Temperature and the Regulation of Enzyme Activity in Poikilotherms

PROPERTIES OF RAINBOW-TROUT FRUCTOSE DIPHOSPHATASE

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1. The properties of fructose diphosphatase from the liver of rainbow trout (*Salmo gairdnerii*) were examined over the physiological temperature range of the organism. 2. Saturation curves for substrate (fructose 1,6-diphosphate) and a cofactor (Mg^{2+}) are sigmoidal, and Hill plots of the results suggest a minimum of two interacting fructose 1,6-diphosphate sites and two interacting Mg^{2+} sites per molecule of enzyme. 3. Mn^{2+} -saturation curves are hyperbolic, and the K_a for Mn^{2+} , which inhibits the enzyme at high concentrations, is 50–100-fold lower than the K_a for Mg^{2+} . 4. Fructose diphosphatase is inhibited by low concentrations of AMP; this inhibition appears to be decreased and reversed by increasing the concentrations of Mg^{2+} and Mn^{2+} . Higher concentrations of AMP are required to inhibit the trout fructose diphosphatase in the presence of Mn^{2+} . 5. The affinities of fructose diphosphatase for fructose diphosphate and Mn^{2+} appear to be temperature-independent, whereas the affinities for Mg^{2+} and AMP are highly temperature-dependent. 6. The pH optimum of the enzyme depends on the concentrations of Mg^{2+} and Mn^{2+} . In addition, pH determines the K_a for Mg^{2+} ; at high pH, K_a for Mg^{2+} is lowered. 7. The enzyme is inhibited by Ca^{2+} and Zn^{2+} , and the inhibition is competitive with respect to both cations. 8. The possible roles of these ions and AMP in the modulation of fructose diphosphatase and gluconeogenic activity are discussed in relation to temperature adaptation.

The enzyme FDPase,* in the presence of a bivalent cation, catalyses the conversion of FDP into fructose 6-phosphate and P_i . The requirement for close regulation of FDPase activity in gluconeogenic tissues is generally recognized, since the simultaneous operation of both FDPase and phosphofructokinase within a single cell would lead to hydrolysis of ATP and thus to a short-circuit in metabolism (Horecker, Pontremoli, Rosen & Rosen, 1966). A number of studies suggest that the concomitant inhibition of FDPase and activation of phosphofructokinase by AMP can prevent the simultaneous function of these two enzymes (Underwood & Newsholme, 1965*a,b,c*; Newsholme & Gevers, 1967). This postulate is supported by studies of liver homogenates insofar as adenylate modulation at this point affects the net direction of carbohydrate metabolism (Gevers & Krebs, 1966). An overlooked feature of FDPase control is a remarkable temperature-dependence of the FDPase-AMP interaction. Thus, at 46°, the K_i of AMP for the rat liver enzyme is about 100 times its value at 2° (Taketa & Pogell, 1965). If AMP modulation were the major controlling mechanism, effi-

ciency of control in a poikilotherm would be unusually sensitive to environmental temperature changes, and glucose production would be essentially 'turned off' at low temperatures, whereas, in salmonid fishes at least, it appears to be favoured at low temperatures (Hochachka, 1967).

This implies that FDPase from salmonid fishes is perhaps less sensitive to AMP control at low temperature or that other regulatory mechanisms are available. We therefore examined the role of cations in addition to those of substrate and AMP in the regulation of rainbow-trout liver FDPase. We chose rainbow trout because these organisms in Nature encounter fairly wide variations in temperature, and thermal adjustments of salmonids are fairly well documented (Hochachka, 1967).

METHODS AND MATERIALS

Rainbow trout were kept at 10° and were fed *ad lib*. Liver samples were excised immediately after death and kept in a deep-freeze until use.

