Na^+ , K^+ and NH_4^+ (for a comprehensive list see Dixon & Webb, 1964). These three ions have been shown to stimulate activity of fructose diphosphatases from a variety of sources (Hubert, Villanueva, Gonzalez & Marcus, 1970) with K^+ and NH_4^+ being the most effective activators. The effectiveness of these cationic stimulators seems to be influenced by temperature. Thus Na^+ stimulates the enzyme about equally at all temperatures, and then causes a partial inhibition at higher concentrations (Table 9). NH_4^+ effects a greater stimulation of the enzyme at $0^{\circ}C$ and $15^{\circ}C$ than at $8^{\circ}C$, at which last-named temperature the king-crab enzyme is largely independent of NH_4^+ . K^+ stimulates king-crab fructose diphosphatase at $0^{\circ}C$, whereas at $8^{\circ}C$ and $15^{\circ}C$ 0.1 mM- K^+ causes an inhibition of approx. 20 and 80% respectively (Table 9). Thus the ability of K^+ to act either as an activator at low temperature or as an inhibitor of enzyme activity at the higher physiological temperatures indicates that the cation could render fructose diphosphatase activity virtually independent of temperature.

Table 7. *Effect of pH on affinity of king-crab muscle fructose diphosphatase for Mn²⁺*

Assays were carried out at 10°C in the presence of 1 mM-fructose 1,6-diphosphate.

pH	$10^5 \times K_s$ for Mn ²⁺ (M)	n_H	Probability of n_H being zero
7.5	1.0		
8.0	1.4	2.33	0.00
9.5	0.32	4.70	0.00

Table 8. *Effect of pH on activity of king-crab muscle fructose diphosphatase at different concentrations of Mn²⁺ and Mg²⁺*

Assays were carried out in the presence of 1 mM-fructose 1,6-diphosphate.

Temp. (°C)	pH	Activity (mm-P _i released/h per mg of protein)			
		0.1 mM-Mn ²⁺	1.0 mM-Mn ²⁺	1 mM-Mg ²⁺	10 mM-Mg ²⁺
0	6.5	0.25	0.05	—	—
	7.0	0.22	0.09	0.025	0.05
	7.5	0.20	0.067	0.126	0.05
	8.0	0.25	0.05	0.076	0.05
	8.5	0.05	0.05	0.10	0.05
	9.0	0.20	0.04	0.025	0.10
	9.5	0.05	0.04	0.025	0.30
10	6.5	0.60	0.60	—	—
	7.0	0.56	0.50	0.55	0.40
	7.5	0.56	0.44	0.55	0.60
	8.0	0.56	0.70	0.60	0.50
	8.5	0.40	0.50	0.55	0.40
	9.0	0.48	0.50	0.50	0.45
	9.5	0.68	0.54	0.50	0.50

Table 9. *Effect of various concentrations of Na⁺, K⁺ and NH₄⁺ on the activity of king-crab muscle fructose diphosphatase*Assays were carried out at pH 7.5 in the presence of 1 mM-fructose 1,6-diphosphate and 0.1 mM-Mn²⁺.

Temp. (°C)	Concn. of univalent cation (mM)	Relative activity (% of control)		
		Na ⁺	K ⁺	NH ₄ ⁺
0	0 (control)	100	100	100
	0.1	150	150	300
	0.5	300	250	800
	1.0	700	300	650
	5.0	650	300	600
	10.0	500	300	300
	50.0	350	300	200
8	0 (control)	100	100	100
	0.1	180	82	107
	0.5	467	82	104
	1.0	111	74	109
	5.0	89	95	112
	10.0	89	121	110
	50.0	45	70	95
15	0 (control)	100	100	100
	0.1	145	22.2	467
	0.5	225	37.5	400
	1.0	535		378
	5.0	562	45	
	10.0	315	45	355
	50.0	225	157	89

of the overall reaction and of the K_i of the inhibitor (ATP) is lowered (P. W. Hochachka & J. E. Lewis, unpublished work). In view of the fact that there is a steep section between pH 7.5 and 8.0 in the pH-activity curve for king-crab fructose diphosphatase (Table 8) and that variations from pH 7.5 at 15–20°C to pH 8.2 at 0°C are within the ranges observed (Reeves & Wilson, 1969; Robin, 1962), this temperature-pH relationship provides a formal mechanism for maintaining the overall regulation of the enzyme activity independent of changes in environmental temperature.

