Fructose Diphosphatase from Rabbit Liver. IX. Isolation and Kinetic Properties of the Enzyme–Substrate Complex*

Sandro Pontremoli, Enrico Grazi, and Augusto Accorsi

ABSTRACT: The enzyme–substrate complex, fructose 1,6-diphosphate–fructose diphosphatase, has been isolated through Sephadex filtration. The data presented are consistent with reactions a and b in the text. The saturated enzyme–substrate complex appears to contain four substrate binding sites per molecule of enzyme. Kinetic analysis of the reaction of formation of the enzyme–substrate complex indicates that at pH 7.5 a cooperative effect is operating among the four binding sites. This effect is not detectable at pH 9.2. Calculation of the microscopic association constants indicates a high affinity of the enzyme for the substrate. The values for the four association constants change at pH 7.5 from 0.77 to 5.8 $\times$ 10^7 M while they are equal for all the four sites at pH 9.2 with a value of 0.5 $\times$ 10^7 M.

Rabbit liver fructose diphosphatase is known to be characterized by the following general properties: absolute requirement for activity of Mg^{2+} or Mn^{2+} (Gomori, 1943), high substrate affinity (Pontremoli *et al.*, 1965c), activity at neutral (7.5) and alkaline (9.2) pH (McGilvery, 1961), and allosteric inhibition by AMP[1] (Gancedo *et al.*, 1965; Taketa and Pogell, 1963, 1965; Opie and Newsholme, 1967).

A number of experiments concerned with chemical substitution of one or two sulfhydryl groups have indicated that the catalytic properties of the enzyme at pH 7.5 and 9.2 are significantly different (Pontremoli *et al.*, 1965a–c). This is further substantiated by the studies on AMP inhibition which is almost complete at pH 7.5 and much less pronounced at pH 9.2.

In previous experiments we have shown that stepwise acetylation of four tyrosyl residues per molecule of enzyme is associated with loss of catalytic activity. Both acetylation and inactivation are prevented by the presence of the substrate, fructose 1,6-diphosphate (Pontremoli *et al.*, 1966a).

The experiments described in the following pages have been carried out in order to gain further information on the number of the substrate binding sites and on the intimate mechanism of the enzyme reaction. Some of these problems have been solved and presented together with the description of the isolation of the enzyme–substrate complex.

Experimental Procedure

Materials

Fructose diphosphatase was prepared as previously described (Pontremoli *et al.*, 1965d). Glucose 6-phosphate dehydrogenase and glucose 6-phosphate isomerase were obtained from Boehringer & Soehne, Germany. Fructose 1,6-diphosphate and TPN^+ (sodium salts) were obtained from Sigma Chemical Corp., St. Louis. Sephadex G-50 (coarse) was supplied by Pharmacia, Uppsala, Sweden. Orthophosphate-^{32}P (carrier free) was purchased from Sorin, Saluggia, Italy, and diluted, as desired, with cold orthophosphate.

Fructose 1,6-diphosphate-^{32}P was isolated from yeast by a modification of the procedure described by Mandl and Neuberg (1957). For this purpose Candida yeast was used (Lake State Yeast Co.) instead of baker's yeast in an incubation mixture containing 2.5 g of yeast, 250 μmoles of sucrose, and 100 μmoles of Na_2HPO_4 (0.5 mCi of ^{32}P) in a total volume of 5 ml. Fructose 1,6-diphosphate-^{32}P, recovered as the calcium salt, was further purified by column chromatography on Dowex 1 as described by Khym and Cohn (1953). In all the experiments the sodium salt of fructose 1,6-diphosphate-^{32}P was used.

Fructose 6-phosphate-^{32}P was prepared from fructose 1,6-diphosphate-^{32}P by acid hydrolysis for 33 min at 100° in 1 N HCl (Leloir and Cardini, 1957) and isolated by column chromatography on Dowex 1 as described by Khym and Cohn (1953). The sodium salt of fructose 6-phosphate-^{32}P was used.