Liver homogenates were prepared in 3 parts of 0.01 M-tris-HCl, pH 7.4, in a VirTis homogenizer at 1–2°. The homogenates were spun at about 30000g for 30 min. at 4° and the pellets were discarded. The supernatant was brought to 15% saturation with solid $(NH_4)_2SO_4$ and stirred for at least 3 hr. at 4°. The suspension was then centrifuged as

* Abbreviations: FDPase, fructose diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11); FDP, fructose 1,6-diphosphate.

above, the pellet discarded and the supernatant brought to 55% saturation with solid $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation and dissolved in a minimal volume of 0.01M-tris-HCl buffer, pH 7.4. The enzyme solution was extensively dialysed against 0.01M-tris-HCl, pH 7.4, at 4° before use in the assays. This procedure separated FDPase from non-specific alkaline phosphatase, which was assayed with *p*-nitrophenyl phosphate as substrate. The reaction medium contained, in 2.0 ml. of 0.05M-tris-HCl buffer, various concentrations of FDP, AMP (Sigma Chemical Co., St Louis, Mo., U.S.A.), Mg^{2+} or Mn^{2+} ions, and Ca^{2+} or Zn^{2+} ions. The reaction was initiated by adding the FDPase preparation and was assayed by measuring P_i release. The P_i was determined by the Fiske & Subbarow (1925) method by using the Technicon AutoAnalyzer. Unless otherwise specified, all kinetic experiments were at 15° and pH 7.4. Enzyme activity was linear with time for at least 30 min. under optimum conditions, and was proportional to enzyme concentration.

RESULTS

Rainbow-trout liver FDPase, unlike most other types of FDPase examined (Horecker *et al.* 1966; Pontremoli, Luppis, Traniello, Wood & Horecker, 1965b), has a low affinity for substrate and a sigmoidal substrate-saturation curve (Fig. 1). Hill plots of the results give slopes of about 2 from 5° to 25°, with 5 mM- Mg^{2+} in the reaction medium. These values may be taken, with reservation, to represent

the degree of interaction between sites that bind the substrate, FDP, and agree with those for the spinach leaf enzyme (Preiss, Biggs & Greenberg, 1967). The slope of the Hill plot rises sharply at low temperature; thus at 0° n equals 4.6, indicating a great increase in FDP site-site interaction, with the result that the enzyme is saturated with substrate much more quickly. The value of n 4.6 that we find in the trout FDPase at 0° does not differ greatly from the number of binding sites calculated by Pontremoli, Grazi & Accorsi (1968) for rabbit liver FDPase.

Substrate inhibition of the trout enzyme is very apparent at pH 7.4 with either Mg^{2+} or Mn^{2+} as the cofactor, as has been reported for the rat liver enzyme (Taketa & Pogell, 1965). The enzyme is inhibited by about 50% at 1 mM-FDP and optimum Mg^{2+} concentration and by about 25% at 1 mM-FDP and optimum Mn^{2+} concentration (Fig. 1), an effect first noted by Krebs (1963). Temperature apparently has little effect on the degree of inhibition by increased FDP, whether Mn^{2+} or Mg^{2+} is the cofactor. There is thus a sharp peak of catalytic activity with a rapid decrease as substrate becomes more concentrated (Fig. 1), suggesting that FDP serves both as a substrate and an effective modulator of the enzyme. We have no information on the mechanism for substrate inhibition; the results are

Fig. 1. Substrate-saturation curves of rainbow-trout FDPase at different temperatures at pH 7.4. Activity is expressed as $\mu\text{moles of } \text{P}_i \text{ released/l./hr./mg. of protein}$: (a) in the presence of 5 mM- Mg^{2+} ; (b) in the presence of 20 μM - Mn^{2+} . Temperatures: $\circ$, 25°; $\blacktriangle$, 20°; $\blacksquare$, 15°; $\triangle$, 10°; $\bullet$, 5°; $\square$, 0°.