Another mechanism for control of fructose diphosphatase activity may involve the univalent cations Na^+ , K^+ and NH_4^+ . The finding that these cations can alter activity of fructose diphosphatases from a variety of mammalian and poikilothermic sources (Marcus, 1970) as well as from skeletal muscle of king-crab (Table 9) is of particular relevance to metabolic control in poikilotherms, because in addition to those of a number of bivalent cations the concentrations of Na^+ and K^+ vary during thermal acclimatization in aquatic poikilotherms (Rao, 1962; Hickman *et al.* 1964; Toews & Hickman, 1969; Umminger, 1969, 1970). Localized changes in concentrations of these cations and compartmentation are known to occur in the cell (Bygrave, 1966, 1967); hence alterations in the phosphatase caused by such changes in cation concentration may well be a major method of influencing the enzyme activity.

As in other allosteric enzymes (for discussion see Atkinson, 1966), the fructose diphosphatase-substrate interaction probably provides another mechanism for control of activity. Similarly to some other enzymes of poikilotherms (see Hochachka, 1970), affinity of king-crab fructose diphosphatase for substrate increases with decreasing temperature, so that at physiological concentrations of substrate the rate of the reaction would be independent of temperature. Indeed, even at $V_{\text{opt.}}$ concentrations of fructose 1,6-diphosphate, between 0°C and 10°C the reaction is virtually independent of temperature and calculated values of E_a approach zero (Table 2). As seen in the fructose 1,6-diphosphate saturation curves (Fig. 1), K_m for fructose 1,6-diphosphate falls with decreasing temperature until at 5°C minimum K_m is obtained; then as temperature is further decreased to 0°C there is an approximately two- to three-fold increase in K_m for fructose 1,6-diphosphate. This effect of temperature of the king-crab fructose diphosphatase-fructose 1,6-diphosphate interaction results in a complex U-shaped K_m -temperature curve (Fig. 1) similar to those seen for a number of regulatory enzymes from various cold- and warm-water poikilotherms. The rather sharp increase in K_m for fructose 1,6-diphosphate at the lower end

of the temperature range carries with it the implication of a diminution in fineness of control by substrate. Thus above and below the temperature at which minimum K_m falls there is an increase in K_m for fructose 1,6-diphosphate and a decrease in efficiency of control by substrate. In other systems in which such complex U-shaped K_m -temperature curves have been observed (Somero, 1969a; Hochachka & Somero, 1968; Baldwin & Hochachka, 1970; Moon, 1970) minimum K_m for substrate occurs at or near the temperature of acclimatization, and it is perhaps significant that the animals used in the present experiments were acclimatized to 5–7°C.

In a comparison of the effects of temperature on enzyme-substrate interaction in pyruvate kinases from two poikilotherms (rainbow trout and an Antarctic fish) and a homiotherm (rat), Somero & Hochachka (1968) found that in the mammalian system temperature exerts a relatively small effect on K_m for substrate; thus in the rat enzyme K_m for substrate is virtually constant over a wide range of temperatures. On the basis of their results, Hochachka & Somero (1968) concluded that 'evolutionary adaptation of enzymes to low temperatures may lead to decreases in K_m for substrate', a kinetic characteristic that would make the rate of reaction at least partially insensitive to changes in temperature. In contrast, the selective pressures involved in maintenance of metabolism and its control at low, and changing, temperatures are not present in a homiothermic animal and hence one would not expect such large temperature effects on enzyme-substrate affinity. In short, judging from the present kinetic data on the king-crab fructose diphosphatase and the findings of Somero & Hochachka (1968), it appears that the enzyme molecules in cold-adapted organisms are better catalysts at low temperatures than are enzymes from warm-adapted (or homiothermic) organisms.

