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Binding of Adenosine 5′-Monophosphate and Substrate by Rabbit Liver Fructose 1,6-Diphosphatase*

M. G. Sarngadharan, Akiharu Watanabe, and Burton M. Pogell

ABSTRACT: The binding of adenosine 5′-monophosphate and fructose 1,6-diphosphate by rabbit liver fructose 1,6-diphosphatase has been studied by the technique of gel filtration. Four binding sites for each ligand per enzyme molecule were estimated from Scatchard plots. In the presence of substrate and absence of divalent cation, the tightness of adenosine 5′-monophosphate binding to the enzyme was greatly increased and positive cooperative interaction was observed among the adenosine 5′-monophosphate binding sites. No cooperative interaction was detectable among the substrate binding sites.

At pH 7.3 and 3°, the respective primary association constants were estimated to be 3.2×10^5 M^{-1} for adenosine 5′-monophosphate and 1.6×10^5 M^{-1} for fructose 1,6-diphosphate.

Numerous kinetic studies have shown that adenosine 5′-monophosphate is a specific inhibitor for fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) from a variety of sources (Taketa and Pogell, 1963; Newsholme, 1963; Scala *et al.*, 1968). The selective removal of AMP inhibition by various treatments of the enzyme from liver, yeast, and kidney (Taketa and Pogell, 1965; Rosen and Rosen, 1966; Marcus and Hubert, 1968) supports the view that AMP is bound at allosteric sites. In the case of the rat liver enzyme, the inhibition is reversible and noncompetitive. The sigmoid nature of the inhibition curve has been explained from theoretical considerations by suggesting cooperative interactions of three or four AMP molecules per molecule of enzyme (Taketa and Pogell, 1965). In general, rabbit liver fructose 1,6-diphosphatase has kinetic properties, including inhibition by AMP, which are very similar to those of the rat liver enzyme (Pontremoli *et al.*, 1965; this paper).

Our preliminary experiments on the direct binding of AMP by rabbit liver fructose 1,6-diphosphatase showed that AMP is bound much more tightly in the presence of the substrate, fructose 1,6-diphosphate (Watanabe *et al.*, 1968). These results also suggested that the enzyme formed intermediate binary and ternary complexes with substrate and inhibitor in the absence of divalent cation. Either Mg^{2+} or Mn^{2+} is needed for over-all catalysis of substrate hydrolysis. Direct evidence for the existence of an enzyme–substrate complex was recently reported by Pontremoli *et al.* (1968a).

Detailed studies by gel filtration of the binding of AMP and substrate to homogeneous preparations of rabbit liver fructose 1,6-diphosphatase are reported in the present communication. These results are consistent with our kinetic observations.

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Experimental Procedures

Materials

Sodium salts of fructose 1,6-diphosphate, TPN^+, β-DPNH, AMP, glucose 6-phosphate, and fructose 6-phosphate, the potassium salt of glucose 1-phosphate, Tris base, and aldolase (rabbit muscle) were obtained from Sigma Chemical Co. Phosphohexoisomerase (yeast) and glucose 6-phosphate dehydrogenase (yeast) were from Boehringer Mannheim Corp. Mixed crystals of α-glycerophosphate dehydrogenase and triose phosphate isomerase (rabbit muscle) were purchased from Calbiochem. Sephadex G-25 (coarse) and G-50 (coarse) were supplied by Pharmacia. Uniformly labeled [^{14}C]AMP (diammonium) and [^{14}C]fructose 1,6-diphosphate (sodium) were obtained from New England Nuclear Corp. The radiochemical purity of these compounds was confirmed by thin-layer chromatography. All other organic or inorganic chemicals used were of reagent grade. All reagents used for enzyme analyses and binding studies were dissolved in glass-distilled water.

Fructose 1,6-diphosphatase was purified from extracts of fresh rabbit livers by substrate elution from CM-cellulose columns (Pogell, 1962; Sarngadharan *et al.*, 1968, and manuscript in preparation). Elution with substrate at pH 6.8 (Pontremoli, 1966) was found to be an improvement over the procedure using a salt gradient at pH 6.0. Unless indicated otherwise, the enzyme used for binding studies had a specific activity of at least 22.5 μmoles of product formed per min per mg of protein at 22°.

Methods

Fructose, 1,6-diphosphatase activity was determined spectrophotometrically by following the rate of TPNH formation at 340 mμ in the presence of excess phosphohexoisomerase and glucose 6-phosphate dehydrogenase as previously described (Pogell *et al.*, 1968). Assays at alkaline pH (9.3) and 22° with 1 mM $MnCl_2$ were used for determination of specific activities.

Enzyme protein concentrations were determined assuming an extinction coefficient of 0.890 at 280 mμ for a 0.1% solution and a molecular weight of 130,000 (Pontremoli, 1966). Concentrations of AMP were determined assuming $\epsilon^{pH7}_{259\,m\mu}$ 15,400. Concentrations of fructose 1,6-diphosphate were determined enzymatically by the decrease in absorbance at 340 mμ in a coupled system containing 0.18 U of aldolase, 0.35 U of triose phosphate isomerase, 0.12 U of α-glycerophosphate dehydrogenase, 175 μg of DPNH, and 0.05 M Tris-HCl buffer (pH 7.5) in a final volume of 1 ml.

The binding of AMP and fructose 1,6-diphosphate by fructose 1,6-diphosphatase was measured by the gel filtration technique (Hummel and Dreyer, 1962; Fairclough and Fruton, 1966; Kemp and Krebs, 1967). All binding studies were carried out at 3°.