Table 1. Effect of temperature on affinity and values of *n* of substrate (FDP) of rainbow-trout FDPase at different pH values and concentrations of cofactor

Temp.	pH7.4						pH9.0		
	5 mM-Mg ²⁺			1 mM-Mn ²⁺			1 mM-Mn ²⁺		
	10 ⁴ <i>K_m</i> (M)	<i>n</i>	Probability of <i>n</i> being zero	10 ⁴ <i>K_m</i> (M)	<i>n</i>	Probability of <i>n</i> being zero	10 ⁴ <i>K_m</i> (M)	<i>n</i>	Probability of <i>n</i> being zero
0°	0.85	4.60	0.000	0.7	4.87	0.017			
5	1.2	2.30	0.001	1.1	4.17	0.003	1.1	4.74	0.000
10	1.2	2.59	0.016				1.2	4.56	0.000
15	1.3	1.71	0.000	1.5	2.39	0.012	1.0	2.73	0.000
20	1.5	2.54	0.000	1.7	2.05	0.002	1.0	2.16	0.000
25	2.4	1.72	0.000	2.1	1.81	0.000	1.4	1.78	0.000

Table 2. Activation energies of rainbow-trout FDPase at several temperature ranges

Temperature range	<i>E_a</i> (kcal./mole)		
	pH7.40		pH9.0
	5 mM-Mg ²⁺	20 μM-Mn ²⁺	1 mM-Mn ²⁺
0-5°	22.90	15.15	
5-10	11.98		7.89
5-15		6.26	
10-15	9.12		19.0
15-20	7.57	8.98	0
20-25	10.67	10.43	13.5

Fig. 2. Substrate-saturation curves of rainbow-trout FDPase at pH 9.0 at a series of temperatures in the presence of 1 mM-Mn²⁺. Activity is expressed as μmoles of P₁ released/l./hr./mg. of protein. Temperatures: ○, 25°; ▲, 20°; ■, 15°; △, 10°; ●, 5°.

Fig. 3. Hill plots for substrate-saturation curves of rainbow-trout FDPase at pH 9.0 at 5°, 10° and 15°. Assay conditions were as described in Fig. 2.

consistent with those of Underwood & Newsholme (1965a) and Taketa & Pogell (1965), who suggested a competitive binding of two identically charged substrate molecules to the active site, resulting in an inactive complex.

Temperature does not appear to affect greatly the ability of the enzyme to bind FDP, although there is a fall in *K_m* from 5° to 0°, and a relatively large rise in *K_m* from 20° to 25°, when either Mg²⁺ or Mn²⁺ is the cofactor (Table 1). Substrate-saturation curves near the alkaline pH optimum (pH 9.0) showed almost the same results. The curves are markedly sigmoidal and there appears to be no substrate inhibition; there is a plateau of activity in each case (Fig. 2). The sigmoidal saturation curves of the trout enzyme at pH 9.0 at all temperatures contrast with the findings of Pontremoli *et al.* (1968), who reported a loss of the co-operative effect between the four FDP-binding sites in the rabbit liver FDPase at pH 9.2, thus suggesting a hyperbolic substrate-saturation curve. As at

Fig. 4. Arrhenius plots for rainbow-trout FDPase at pH 7.4 and pH 9.0. Values are calculated from reaction velocities under optimum conditions ($v_{opt.}$). (a) pH 9.0: Δ , 1 mM- Mn^{2+} . (b) pH 7.4: $\circ$, 20 μM - Mn^{2+} ; $\bullet$, 5 mM- Mg^{2+} .

Fig. 5. Inhibition of rainbow-trout FDPase by AMP. (a) pH 7.4, 30 mM-FDP, 1 mM- Mg^{2+} , various temperatures: $\circ$, 25°; Δ , 20°; $\blacksquare$, 15°; $\triangle$, 10°; $\bullet$, 5°; $\square$, 0°. (b) pH 9.5, 15 mM-FDP, various concentrations of Mg^{2+} : Δ , 5 mM; $\bullet$, 1 mM; $\circ$, 0.6 mM.

pH 7.4, the slopes of Hill plots increase at low temperature, so that at pH 9.0 at 5° n is 4.74 and at 25° n is 1.78 (see Fig. 3 and Table 1).