Methods

D-Fructose 1,6-diphosphatase activity was measured spectrophotometrically by following the rate of TPNH formation at 340 mμ in the presence of excess glucose 6-phosphate isomerase and glucose 6-phosphate dehydrogenase. The test system (1 ml) contained 0.1 mM fructose 1,6-diphosphate, 1 mM $MnCl_2$, 40 mM glycine buffer (pH 9.2), 0.15 mM TPN^+, 2 units of glucose 6-phosphate isomerase, and 0.3 unit of glucose 6-phosphate dehydrogenase. The temperature was 22°. One unit of the enzyme was defined as the amount which would cause an optical density change of 1/min under

* From the Istituto di Chimica Biologica dell'Università di Ferrara, Ferrara, Italy. *Received January 15, 1968.* This work was supported by a grant from the Italian Consiglio Nazionale delle Ricerche, Impresa di Enzimologia, and by Grant 12991-03 from the National Institutes of Health.

[1] The abbreviations used are as listed in *Biochemistry 5*, 1445 (1966).

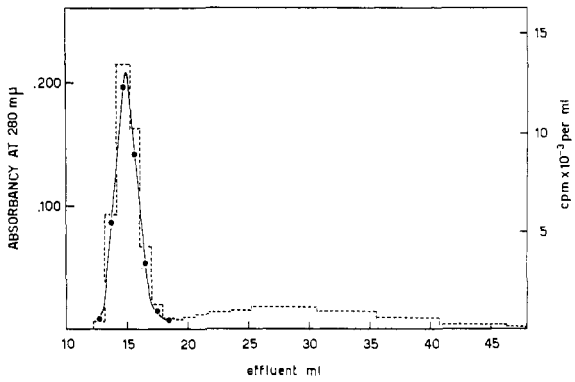

FIGURE 1: Isolation of a fructose 1,6-diphosphate–enzyme complex. The incubation mixture (0.5 ml) contains 0.65 mg of fructose diphosphatase (specific activity 120 units/mg of protein), 25 mμmoles of fructose 1,6-diphosphate-^{32}P (specific activity 2,300,000 cpm/μmole), and triethanolamine–HCl buffer (0.02 M, pH 7.5). After 10-min incubation at 2° the mixture is applied to a Sephadex G-50 column (1.2 × 37 cm) equilibrated with 0.02 M triethanolamine–HCl buffer (pH 7.5). Elution is performed with the same buffer at a rate of 4 ml/min. The temperature is 2°. (●—●) Protein concentration expressed as absorbance at 280 mμ. (-----) Radioactivity in counts per minute.

the above conditions. Specific activity was expressed as units per milligram of protein.

Protein concentration was measured from the absorbance at 280 mμ, the method being standardized against a known dry weight of dialyzed fructose diphosphatase.

Sephadex G-50 filtration was performed at 2° on columns (1.2 × 37 cm) equilibrated with triethanolamine buffer (0.02 M, pH7.5) or with glycine buffer (0.02 M, pH 9.2) (the pH values of both buffers were determined at 2°). For the equilibrium experiments the columns were equilibrated with the buffers containing the radioactive substrate at various concentrations. Flow rate was 4 ml/min. Fractions of 1 ml were collected for the first 22 ml; later on the volume of the fraction was 5 ml.

Radioactivity determinations on the effluent of the columns were made in a Packard Tri-Carb liquid scintillation counter in 10 ml of Bray's solution (Bray, 1960).

Chromatographic separation of fructose 1,6-diphosphate-^{32}P, fructose 6-phosphate-^{32}P, and orthophosphate-^{32}P was performed by descending chromatography at 26° for 24 hr on Whatman No. N1 paper with the solvent system, methyl Cellosolve–methyl ethyl ketone–3 N NH_4OH (7:2:3) (Mortimer, 1952). Before use the paper was washed with 0.1 N HCl followed by 1 mM EDTA and finally by water to remove metal impurities (Mortimer, 1952). Location of radioactive spots on paper chromatograms was made by cutting the paper into pieces 0.5 cm wide and assaying them in a low-background gas-flow Geiger-Müller counter purchased from the Società Elettronica Lombarda, Milan, Italy. The internal standards of fructose 1,6-diphosphate and fructose 6-phosphate were detected with the diphenylamine reagent (Ashwell, 1957); orthophosphate with the molybdate reagent (Hanes and Isherwood, 1949).

Results

Isolation of a Fructose 1,6-Diphosphate–Enzyme Complex. In the absence of Mn^{2+}, fructose diphosphate reacts with fructose 1,6-diphosphate-^{32}P to form a fructose 1,6-diphosphate–enzyme complex. The complex can be isolated by gel filtration.