For studies of AMP binding, columns of 1-cm diameter containing 25 ml of Sephadex G-25 (coarse, Pharmacia) were equilibrated with 0.05 M Tris-HCl buffer (pH 7.3 at 3°) containing radioactive AMP of varying concentrations and any other additions indicated. The gel surface was stabilized using a small disk of glass filter paper. Aliquots of fructose 1,6-diphosphatase were lyophilized and dissolved in 0.17 ml of the solutions used for column equilibration. After standing 2 min, 0.15 ml of the protein solutions was transferred to the top of the columns and allowed to enter the gel. The columns were eluted with more of the same solutions and fractions of 1 ml were collected at a flow rate of 35–40 ml/hr; 0.2 ml of each fraction was mixed with 1.5 ml of NCS solubilizer (Nuclear-Chicago) and, after obtaining a homogeneous phase, was counted in a Nuclear-Chicago Mark I scintillation spectrometer employing 10 ml of a scintillation fluid prepared by diluting 42 ml of Spectrafluor (Nuclear-Chicago) with 1 l. of toluene.

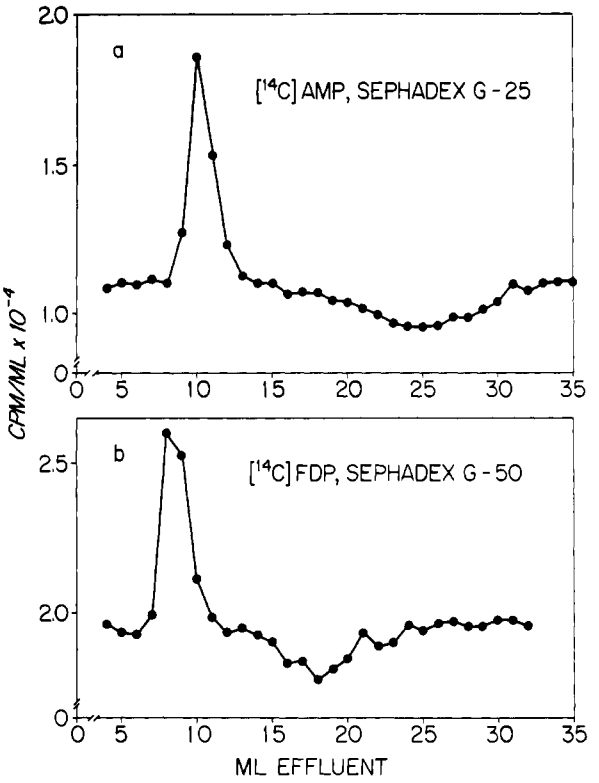

FIGURE 1: Elution profiles for the binding of AMP and fructose 1,6-diphosphate by fructose 1,6-diphosphatase. Gel filtrations were carried out as described under Methods. (a) The Sephadex G-25 column was equilibrated with 0.05 M Tris-HCl buffer (pH 7.3 at 3°) containing 0.1 mM EDTA, 0.2 mM fructose 1,6-diphosphate, and 5.5 μM [^{14}C]AMP (specific radioactivity 2.0×10^6 cpm/μmole). Fructose 1,6-diphosphatase (0.37 mg) had a specific activity of 15.3 U/mg of protein. (b) The Sephadex G-50 column was equilibrated with 0.05 M Tris-HCl buffer containing 9.2 μM [^{14}C]fructose 1,6-diphosphate (specific radioactivity 2.2×10^6 cpm/μmole). The enzyme used (0.33 mg) was treated as follows. $MgSO_4$ (20 mM) was added to pure fructose 1,6-diphosphatase (10 mg/ml) obtained by substrate elution from CM-cellulose columns (Sarngadharan *et al.*, 1968). After standing 20 min at room temperature and 1 hr at 3°, the solution was dialyzed overnight against 5 mM sodium malonate (pH 6.0).

The results of a representative experiment demonstrating the binding of AMP by fructose 1,6-diphosphatase are shown in Figure 1a. Return of the concentration of AMP to its original level after the protein peak and

TABLE I: Effect of Various Sugar Phosphates and Orthophosphate on the Binding of AMP by Fructose 1,6-Diphosphatase.[a]

Expt	Addition (mM)	r^b [AMP] = 2.7 μM	r^b [AMP] = 5.5 μM
1[c]	None		0.34
	Fructose 1,6-diphosphate (0.2)		3.68
	Glucose 1-phosphate (10)		0.62
	Fructose 6-phosphate (10) + glucose 6-phosphate (1.3)		2.56
2[d]	None		0.43
	Fructose 1,6-diphosphate (0.2)		2.67
	Fructose 1,6-diphosphate (2)		2.75
	Phosphate (2)		0.41
	Glucose 6-phosphate (0.2)		1.00
	Glucose 6-phosphate (2)		1.72
3	None	0.05	0.40
	Fructose 1,6-diphosphate (0.1)	3.04	3.22
	Fructose 6-phosphate (0.1)	1.25	1.82
	Glucose 6-phosphate (0.1)		1.07
	Glucose 6-phosphate (1)		2.34

[a] Enzyme was treated with Mg^{2+} and dialyzed as described under Figure 1b. Experiments 1 and 2 were carried out on 1-cm diameter columns containing 15 ml of Sephadex G-25 in the presence of 0.1 mM EDTA. Other conditions were as described under Methods. [b] In expt 1 and 2, partially purified enzyme was used. For calculation of values of r, amounts of enzyme were estimated from activity measurements, assuming a specific activity of 22.5 U/mg for homogeneous enzyme. In expt 3, the enzyme was homogeneous and values of r were calculated from the protein content. [c] Amount of protein, 0.41–0.47 mg (15.6 U/mg). [d] Protein, 0.74–0.99 mg (6.3 U/mg).

after the trough ensures that the operation of the column is under equilibrium conditions. This is an essential requirement for the validity of this method for measurements of protein–small molecule interactions (Fairclough and Fruton, 1966).