Table 3. AMP inhibition of rainbow-trout FDPase at a series of temperatures: effect of temperature on values of n and K_i for AMP

Assays were carried out in 0.05 M-tris-HCl buffer, pH 7.40, in the presence of 0.3 mM-FDP and 1 mM- Mg^{2+} .

Temp.	$10^5 K_i$ for AMP (M)	n	Probability of n being zero
0°	2.5	1.10	0.000
5	4.5	1.49	0.000
10	13	0.97	0.000
15	4.5	2.39	0.000
20	33	1.52	0.000
25	80	1.65	0.000

The activation energy of the enzyme at 0–5° is approx. 1½–2 times that at higher temperatures (Table 2), and there is a break in the Arrhenius plot at 5° at pH 7.4 (Fig. 4b). At pH 9.0, there is a plateau between 15 and 20° in the Arrhenius plot, with a rise in activity at 20° (Fig. 4a). The enzyme thus appears to be temperature-insensitive between 15° and 20° at pH 9.0.

Inhibition by AMP. FDPase is strongly inhibited by AMP (Horecker *et al.* 1966; Taketa & Pogell, 1963, 1965; Underwood & Newsholme, 1965a). At pH 7.4 and at optimum concentrations of Mg^{2+} and FDP, the inhibition curve of FDPase is characteristically sigmoidal (Fig. 5), and there appear to be two interacting sites that bind AMP per molecule of enzyme. This number of sites differs from the value of three estimated by Taketa & Pogell (1965) for the rat liver enzyme. Temperature has a strong effect on the affinity of the trout enzyme for AMP (Table 3). At 0°, K_i is 2.5×10^{-5} M, and at 25° the apparent K_i is 8×10^{-4} M, approximately a 30-fold increase. The above relationship between temperature and affinity of the enzyme for the inhibitor was also reported in rat liver FDPase (Taketa & Pogell, 1965), in which there is about 100-fold increase in K_i with a rise in temperature from 2° to 46°. These inhibition constants for the rainbow-trout enzyme agree closely with those for the K_i of the lake-trout enzyme (Hochachka, 1967) and the brook-trout liver enzyme (M. Bosomworth, H. W. Behrisch & P. W. Hochachka, unpublished work). Further experiments with the lake-trout FDPase yielded confusing results: the enzyme from two separate preparations was almost totally insensitive to AMP inhibition. Variability in AMP sensitivity has also been noted for the ox liver enzyme (W. L. Byrne, personal communication); it thus appears that there may be both sensitive and insensitive forms of the enzyme.

There is a conspicuous decrease in K_i for AMP in the rainbow-trout liver enzyme at 15° (Table 3) and a corresponding rise in the slope of the Hill plot at

Table 4. Effects of various concentrations of cofactor and pH values on values of *n* and *K_i* for AMP

Assays were carried out at 15° in the presence of 1mM-FDP.

pH 7.4				pH 9.5			
Concn. of cofactor (mM)	10 ⁵ <i>K_i</i> for AMP (M)	<i>n</i>	Probability of <i>n</i> being zero	Concn. of cofactor (mM)	10 ⁴ <i>K_i</i> for AMP (M)	<i>n</i>	Probability of <i>n</i> being zero
Mg ²⁺ 0.6	5	1.58	0.008	Mg ²⁺ 0.6	1.3	1.89	0.056
1.0	6.5	1.24	0.000	1.0	2.1	1.49	0.081
5.0	9	1.08	0.000	5.0	No inhibition		
Mn ²⁺ 2.02	11	2.14	0.000				
0.01	13	0.906	0.002

Fig. 6. Reversal of AMP inhibition of rainbow-trout FDPase with both Mg²⁺ and Mn²⁺ as cofactors. Assays were carried out at pH 7.4 at 15° in the presence of 1mM-FDP. ○, Control, 20 μM-Mn²⁺; ●, control, 1mM-Mg²⁺; ×, 20 μM-Mn²⁺, + 0.25 mM-AMP at 10 min., + 1mM-Mn²⁺ at 20 min.; ▲, 1mM-Mg²⁺, + 80 μM-AMP at 10 min., + 10 mM-Mg²⁺ at 20 min.; △, 1mM-Mg²⁺, + 80 μM-AMP at zero time.