The elution pattern of the reaction mixture from a Sephadex G-50 column is shown in Figure 1. A radioactive protein peak is eluted first followed by the excess substrate. The radioactive material bound to the protein has been identified by paper chromatography as fructose 1,6-diphosphate (Table I).

TABLE I: Identification as Fructose 1,6-Diphosphate of the Radioactive Material Released after Perchloric Acid Treatment of the Fructose 1,6-Diphosphate–Enzyme Complex.[a]

	Distance Traveled from the Origin (cm)
Fructose 1,6-diphosphate	0.5
Fructose 6-phosphate	7.5
Orthophosphate	12.0
Radioactive material	0.5

[a] Fractions 14–17 (4 ml), eluted from the column described in Figure 1 and containing 0.58 mg of the fructose 1,6-diphosphate–enzyme complex (specific activity 55,800 cpm/mg of protein), are pooled and treated with 0.4 ml of 70% perchloric acid. The protein precipitate is discarded by centrifugation. The radioactive supernatant is neutralized with 1 N KOH and kept at 0° for 4 hr. Potassium perchlorate crystallizes and is removed by centrifugation. The supernatant is concentrated under reduced pressure to approximately 0.4 ml and further crystallization of potassium perchlorate occurs. To an aliquot of the liquid phase (0.1 ml, 7500 cpm) are added as internal standards 0.05 μmole of fructose 1,6-diphosphate and 0.05 μmole of fructose 6-phosphate; to a second aliquot (0.1 ml, 7500 cpm), 0.05 μmole of orthophosphate is added. The two aliquots are placed on Whatman No. 1 paper and chromatographed with the solvent system methyl Cellosolve–methyl ethyl ketone–3 N NH_4OH (7:2:3). Location of the radioactive material and of the internal standards is performed as described under Methods.

The binding between the enzyme and fructose 1,6-diphosphate is specific; in fact both fructose 6-phosphate-^{32}P and orthophosphate-^{32}P fail to give a complex with the enzyme under the same condition (Table II).

The rate of formation of the enzyme–substrate complex is, as expected, too high to be studied with our technique. At 2°, after 2 min the reaction already attains an equilibrium.

TABLE II: Specific Binding of Fructose 1,6-Diphosphate to Fructose Diphosphatase.[a]

Additions	Radioact. Assocd with the Protein (cpm/mg)
Fructose 1,6-diphosphate-^{32}P	49,040
Fructose 6-phosphate-^{32}P	980
Orthophosphate-^{32}P	0

[a] The incubation mixtures (0.5 ml) contain 0.6 mg of fructose diphosphatase (specific activity 120 units/mg of protein), triethanolamine–HCl buffer (0.02 M, pH 7.5), and either 25 mμmoles of fructose 1,6-diphosphate-^{32}P (specific activity 2,300,000 cpm/μmole) or 75 mμmoles of fructose 6-phosphate-^{32}P (specific activity 1,150,000 cpm/μmole) or 75 mμmoles of orthophosphate-^{32}P (specific activity 4,500,000 cpm/μmole). After 10 min at 2°, the three incubation mixtures are submitted to Sephadex G-50 filtration with the procedure described in Figure 1. The protein-containing fractions are pooled and the radioactivity associated with the protein is determined.

Mn^{2+}-Catalyzed Dephosphorylation of the Fructose 1,6-Diphosphate–Enzyme Complex. Enzyme-bound fructose 1,6-diphosphate undergoes hydrolysis to fructose 6-phosphate and orthophosphate when Mn^{2+} is added. The experiment is described in Figure 2. A sample of the enzyme–substrate complex, isolated as described in Figure 1, is divided in three aliquots. The first receives no additions; to the second and to the third $MnCl_2$ (2×10^{-6} and 2×10^{-5} M, final concentration) is added.

TABLE III: Identification as Fructose 6-Phosphate and Orthophosphate of the Radioactive Material Released, in the Presence of Mn^{2+}, from the Fructose Diphosphate–Enzyme Complex.[a]

	Distance Traveled from the Origin (cm)
Fructose 1,6-diphosphate	0.7
Fructose 6-phosphate	7.0
Orthophosphate	12.3
Radioactive compound I	7.0
Radioactive compound II	12.3

[a] A fraction (5 ml) containing 8500 cpm of the radioactive material released from the fructose 1,6-diphosphate–enzyme complex after 10-min incubation with 2×10^{-5} M $MnCl_2$ and isolated by Sephadex G-50 filtration (experiment of Figure 2) is prepared and submitted to paper chromatography with the procedure described in Table I. Fructose 1,6-diphosphate, fructose 6-phosphate, and orthophosphate are added as internal standards.