Binding of fructose 1,6-diphosphate was measured on columns of Sephadex G-50 (coarse, Pharmacia) of the same size used for the AMP binding. Columns were equilibrated with Tris-HCl buffer containing radioactive fructose 1,6-diphosphate of varying concentrations and any other addition indicated. Samples of the enzyme dissolved in appropriate solutions were applied on the columns and eluted as described above. Fractions were collected and radioactivity was determined as detailed for AMP binding. The elution profile of a typical experiment is shown in Figure 1b.[1]

In the studies of both AMP and substrate binding, the peak of radioactivity corresponded with the protein peak as determined by absorbance at 280 mμ. All results were calculated from the excess radioactivity present in the protein peak. Control experiments showed that there was no quenching of radioactivity by protein.

[1] Fructose 1,6-diphosphate is partially excluded from both Sephadex G-25 and G-50 gels and appears in the eluate before AMP. However, as illustrated in Figure 1b, a complete separation of substrate from enzyme was obtained with Sephadex G-50.

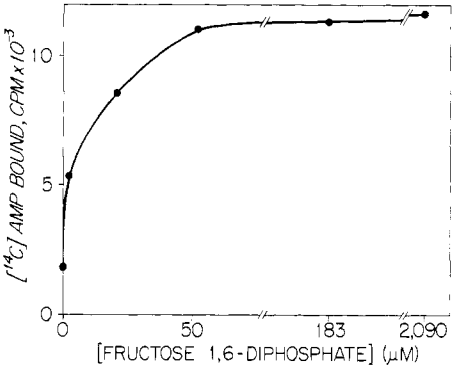

FIGURE 2: Effect of fructose 1,6-diphosphate concentration on the binding of AMP by fructose 1,6-diphosphatase. The binding was measured on Sephadex G-25 columns equilibrated with Tris-HCl buffer containing 0.1 mM EDTA, 5.5 μM [^{14}C]AMP (specific radioactivity 2.0–2.1 × 10^6 cpm/μmole), and varying concentrations of fructose 1,6-diphosphate. Enzyme used (0.27 mg, 6.3 U/mg of protein) was treated with Mg^{2+} and dialyzed as described for Figure 1b. The numbers on the ordinate are corrected for slight variations in specific radioactivity of AMP among different experiments and calculated using the value, 2.0 × 10^6 cpm/μmole.

TABLE II: Effect of AMP Concentration on Formation of Enzyme–AMP Complex.

Expt	Total Enzyme (mμmoles)	AMP Concn (μM)	AMP Sp Radioactivity (cpm × 10^{-6}/μmole)	Bound AMP cpm	Bound AMP mμmole	r^a
1	2.52	0.29	40.3	50,400	1.25	0.50
2	2.52	0.47	22.2	45,000	2.03	0.81
3	2.52	0.65	18.0	54,000	3.00	1.19
4	2.52	0.92	12.4	59,500	4.78	1.90
5	2.52	1.38	7.4	46,500	6.27	2.49
6	2.52	1.83	6.2	43,200	7.00	2.78
7	2.52	2.74	3.8	29,100	7.67	3.04
8	2.52	5.47	1.9	15,400	8.06	3.20
9	5.03	9.11	1.2	19,400	15.7	3.12

[a] r = moles of [^{14}C]AMP bound per mole of fructose 1,6-diphosphatase.

TABLE III: Effect of Substrate Concentration on Formation on Enzyme–Substrate Complex.

Expt	Total Enzyme (mμmoles)	Fructose 1,6-Diphosphate Concn (μM)	Fructose 1,6-Diphosphate Sp Radioactivity (cpm × 10^{-6}/μmole)	Bound Fructose 1,6-Diphosphate cpm	Bound Fructose 1,6-Diphosphate mμmoles	r[a]
1	3.28	1.03	11.26	19,700	1.75	0.53
2	3.28	2.03	5.76	17,700	3.07	0.94
3	3.28	3.03	3.86	16,800	4.35	1.33
4	3.28	4.02	2.87	14,300	4.99	1.52
5	3.28	6.02	1.93	12,500	6.51	1.99
6	3.28	15.99	0.73	5,700	7.76	2.36
7	3.28	23.97	0.48	4,100	8.40	2.56
8	3.28	29.96	0.39	3,500	8.97	2.74

[a] r = moles of [^{14}C]fructose 1,6-diphosphate bound per mole of fructose 1,6-diphosphatase.

Results and Discussion

Binding of AMP by Fructose 1,6-Diphosphatase. The results shown in Figure 2 confirm our earlier report (Watanabe *et al.*, 1968) that the binding of AMP by fructose 1,6-diphosphatase is enhanced in the presence of substrate. Half-maximal binding of AMP was found with 4 μM fructose 1,6-diphosphate and saturation of binding with about 50 μM fructose 1,6-diphosphate. These results also extend earlier kinetic studies showing that inhibitor binding to enzyme is noncompetitive with respect to substrate (Taketa and Pogell, 1965). Pontremoli *et al.* (1968b) have recently published studies of AMP binding to rabbit liver fructose 1,6-diphosphatase and also obtained increased binding in the presence of substrate.