Fig. 7. Mg²⁺-saturation curves of rainbow-trout FDPase at a series of temperatures. Assay conditions were as described in Fig. 6 except that the concentration of FDP was 0.3 mM. Activity is expressed as μmoles of P₁ released/l./hr./mg. of protein. Temperatures: ○, 25°; ▲, 20°; ■, 15°; △, 10°; ●, 5°; □, 0°.

this temperature, a relationship not seen at the other temperatures, although *K_i* does change with temperature. In an attempt to find a possible explanation for this behaviour, we noted that *K_a* for Mg²⁺ was slightly higher at 15° than at any of the other temperatures tested, with the exception of 25°. The powerful inhibition of rainbow-trout liver FDPase at this temperature may be related to the relative decrease in affinity for the cofactor. Also, the nature of the enzyme-modulator or the enzyme-substrate complex, or both, may change with temperature, with the result that catalytic activity becomes more sensitive to AMP at 15°. Higher concentrations of Mg²⁺ or Mn²⁺ cause an increase in *K_i* and so protect against inhibition by AMP (Table 4).

In addition, Hill plots of these results demonstrate a definite fall in the value of *n* with increasing concentration of cofactor (Table 4). It thus seems that higher concentrations of Mg²⁺ or Mn²⁺ cause a fall in AMP site-site interaction, in this way regulating the effectiveness of AMP as an inhibitor. These results are obtained with either Mn²⁺ or Mg²⁺ in the reaction medium and provide a model for the control of FDPase activity.

Inhibition of the rat liver enzyme by AMP can be reversed by additional Mg²⁺ (Underwood & News-holme, 1965*a*), and it appears that both Mn²⁺ and Mg²⁺ can partially, if not wholly, reverse AMP inhibition of the trout liver enzyme (Fig. 6). Thus 10mM-Mg²⁺ restores about 70% of the former activity of FDPase that has been 80% inhibited by the addition of 80 μM-AMP, and 1mM-Mn²⁺ restores about 50% of the original activity of FDPase that has been 80% inhibited by 0.25mM-AMP (Fig. 6).

Table 5. *Effect of temperature on values of K_a and n of Mg^{2+}*

Assays were carried out in 0.05M-tris-HCl, pH 7.40.

Temp.	0.3mm-FDP			1mm-FDP $10^6 K_a$ for Mn^{2+} (M)
	$10^4 K_a$ for Mg^{2+} (M)	n	Probability of n being zero	
0°	5.2	0.924	0.006	
5	1.2	1.90	0.000	7.5
10	1.5	1.82	0.000	3.5
15	2.7	2.42	0.000	4.5
20	0.95	1.91	0.000	4.0
25	0.60	1.86	0.005	

In contrast with FDPase from most other organisms (Horecker *et al.* 1966), rainbow-trout liver FDPase is inhibited by AMP at alkaline pH (Fig. 5b). This result is not surprising, since the pH of the body fluids of fishes does not appear to be as rigidly controlled as that of mammals (see Steen, 1963). Here again the concentration of cofactor determines the apparent K_i (Table 4) and the degree of homotropic interaction between AMP-binding sites. The values of n at 1mm- Mg^{2+} and 0.6mm- Mg^{2+} are 1.49 and 1.89 respectively, which are in agreement with the calculated number of AMP-binding sites at pH 7.4.

Search for a metabolite activator of trout liver FDPase. A search was made for metabolic intermediates that would activate rainbow-trout liver FDPase. Considerable interest centred on phosphoenolpyruvate and ATP, since the substrate of FDPase, FDP, is a positive modulator of rainbow-trout pyruvate kinase (Somero & Hochachka, 1968), and ATP is a negative modulator for fish phosphofructokinase (J. Freed, personal communication) and mammalian phosphofructokinase (Newsholme & Gevers, 1967).