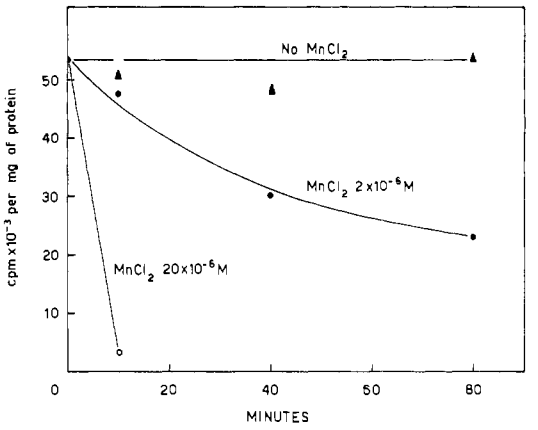

FIGURE 2: Mn^{2+}-catalyzed dephosphorylation of the fructose 1,6-diphosphate–enzyme complex. The fructose 1,6-diphosphate–enzyme complex (9.75 mg) (specific activity 53,000 cpm/mg of protein) is prepared and isolated as described in Figure 1. The fructose 1,6-diphosphate–enzyme complex solution (7.5 ml) is divided in three fractions, 2.5 ml each, containing 3.25 mg of radioactive protein in 0.02 M triethanolamine buffer (pH 7.5). To one fraction 0.025 ml of water is added, to the second and third fractions 0.025 ml of 0.2 and 2 mM, respectively, $MnCl_2$ is added. The temperature is 2°. At time intervals 0.5-ml aliquots are taken and submitted to Sephadex G-50 filtration as described under Figure 1. The protein-containing fractions of the eluate are pooled and the radioactivity associated with the protein is determined.

At time intervals samples are taken and submitted to Sephadex G-50 filtration. In the absence of Mn^{2+}, approximately 95% of the radioactivity is still bound to the enzyme. In the presence of Mn^{2+} radioactivity dissociates from the protein, the rate of the process depending on cation concentration. The radioactive material which dissociates from the protein is separated by the Sephadex filtration and resolved by paper chromatography into two radioactive compounds identified as fructose 6-phosphate-^{32}P and orthophosphate-^{32}P (Table III).

Equilibrium Constants of the Formation Reaction of the Enzyme–Substrate Complex at pH 7.5. The equilibrium of the formation reaction of the enzyme–substrate complex has been studied at pH 7.5 by gel filtration on Sephadex G-50 columns equilibrated with the radioactive substrate. Before the filtration the enzyme (from 4.4 to 8.8 mμmoles) is allowed to react with fructose 1,6-diphosphate-^{32}P (from 1.25 to 40 mμmoles). This has been done because the total amount of substrate available on the column (total volume of the column × substrate concentration) for most concentrations used is, on a molar basis, smaller than the total amount of the enzyme.

The columns are operated at a rate of 4 ml/min, and the protein peak travels from the top to the bottom of the column in 3 min. This time is sufficient to allow equilibration of the substrate bound to the enzyme with the substrate of the medium. This is proved by the experiment described in Figure 3 which shows that labeled enzyme–substrate complex travelling in 3 min through a Sephadex G-50 column equilibrated with cold substrate exchanges completely the bound, radio-

TABLE IV: Fructose 1,6-Diphosphate–Enzyme Complex Formation at pH 7.5 as a Function of Substrate Concentration.[a]

Expt	Total[c] FDPase (mμmoles)	[^{32}P]FD Added to the Enzyme before the Filtration cpm	[^{32}P]FD Added to the Enzyme before the Filtration mμmoles	[^{32}P]FD Equil Concn[d] ($\times 10^{-7}$ M)	[^{32}P]FD Bound[a] to the Enzyme cpm	[^{32}P]FD Bound[a] to the Enzyme mμmoles	$\bar{\nu}$[b]
1	4.4	2,875	1.25	0.1	3,340	1.45	0.33
2	4.4	5,750	2.50	0.19	5,960	2.59	0.59
3	4.4	8,626	3.75	0.23	8,630	3.75	0.85
4	4.4	11,500	5.00	0.31	10,670	4.64	1.05
5	4.4	17,250	7.50	0.28	16,800	7.30	1.66
6	4.4	23,000	10.00	0.50	21,780	9.51	2.16
7	4.4	34,500	15.00	1.00	33,350	14.50	3.30
8	4.4	46,000	20.00	1.70	36,750	15.90	3.60
9	4.4	46,000	20.00	4.50	37,950	16.50	3.75
10	4.4	46,000	20.00	7.50	38,410	16.70	3.80
11	8.8	92,000	40.00	100.00	81,880	35.60	4.04