In our initial experiments, the effect of Mg^{2+} on AMP binding was studied in the presence of high levels of substrate (Watanabe *et al.*, 1968). A significant decrease in binding was observed in the presence of Mg^{2+}, probably because of the formation of a Mg^{2+}–AMP chelate. Opie and Newsholme (1967) have reported that the decreased inhibition by AMP of muscle fructose 1,6-diphosphatase observed with increasing Mg^{2+} concentration can be explained on the basis of this chelation.

The relative specificity of fructose 1,6-diphosphate as effector for enhancing AMP binding is summarized in Table I. Glucose 1-phosphate and P_i at high concentrations had little or no effect on AMP binding. A small enhancement of binding was produced by either fructose 6-phosphate or glucose 6-phosphate, suggesting that the 6-phosphate group is involved in enzyme–substrate interaction. Their efficiency for increasing the affinity of the enzyme for AMP was considerably lower than that of fructose 1,6-diphosphate, both with respect to the concentrations necessary and the observed maximal effects on binding of nucleotide. Binding of AMP was considerably lower when phosphate buffer (0.05 M, pH 7.5) was used in place of Tris buffer.

A summary of our detailed studies by gel filtration of the binding of AMP by pure fructose 1,6-diphosphatase in the presence of 0.1 mM fructose 1,6-diphosphate is given in Figure 3 and Table II. The data were analyzed

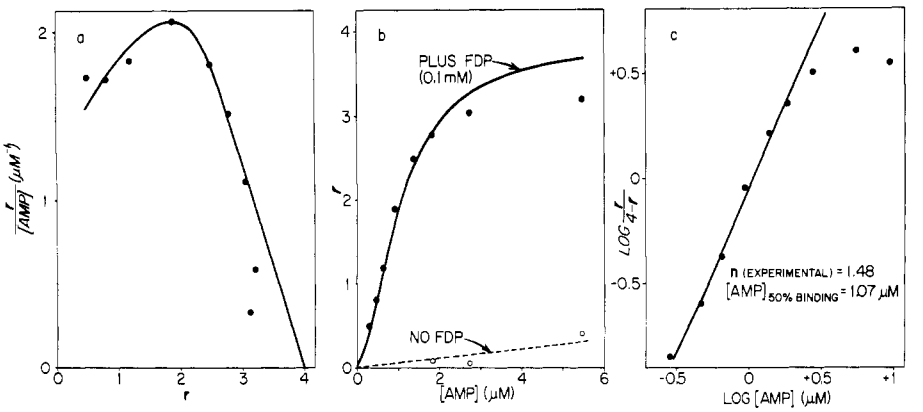

FIGURE 3: Plots of experimental data. (a) Scatchard plot of $r/[AMP]$ *vs.* r for the binding of AMP by fructose 1,6-diphosphatase at different concentrations of AMP. Gel filtration experiments were carried out on Sephadex G-25 columns equilibrated with Tris-HCl buffer containing 0.1 mM fructose 1,6-diphosphate and 0.29–5.5 μM [^{14}C]AMP. Enzyme (0.33 mg) was treated with Mg^{2+} and dialyzed as described under Figure 1b. (b) Plot of r as a function of AMP concentration. The solid line represents a theoretical curve calculated as described in the text ($r = 4y$). The experimental points represent the same data given in part a. The lower curve shows the binding of AMP in the absence of substrate. Other details are as described for part a. (c) Hill plot of log $(r/(4 - r))$ *vs.* log [AMP]. The solid line represents a theoretical curve calculated as in part b.

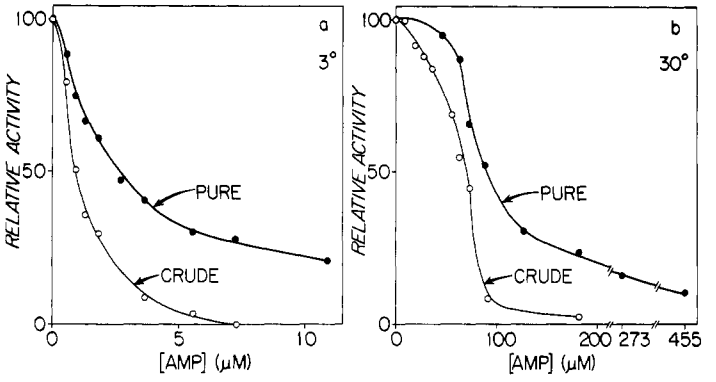

FIGURE 4: Inhibition of fructose 1,6-diphosphatase by AMP. Enzyme was assayed as described by Pogell *et al.* (1968) in a final volume of 0.5 ml. Dialyzed rabbit liver supernatant (crude enzyme) or pure fructose 1,6-diphosphatase was preincubated for 10 min at 3° (a) or 5 min at 30° (b) with 0.05 M Tris-HCl buffer (pH 7.3 at respective temperatures), 10 mM $MgSO_4$, 0.1 mM EDTA, 0.015 mM TPN^+, phosphohexoisomerase (7.8 U/ml), and glucose 6-phosphate dehydrogenase (1.4 U/ml). The reaction was started by addition of 0.1 mM (a) or 0.05 mM (b) fructose 1,6-diphosphate followed immediately by AMP at concentrations indicated. Protein concentrations: 3°, 817 μg/ml of crude enzyme or 6.8 μg/ml of pure enzyme; 30°, 58 μg/ml of crude enzyme or 0.55 μg/ml of pure enzyme. The rates at 3° were measured after a time lag of 15–20 min and represent maximal inhibitions.