The ability of phosphoenolpyruvate to act as a positive modulator towards FDPase would have made possible the realization of a coupling of two control sites in glycolysis and gluconeogenesis. Likewise, ATP activation of FDPase would couple with ATP inhibition of phosphofructokinase. However, the enzyme was not affected by either phosphoenolpyruvate or ATP, or by any other of the following metabolites tested: glucose 6-phosphate, 3-phosphoglycerate, pyruvate, lactate, oxaloacetate, malate, citrate, succinate, ADP, NADH. We therefore examined possible cationic control of the functions of rainbow-trout FDPase.

Cation requirements and cation control. Trout liver FDPase, like other types of FDPase examined (Gomori, 1943), shows an absolute requirement for a bivalent cation, which can be fulfilled by Mg^{2+} or Mn^{2+} . The Mg^{2+} -saturation curve is sigmoidal in the presence or absence of EDTA in the reaction

medium (Fig. 7). At high FDP concentration (1mm) at 15°, K_a for Mg^{2+} of the rainbow-trout FDPase is 5×10^{-4} M. At 0.3mm-FDP ($v_{opt.}$) the slopes of Hill plots for the rainbow-trout enzyme approximate to 2 at all temperatures except 0°, where n equals 0.924 (Table 5). These kinetics suggest that FDPase contains interacting binding sites for Mg^{2+} and that Mg^{2+} serves both as a cation cofactor and as a positive modulator of the enzyme. The K_a for Mg^{2+} changes conspicuously with temperature (Table 5); there is a gradual fall in the value of K_a as the temperature is lowered from 15° to 5°, and then a sharp increase in K_a at 0°. The value of n for Mg^{2+} falls from 2 between 20° and 5° to less than 1 at 0°, indicating considerable decrease in the co-operative interaction between the Mg^{2+} -binding sites, a finding that may, in part, explain the large increase in K_a for Mg^{2+} at 0°. Then, as temperature rises from 15° to 25°, there is a rapid decrease in K_a for Mg^{2+} .

Under identical conditions (0.3mm-FDP) the relationship between temperature and K_i for AMP is almost exactly opposite to the relationship between temperature and the K_a for Mg^{2+} (Tables 3 and 5). The concentration of FDP also appears to affect the K_a for Mg^{2+} ; thus, at 1mm-FDP and 15°, the K_a for Mg^{2+} is 4.5×10^{-4} M, whereas, at 0.3mm-FDP and 15°, K_a for Mg^{2+} is 2.7×10^{-4} M.

The cation requirements of the enzyme can be satisfied by very much lower concentrations of Mn^{2+} . For rainbow-trout FDPase the Mn^{2+} -saturation curve is strictly hyperbolic and the K_a for Mn^{2+} (Fig. 8) is about 50- to 100-fold lower than the K_a for Mg^{2+} (Fig. 7) at all temperatures. The difference between Mg^{2+} - and Mn^{2+} -saturation curves is not understood. The results are similar to the effect of Mn^{2+} on the FDP-activation curve of ADP pyrophosphorylase from *E. coli* B (Gentner, 1968): FDP is a positive modulator of this enzyme, and in the presence of Mg^{2+} the activation curve is sigmoid; when Mn^{2+} is present in the reaction mixture, the curve becomes hyperbolic. The concentrations of FDP affect the affinity of the rainbow-

Fig. 8. Mn^{2+} -saturation curves of rainbow-trout FDPase at a series of temperatures. Assay conditions were as described in Fig. 6, except that the temperature was varied as indicated. Activity is expressed as μ moles of P_i released/l./hr./mg. of protein. Temperatures: $\times$, 30° ; $\blacktriangle$, 20° ; $\blacksquare$, 15° ; $\triangle$, 10° ; $\bullet$, 5° .