A fourth mechanism, perhaps the most striking, is the enhancement of the fructose diphosphatase-fructose 1,6-diphosphate interaction by phosphoenolpyruvate. At 0°C and 15°C 0.5 mM-phosphoenolpyruvate effects a fall in K_m for fructose 1,6-diphosphate to 6×10^{-6} and 19×10^{-6} M respectively, values well within the observed physiological concentration range of fructose 1,6-diphosphate [P. W. Hochachka, J. M. Freed, G. N. Somero & C. L. Prosser (unpublished work) found concentrations of about 0.015–0.025 mmol of fructose 1,6-diphosphate/l of total water for king-crab muscle]. In this connexion fructose 1,6-diphosphate has been shown to be a feedforward activator of pyruvate kinase (Haeckel, Hess, Lauterborn & Wuester, 1968; Hess, 1968; Somero, 1969a), and the identification of phosphoenolpyruvate as a regulator of fructose diphosphatase activity in king-crab skeletal muscle

permits the suggestion of a coupling of the control mechanisms of several regulatory steps in the glycolytic and gluconeogenic chains. Thus under conditions of decreased energy charge (high AMP and low ATP concentrations) both the phosphofructokinase and pyruvate kinase steps are open, a situation characterized by increased concentrations of fructose 1,6-diphosphate and decreased concentrations of phosphoenolpyruvate (Hess, 1968). The activation by fructose 1,6-diphosphate of the pyruvate kinase step further enhances the rate of glycolysis and causes an increase in the energy charge of the cell. The resultant increase in ATP concentrations will in turn tend to inhibit both rate-limiting reactions of glycolysis, phosphofructokinase and pyruvate kinase (Haeckel *et al.* 1968; Hess, 1968) and thus lead to a transient increase in phosphoenolpyruvate concentration and decrease in that of fructose 1,6-diphosphate. Phosphoenolpyruvate may now act as an activator of gluconeogenesis by enhancing the affinity of fructose diphosphatase for its substrate (fructose 1,6-diphosphate). Such modulator-induced changes in K_m for substrate have also been observed for king-crab muscle phosphofructokinase (J. Freed, personal communication) and appear to be a regulatory mechanism common to many organisms (see Scrutton & Utter, 1969).

In this connexion reports (Newsholme & Gevers, 1967; Newsholme & Crabtree, 1970) of cycling of intermediates at the fructose diphosphatase-phosphofructokinase step in skeletal muscle suggest that negative modulation of fructose diphosphatase activity by AMP is not the sole mechanism of control at this level. The apparently allosteric effect of phosphoenolpyruvate on fructose diphosphatase at low concentrations of substrate (see Fig. 4) as well as the observed phosphoenolpyruvate-induced 'protection' against inhibition by AMP (Fig. 5) offer a formal mechanism for the activation of fructose diphosphatase and control of activity under conditions that favour concomitant activity of phosphofructokinase.

It has been suggested (Noble, 1969) that co-operative allosteric interactions, such as the haem-haem interactions in haemoglobin, require that the change in inter-subunit bonding energy accompanying the attachment of a ligand molecule be not uniform but depend rather on the extent to which the protein is already 'liganded'. Thus the energy of subunit bonding might be the primary co-operative parameter (Noble, 1969). It seems reasonable to accept that the allosteric properties of enzymes confer selective advantage to the cell or organism only when they are effective under physiological concentrations of substrate, modulator and cofactor. In an enzyme from a poikilotherm the binding of ligand at low temperature may

result in a considerable increase in the free energy of the conformation (relative to the proposed low bonding energy). This would lead to enhanced subunit-subunit (or site-site) interaction at low temperature, as suggested by the increased Hill constants of substrate saturation in trout fructose diphosphatase at low temperature (Behrisch & Hochachka, 1969a) and in the king-crab muscle enzyme at low temperature in the presence of an allosteric activator (see Table 4). It is therefore suggested that two sites at which selective pressure has been large during evolution to cold are (1) enhanced enzyme-ligand affinity at low temperatures and (2) diminished subunit bonding energy, which permits allosteric behaviour at low temperatures.

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