in accordance with the eq 1, where r = moles of AMP

$$\frac{r}{[AMP]_{free}} = kn - kr \qquad (1)$$

bound per mole of enzyme, n = number of binding sites for AMP per molecule of enzyme, and k = association constant for the enzyme–AMP complex (Klotz, 1953). This equation is valid for the binding of small molecules by a protein with multiple binding sites, and a plot of $r/[AMP]_{free}$ *vs.* r will be linear if the sites are independent and noninteracting. But, as shown in Figure 3a, the binding of AMP by fructose 1,6-diphosphatase in the presence of substrate did not conform to this situation and the plot was nonlinear. A similar Scatchard plot was reported by Frieden and Colman (1967) for the binding of GTP by glutamate dehydrogenase in the presence of DPNH. Extrapolation to infinitely high concentration of AMP ($r/[AMP]_{free} = 0$) gave a value of four binding sites for AMP per molecule of enzyme. The nonlinearity of the plot indicates that there is cooperative interaction among the AMP binding sites. As expected, the plot of r *vs.* [AMP] was sigmoidal (Figure 3b), and an apparent n value of 1.48 could be estimated from a Hill plot (Figure 3c). Similar results were obtained in the presence of 0.1 mM EDTA.

At subsaturating levels of AMP, the r values for AMP binding were found to decrease somewhat with increasing protein concentration. In the presence of 1.4 μM AMP and 0.2 mM fructose 1,6-diphosphate, increasing the amount of enzyme from 0.14 mg to 0.27, 0.37, 0.73, and 1.1 mg decreased the binding by 6, 7, 16, and 28%, respectively. The studies reported were carried out with low enough levels of enzyme so that this effect was minimized.

The concentration of AMP at which 50% of maximal theoretical binding occurred at 3° was 1.07 μM (Figure 3c). In the presence of 10 mM Mg^{2+}, 50% inhibition of

pure enzyme was found at a total AMP concentration of 2.70 μM at the same temperature (Figure 4a). Assuming that AMP forms a chelate with Mg^{2+} under these conditions and that the association constant, k, of the complex is 89 (Walaas, 1958), the concentration of free AMP would be 1.43 μM, as calculated from eq 2. Thus

$$[AMP]_{free} = \frac{[AMP]_{total}}{k[Mg^{2+}] + 1} \quad (2)$$

the concentrations of AMP necessary for half-maximal binding and inhibition are in good agreement, if one assumes that the AMP–Mg^{2+} complex is not bound to the enzyme. Studies by Opie and Newsholme (1967) and Pontremoli *et al.* (1968b) have also indicated that the decreased inhibition by AMP and the reduction in binding observed with increasing concentrations of Mg^{2+} can be explained by this assumption.

The inability to experimentally obtain the maximum value of 4 for r parallels the observation that pure fructose 1,6-diphosphatase could not be completely inhibited even at high concentrations of AMP (Figure 4a, b). Under identical conditions, the pure enzyme was much less inhibited by AMP than fructose 1,6-diphosphatase in crude extracts, both at 3 and 30°. Lack of complete inhibition of pure liver enzyme by AMP at pH 7.5 and variability in results have also been indicated by Horecker *et al.* (1966). Differences in sensitivity of the pure and crude preparations to AMP were apparent both in the initial and final enzyme rates at 3° (Watanabe *et al.*, 1968). As previously observed with rat liver enzyme (Taketa and Pogell, 1965), the extent of inhibition was greater at the lower temperature. The binding of AMP also followed a similar pattern with respect to temperature, namely, an increased binding at 3° over that observed at 22°.

The variation in degree of inhibition by AMP observed upon purification of the enzyme suggests that other factors, such as the natural protein activator found in liver (Pogell *et al.*, 1968), may play a role in maintaining the proper physiological conformation of the enzyme.

It is possible that a weakening of the enzyme subunit interactions occurs upon purification, and thus a maximal binding of four AMP molecules per enzyme molecule was not demonstrable. A somewhat analogous situation appears to occur with aspartate transcarbamylase from *Escherichia coli* (Weitzman and Wilson, 1966). The changes in enzyme activity and CTP inhibition after treatment with various desensitizing agents have been interpreted in terms of a weakening of subunit interactions.

The sigmoid nature of the AMP inhibition curves is apparent both with crude and pure fructose 1,6-diphosphatase (Figure 4). However, a detailed comparison of apparent n values will have to await resolution of the basis for desensitization to AMP during purification. The apparent n values of 2–2.3 obtained with crude rabbit liver enzyme were in close agreement with values previously found with crude rat liver preparations (Taketa and Pogell, 1965).