Fig. 9. Lineweaver-Burk plots of Ca^{2+} inhibition of rainbow-trout FDPase showing the inhibition to be competitive with respect to the cofactor. Assay conditions were as described in Fig. 6. v , Activity (μ moles of P_i released/l./hr./mg. of protein). Concentrations of Ca^{2+} (mM): $\blacktriangle$, 0.8; $\circ$, 0.6; $\triangle$, 0.1; $\bullet$, 0.

Table 6. Inhibition of FDPase by Ca^{2+} and Zn^{2+} at various concentrations of cofactor

Assays were carried out at 15° in 0.05 M-tris-HCl, pH 7.40, with 1 mM-FDP in the reaction mixture.

Concn. of cofactor	$10^5 K_i$ for Ca^{2+} (M)	Concn. of cofactor	$10^7 K_i$ for Zn^{2+} (M)
Mg^{2+} 0.6 mM	2.8	Mg^{2+} 0.6 mM	1.5
1 mM	4.6	1 mM	4.8
5 mM	10.2	5 mM	5.0
10 mM	40.0		
Mn^{2+} 5 μ M	23	Mn^{2+} 20 μ M	7.4
10 μ M	38	0.1 mM	9.6
20 μ M	65		

trout enzyme for Mn^{2+} . At all temperatures between 0° and 25° , K_a for Mn^{2+} is 3.0×10^{-6} – 3.5×10^{-6} M at 0.3 mM-FDP ($v_{opt.}$). At high concentrations (1 mM) of FDP, K_a for Mn^{2+} varies slightly; at 5° K_a for Mn^{2+} rises to 7.5×10^{-6} M from a value of about 4×10^{-6} M at all other temperatures (Table 5). The enzyme is inhibited by higher concentrations of Mn^{2+} (Fig. 8); 0.1 mM- Mn^{2+} results in about 25% inhibition of activity at all temperatures.

Ca^{2+} is inhibitory to trout FDPase and is competitive with respect to both Mn^{2+} and Mg^{2+} (Fig. 9). The K_i for Ca^{2+} depends on the Mn^{2+} or Mg^{2+} concentration (Table 6). At saturating, but not inhibitory, values of Mg^{2+} , the K_i for Ca^{2+} is about 4.5×10^{-5} M at 15° . Much higher concentrations of Ca^{2+} are required to compete with Mn^{2+} , presumably because of the high affinity of FDPase for Mn^{2+} ; at saturating but not inhibitory concentrations of Mn^{2+} at 15° , the K_i for Ca^{2+} is about 8×10^{-4} M. Thus a given percentage inhibition in the presence of Mg^{2+} can be achieved by one-twentieth of the amount of Ca^{2+} that is required when Mn^{2+} is the cofactor.

The affinity of FDPase for Zn^{2+} is also very high. In the presence of saturating concentrations of Mn^{2+} , the K_i for Zn^{2+} is about 7×10^{-7} M, and in the presence of Mg^{2+} , the K_i for Zn^{2+} is about 5×10^{-7} M (see Table 6), values that are well below the concentrations reported for tissue fluids (Krebs & Woodford, 1965).

As with most types of FDPase (Horecker *et al.* 1966; Byrne, 1964; Pontremoli, Luppis, Wood, Traniello & Horecker, 1965a; Pontremoli *et al.* 1965b), the pH curves are complex and the pH optimum for the rainbow-trout FDPase is alkaline and well beyond the expected physiological range at all concentrations of Mg^{2+} . High concentrations of Mg^{2+} tend to shift the pH optimum towards the physiological range (pH 7.5–7.8 for fish) and definitely stimulate FDPase activity at these pH values (Fig. 10a). This effect of Mg^{2+} on pH optima is even more marked for spinach leaf FDPase

Fig. 10. Effects of changing concentrations of Mg^{2+} and Mn^{2+} on pH optima of rainbow-trout FDPase. Assay conditions were as described in Fig. 6, except that the pH was varied as indicated. (a) Mg^{2+} (mM): $\circ$, 20; $\blacktriangle$, 5; $\bullet$, 0.6. (b) Mn^{2+} (μM): $\circ$, 100; $\triangle$, 50; $\bullet$, 20.