[a] Calculated from the specific activity of fructose 1,6-diphosphate-^{32}P. [b] $\bar{\nu}$ = moles of fructose 1,6-diphosphate-^{32}P bound per mole of enzyme. [c] Calculated on the basis of a molecular weight of 130,000 (Pontremoli *et al.*, 1965d). [d] Determined by dividing the number of counts per minute per milliliter of the fractions immediately following the protein peak by the specific radioactivity of fructose 1,6-diphosphate-^{32}P. [e] The incubation mixtures (0.5 ml) contain fructose 1,6-diphosphatase (specific activity 120 units/mg of protein), fructose 1,6-diphosphate-^{32}P (specific activity 2,300,000 cpm/μmole) as indicated in the table, and 0.02 M triethanolamine–HCl buffer (pH 7.5). After 10-min incubation at 2° the incubation mixtures are submitted to gel filtration on Sephadex G-50 columns (1.2 × 37 cm) equilibrated with 0.02 M triethanolamine–HCl buffer (pH 7.5) plus the radioactive substrate as indicated in the table. The columns are operated as described under Methods. The fractions collected are analyzed for the radioactivity and for the protein content.

active substrate with the unlabeled substrate of the medium. The results of the equilibrium experiments are reported in Table IV. In Figure 4 the data of Table IV are plotted as a function of the free substrate concentration against the number of moles of substrate bound per mole of enzyme ($\bar{\nu}$). Inspection of the figure indicates that $\bar{\nu}$ approaches a limiting value of four at high concentration of substrate. Furthermore, the shape of the curve is sigmoid. This indicates that the presence of fructose 1,6-diphosphate on some of the sites modifies the affinity of the others for fructose 1,6-diphosphate. The sites are therefore interacting. The interaction is positive as is shown from the value of $n = 1.71$ obtained from the Hill plot, $\log \bar{\nu}/(4 - \bar{\nu})$ *vs.* $\log S$ (Figure 5).

Assuming that the four binding sites for fructose 1,6-diphosphate are intrinsically equivalent, a rough

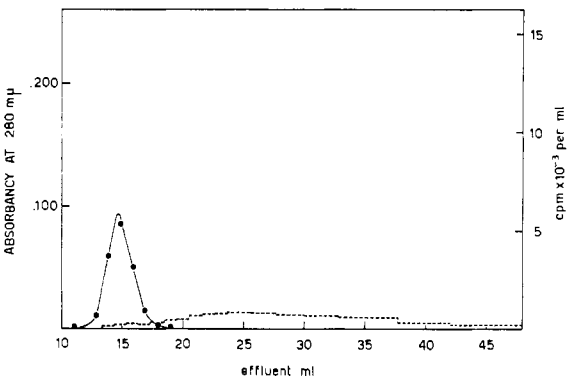

FIGURE 3: Exchange reaction between fructose 1,6-diphosphate-^{32}P–enzyme complex and fructose 1,6-diphosphate in the medium. Fructose 1,6-diphosphate–enzyme complex (0.29 mg) (specific activity 51,000 cpm/mg of protein), prepared and isolated as described under Figure 1 and dissolved in 0.5 ml of 0.02 M triethanolamine–HCl buffer (pH 7.5), is applied to a Sephadex G-50 column (1.2 × 37 cm) equilibrated with triethanolamine buffer (0.02 M, pH 7.5) plus 10^{-5} M fructose 1,6-diphosphate. Elution is performed at 2° with the same buffer solution. (●—●) Protein concentration expressed as absorbance at 280 mμ. (----) Radioactivity in counts per minute.