Theoretical Considerations. The experimental data on AMP binding can be reasonably explained according to the theory originally developed by Coryell *et al.* (1939) and Coryell (1939) for the cooperative interactions of O_2 molecules with hemoglobin. Only two binding constants are involved in their formulation. The AMP molecules are assumed to interact as if placed at the corners of a square, each of the four AMP molecules being bound to an enzyme subunit by essentially the same forces. The presence of an AMP molecule on the enzyme produces a conformational alteration which lowers the free-energy change accompanying each additional AMP binding by $RT \ln \alpha$. Our earlier kinetic studies on AMP inhibition of rat liver fructose 1,6-diphosphatase were compatible with this theory, assuming either three or four inhibitor binding sites per enzyme molecule (Taketa and Pogell, 1965).

$$y = \frac{k_1[AMP] + (2\alpha + 1)k_1^2[AMP]^2 + 3\alpha^2k_1^3[AMP]^3 + \alpha^4k_1^4[AMP]^4}{1 + 4k_1[AMP] + (4\alpha + 2)k_1^2[AMP]^2 + 4\alpha^2k_1^3[AMP]^3 + \alpha^4k_1^4[AMP]^4} \quad (3)$$

The solid line in Figure 3b represents the theoretical curve calculated using eq 3, where y = fractional saturation of enzyme molecules with AMP (Taketa and Pogell, 1965). It is assumed that a maximum of four AMP molecules are bound per enzyme molecule. The interaction constant, α, was estimated to be 2.9 from the apparent n value of 1.48 obtained in Figure 3c. The primary association constant, k_1, was estimated to be 0.32 μM^{-1}, assuming that half-saturation with AMP occurred at 1.07 μM AMP (Figure 3c) and that $k_1 = 1/\alpha[AMP]_{50\%satn}$ (Coryell, 1939). It may be seen that the experimental points agree quite well with the calculated values in the lower range of AMP concentrations. Deviations are more marked at higher AMP levels.

AMP Binding in the Absence of Substrate. Figure 3b (lower curve) shows that the affinity of fructose 1,6-diphosphatase for AMP was considerably lower in the absence of substrate. Pontremoli *et al.* (1968b) reported about a threefold higher binding of AMP in the absence of substrate. The increased binding may have been caused by the presence of small amounts of bound fructose 1,6-diphosphate in their preparations or differences resulting from crystallization of the enzyme.

Binding of Fructose 1,6-Diphosphate by Fructose 1,6-Diphosphatase. Evidence obtained by gel filtration on Sephadex G-50 for the formation of a complex between enzyme and substrate in the absence of divalent cations is summarized in Figure 5 and Table III. A linear Scatchard plot was observed, which upon extrapolation to infinitely high concentration of substrate ($r/[FDP]_{free} = 0$), indicated a maximum of four binding sites for fructose 1,6-diphosphate per enzyme molecule. An association constant for the enzyme-substrate complex of 0.16 μM^{-1} (dissociation constant = 6.4 μM) was obtained from the slope of Figure 5a. The close agreement of the dissociation constant with the K_m (7.6 μM) determined kinetically at 3° under similar conditions (Figure 6) suggests that hydrolysis of enzyme–sub-

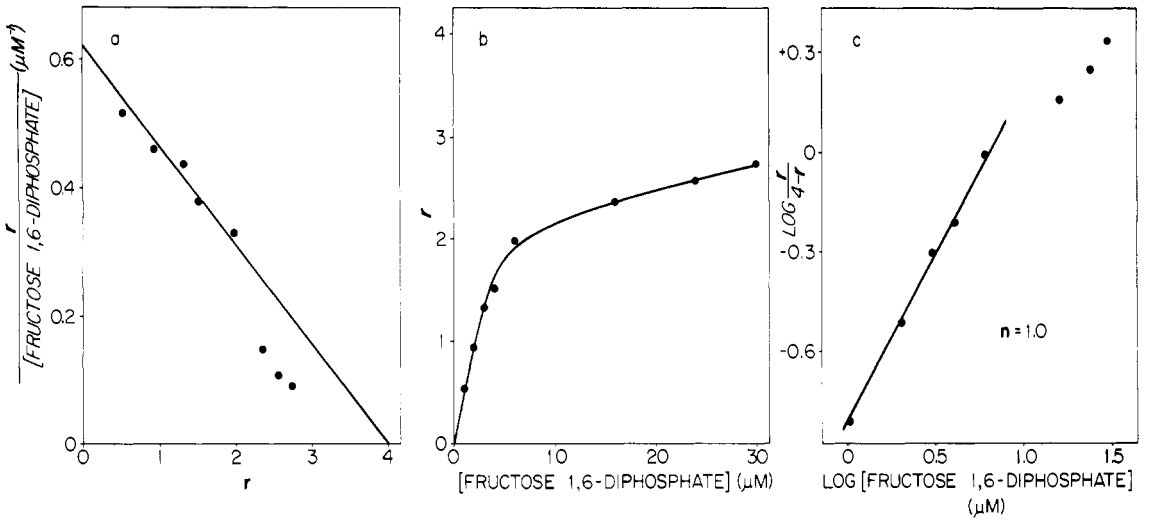

FIGURE 5: Plots of experimental data. (a) Scatchard plot of r/[fructose 1,6-diphosphate] *vs.* r for the binding of fructose 1,6-diphosphate by fructose 1,6-diphosphatase at varying concentrations of substrate. Gel filtration experiments were performed on Sephadex G-50 columns equilibrated with Tris-HCl buffer containing 0.1 mM EDTA and 1.0–30.0 μM [^{14}C]fructose 1,6-diphosphate. Enzyme (0.43 mg) was dialyzed against 5 mM sodium malonate (pH 6.0). (b) Plot of r *vs.* concentration of fructose 1,6-diphosphate. (c) Hill plot of log $(r/(4-r))$ *vs.* log [fructose 1,6-diphosphate].

strate complex into enzyme and products is a much slower process than its breakdown into enzyme and substrate. The presence of AMP had no significant effect on binding of substrate.