Fig. 11. Effect of pH on K_a values of Mg^{2+} and Mn^{2+} for rainbow-trout FDPase. Assay conditions were as described in Fig. 6. FDPase activity is expressed as μ moles of P_i released/l./hr./mg. of protein. $\circ$, pH 7.0; $\bullet$, pH 7.4; Δ , pH 8.2; $\blacktriangle$, pH 9.0. (a) Mg^{2+} ; (b) Mn^{2+} .

(Preiss *et al.* 1967), in which high Mg^{2+} concentrations decrease the optimum to about pH 7.4. The K_a for Mg^{2+} is also affected by pH (Fig. 11); thus increasing pH tends to lower the K_a for Mg^{2+} . Hill plots of these data indicate n values for Mg^{2+} of about 2 at pH 7.0, pH 7.4 and pH 9.0. However, at pH 8.2, which appears to be an optimum for some of our preparations of the trout enzyme, the slope

Table 7. Effect of pH on affinity of rainbow-trout FDPase for cofactor

Assays were carried out at 15° in the presence of 1mM-FDP. Hill plots for Mn^{2+} -saturation curves were not made because the Mn^{2+} curve is hyperbolic (see Fig. 8).

pH	$10^4 K_a$ for Mg^{2+} (M)	n	Probability of n being zero	$10^6 K_a$ for Mn^{2+} (M)
7.0	3.7	1.81	0.000	6.0
7.4	2.3	2.88	0.008	3.2
8.2	0.7	3.04	0.014	3.6
9.0	0.7	1.47	0.001	6.4

of the Hill plot is 3.04, indicating increased co-operation between the Mg^{2+} -binding sites (Table 7). In the presence of Mn^{2+} , there is a distinct pH optimum at pH 9.0–9.5 at all Mn^{2+} concentrations (Fig. 10b). At lower concentrations of Mn^{2+} , another optimum is seen at pH 7.0, which disappears at inhibitory concentrations of the cofactor (Fig. 10b).

DISCUSSION

These results clearly show that modulation of the concentrations of FDP, AMP, Mg^{2+} , Mn^{2+} and Ca^{2+} in the liver cell can markedly affect the activity of rainbow-trout liver FDPase and thus ultimately the production of glucose. The inhibition of FDPase and concomitant activation of phosphofructokinase by AMP (Underwood & Newsholme, 1965a,b; Horecker *et al.* 1966) is important in the regulation of gluconeogenesis in pigeon liver homogenates (Gevers & Krebs, 1966). However, the remarkable temperature-dependence of FDPase-AMP interactions further complicates the problem in a poikilotherm. If AMP modulation were the major con-

trolling mechanism, efficiency of metabolic control would be extremely sensitive to environmental temperature, and glucose production would be strongly inhibited at low temperatures. On the contrary, gluconeogenesis appears to be favoured during low-temperature acclimatization in fish (Hochachka, 1967). These conditions create a requirement for the control of sensitivity to AMP that can be satisfied by the ability of Mg^{2+} and Mn^{2+} to protect against AMP inhibition, to regulate AMP site-site interactions and to alter the pH optimum of the enzyme. These findings are of particular relevance to metabolic control in poikilotherms because Ca^{2+} , Mg^{2+} and probably Mn^{2+} are regulated during thermal acclimatization in fish (Rao, 1962; Hickman, McNabb, Nelson, Van Breemen & Comfort, 1964; Houston & Madden, 1968). It is known that localized changes in cation concentration and compartmentation do occur in the cell (see Bygrave, 1967); hence changes in the kinetic characteristics of FDPase caused by such variations in cation concentrations may well be a major method of control of FDPase activity and gluconeogenesis.

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