No suggestion of cooperative interactions among the fructose 1,6-diphosphate binding sites was evident in the three different plots of Figure 5; in fact, the apparent n obtained from the Hill plot was 1.0 (Figure 5c). The maximal experimental value for r found in these experiments was only 2.74. There is no obvious explanation for the apparent inhibition of binding at higher substrate concentrations, other than the possibility that the presence of two fructose 1,6-diphosphate molecules per enzyme molecule might somehow inhibit further binding.

The finding of four substrate binding sites per enzyme molecule could explain the marked inhibition of enzyme at low substrate concentrations (Taketa and Pogell, 1965). Thus, the simultaneous binding of several molecules of fructose 1,6-diphosphate may result in lowered catalytic activities. Also, formation of this enzyme–substrate complex with resultant changes both in conformation and charge probably accounts for the specific elution of fructose 1,6-diphosphatase obtained with substrate from substituted cellulose columns (Pogell, 1962).

Comparison with Binding Data of Pontremoli, Grazi, and Accorsi. Our present experiments confirm evidence first reported by Pontremoli *et al.* (1968a) for the formation of enzyme–substrate complex containing four molecules of fructose 1,6-diphosphate per enzyme molecule of molecular weight 130,000. However, some of our experimental evidence is at variance with their report. We found no evidence for the cooperative interaction among the substrate sites reported by them and also observed a much lower association constant for the complex. No available kinetic studies have given any evidence for cooperative interactions among substrate sites (Taketa and Pogell, 1965; present report).

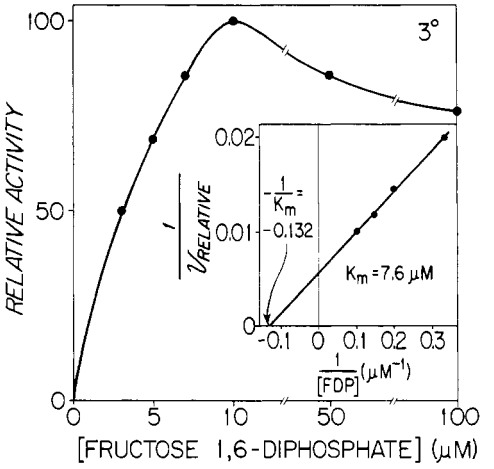

FIGURE 6: Effect of substrate concentration on fructose 1,6-diphosphatase activity at 3°. Pure fructose 1,6-diphosphatase (0.91 μg/ml) was assayed as described under Figure 4a. Reactions were started by addition of varying concentrations of fructose 1,6-diphosphate to the system after preincubation for 10 min. The *inset* shows a Lineweaver–Burk plot of data from this figure for the range of non-inhibitory substrate concentrations. Enzyme activity was expressed relative to the maximum rate observed at 10 μM substrate.

We feel that the conclusions drawn from their observations in this regard are open to question, primarily because the binding studies were not done under equilibrium conditions. In their experiments, enzyme–substrate complex was first formed at high substrate concentrations and then filtered through Sephadex G-50 columns equilibrated with much lower substrate concentrations. Under these conditions, the enzyme–substrate complex would be constantly dissociating as it moved into areas of lower substrate concentration, and it would be impossible to determine the free

substrate concentrations on the column. Also, the measured values for binding included small amounts of the substrate dissociating at the final stages of the filtration. In their first demonstration of enzyme–substrate complex formation shown in their Figure 1 and Table I, the Sephadex column contained no added fructose 1,6-diphosphate, and yet an r of 3.2 can be estimated from their data. Furthermore, if their system was indeed in equilibrium, refiltration of the isolated complex in the absence of added substrate should have resulted in considerable loss of radioactivity. They, however, state that, under these conditions in the absence of Mn^{2+}, approximately 95% of the radioactivity was still bound to the enzyme. These experiments imply that enzyme–substrate formation was irreversible during the time of passage through the column. In the face of this information, the exchange of radioactivity of the enzyme–substrate complex with cold fructose 1,6-diphosphate on the column cannot be interpreted as evidence for equilibrium, and the mechanism of this exchange would have to be independent of the mode of formation of the complex.

In our experiments, all the radioactive fructose 1,6-diphosphate bound to enzyme in the isolated complex was lost upon overnight dialysis, as would be expected in a completely reversible system. Also, in the column illustrated in Figure 7, which was run under conditions similar to those of the above authors except for a much slower flow rate, an r of 1.62 was found; a value greater than 3 would be expected according to Pontremoli *et al.* (1968a). It also may be seen in Figure 7 that the free radioactivity level did not drop to the initial equilibrium value after the protein peak, another complication with their experimental approach.

Role of Divalent Cation. The role of the divalent cations, Mg^{2+} and Mn^{2+}, in the enzymatic process is apparently twofold: (1) to permit the hydrolysis of the enzyme–substrate complex into products and (2) to modify the conformation of the enzyme in the presence of substrate and AMP. The marked time-dependent increase in enzyme inhibition observed at 3° in the presence of Mg^{2+} and AMP (Watanabe *et al.*, 1968) and the modification of AMP binding in the presence of Mn^{2+} (Pontremoli *et al.*, 1968b) could be explained by the latter type of structural alteration.

Concluding Comment. The present experimental evidence is consistent with the presence of four binding sites for both AMP and fructose 1,6-diphosphate, respectively, per enzyme molecule of molecular weight 130,000. Since evidence for the splitting of fructose 1,6-diphosphatase into four subunits has been indicated (Pontremoli *et al.*, 1968b), the occurrence of one binding site for each ligand per subunit appears probable. In the presence of substrate, a conformational change of enzyme occurs with a resultant increased association constant for AMP and an induced positive cooperative interaction among the AMP binding sites. The basic assumption in the model proposed by Monod *et al.* (1965) for a regulatory protein, namely, the existence of two conformations of the enzyme with different affinities for modifiers, is thus clearly satisfied by the described behavior of fructose 1,6-diphosphatase.

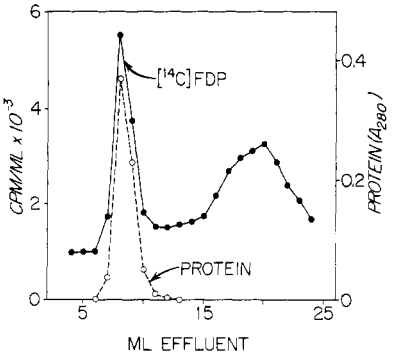

FIGURE 7: Gel filtration of preformed enzyme–substrate complex. Pure enzyme was dialyzed against 5 mM sodium malonate (pH 6.0). The protein (0.97 mg, 7.5 mμmoles) was dissolved in 0.34 ml of 114 μM [^{14}C]fructose 1,6-diphosphate (specific radioactivity 8.8×10^5 cpm/μmole) in 0.05 M Tris-HCl buffer. After standing 10 min at 3°, 0.3 ml of the solution was transferred to a Sephadex G-50 column equilibrated with 0.05 M Tris-HCl buffer containing 1.14 μM substrate of the same specific radioactivity. The column was eluted with the same dilute substrate solution.

Added in Proof

In some recent experiments, we have found an explanation for the higher, but variable, binding of AMP in the absence of substrate by fructose 1,6-diphosphatase which was not previously treated with Mg^{2+} (see Watanabe *et al.*, 1968). It was very difficult to remove last traces of bound fructose 1,6-diphosphate from the phosphatase at pH 6, even by exhaustive dialysis after gel filtration on Sephadex G-200. The rate of loss of added radioactive substrate by dialysis was much slower at pH 6.0 than at pH 7.3. Treatment with Mg^{2+}, as described in Figure 1b, serves to hydrolyze all remaining substrate and reduces the binding of AMP in the absence of substrate to a constant low level. Repetition of the fructose 1,6-diphosphate binding experiments with enzyme after Mg^{2+} treatment still showed four binding sites with an apparent n of 1. However, the maximal experimental value for r was now increased to 3.6.

Also, there was a very high specificity for AMP binding. Under conditions where an r value of 2.8 was observed for AMP in the presence of substrate, less than 0.2 mole of ^{14}C-labeled ATP, ADP, or adenosine was bound per mole of enzyme.

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Purification and Properties of Rabbit Muscle Amylo-1,6-glucosidase–Oligo-1,4→1,4-transferase*

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ABSTRACT: The glycogen phosphorylase limit dextrin-debranching system of muscle amylo-1,6-glucosidase–oligo-1,4→1,4-transferase was purified twofold over previous preparations by a new procedure and its properties reinvestigated. The preparation was found to be homogeneous by both physical and biological criteria and apparently contains two distinct enzymatic activities.

The pH optima in cationic buffers was found to be at 6.6 whereas that in anionic buffers was shifted to 7.2. Protonated hydroxylalkyl-substituted amines were found to inhibit the glucosidase–transferase in a simple linear noncompetitive manner. A mechanism for this type of inhibition is suggested.

The classical mammalian debranching system of glycogen phosphorylase limit dextrin (Cori and Larner, 1951; Larner and Schliselfeld, 1956) consists of two enzymatic activities. Oligo-1,4-transferase (EC 2.4.1.24 α-1,4-glucan:α-1,4-glucan-4-oligoglucan transferase) disproportionates the symmetric phosphorylase limit dextrin of glucogen to form an asymmetric structure having a single glucosyl residue branch and amylo-1,6-glucosidase (EC 3.2.1.33 dextrin:6-glucohydrolase) then liberates the glucosyl residue. This generates a linear chain which is further susceptible to the action of phosphorylase (EC 2.4.1.1 α-1,4-glucan:orthophosphate glucosyltransferase) (Walker and Whelan, 1960; Abdullah and Whelan, 1963; Brown *et al.*, 1963; Illingworth and Brown, 1962; Abdullah *et al.*, 1964; Brown and Illingworth, 1964; Brown and Brown, 1966; Hers *et al.*, 1964).

There have however been discrepancies in the reported properties of the glucosidase–transferase system. The pH optimum for hydrolysis has been shown to be 7.2–7.4 in one case (Larner and Schliselfeld, 1956) and 5.8–6.5 in others (Brown and Illingworth, 1964; Brown and Brown, 1966; Hers *et al.*, 1964, 1967; Taylor and Whelan, 1966). In addition, Tris and glycylglycine have been found to be inhibitory in some cases and not in others (Brown and Brown, 1966; Hers, 1964; Brown, 1964). The pH optimum for incorporation of glucose-^{14}C into glycogen has likewise been reported as not coinciding with that of hydrolysis (Hers *et al.*, 1964, 1967).

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