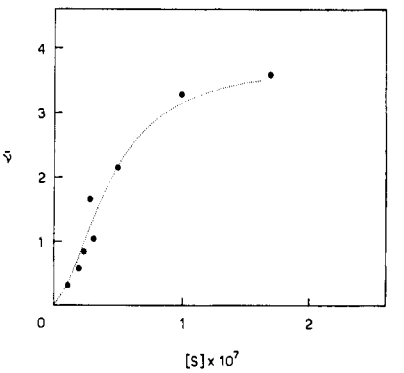

FIGURE 4: Fructose 1,6-diphosphate–enzyme complex formation as a function of substrate concentration (pH 7.5). (●) Experimental points; (------) curve calculated from eq 1 with the values of the microscopic association constants reported in Table V.

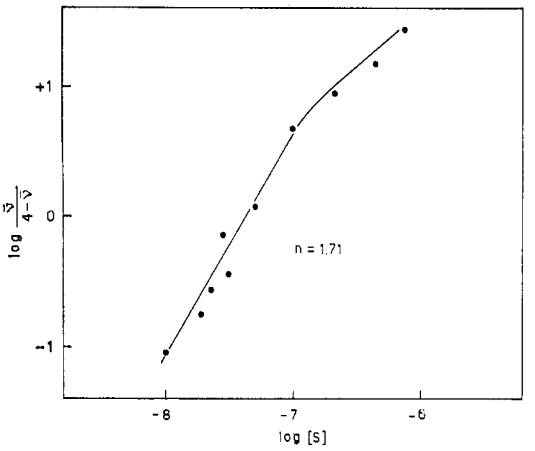

FIGURE 5: Plot of log $\bar{\nu}/(4 - \bar{\nu})$ *vs.* log S.

estimation can be obtained of the four microscopic association constants k_1, k_2, k_3, and k_4 which are defined by

$$[ES_1]/[E][S] = K_1 = 4k_1$$
$$[ES_2]/[ES_1][S] = K_2 = (3/2)k_2$$
$$[ES_3]/[ES_2][S] = K_3 = (2/3)k_3$$
$$[ES_4]/[ES_3][S] = K_4 = k_4/4$$

where K_1, K_2, K_3, and K_4 are the apparent macroscopic association constants (Edsall and Wyman, 1958). As suggested by Edsall *et al.* (1954), k_1 has been estimated from the limiting slope of the curve for $\bar{\nu}$ as a function of S as S approaches zero, $\lim_{S\to 0} d\bar{\nu}/dS = 4k_1$ (Figure 6B); k_4 from the limiting slope of the curve for $\bar{\nu}$ as a function of $1/S$ as $1/S$ approaches zero, $\lim_{1/S\to 0} d\bar{\nu}/d(1/S) = -4/k_4$ (Figure 7B). After determination of k_1 and k_4, k_2 and k_3 have been, respectively, estimated from the limiting slopes of the curves for log Q (where $Q = \bar{\nu}/(4 - \bar{\nu})S$) as a function of S as S approaches zero, $\lim_{S\to 0}$ d log $Q/dS = 3(k_2 - k_1)$ (Figure 6A); and for log Q as a function of $1/S$ as $1/S$ approaches zero, $\lim_{1/S\to 0}$ d log $Q/d(1/S) = 3(k_3 - k_4)/k_3k_4$ (Figure 7A). The values of the microscopic association constants obtained with the above procedure (Table V) have been used to calculate the theoretical curve of the binding of fructose 1,6-diphosphate to the enzyme from

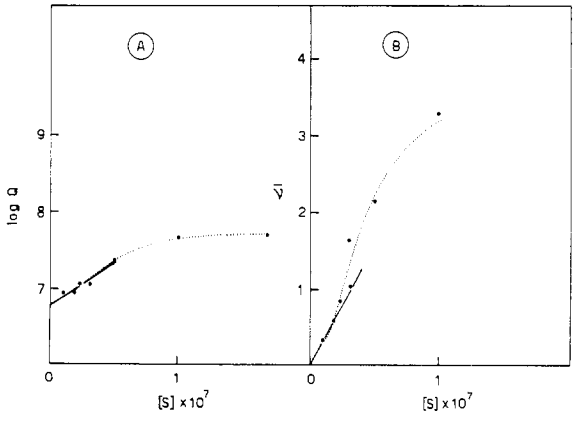

FIGURE 6: Plot of log Q *vs.* S (A) and plot of $\bar{\nu}$ *vs.* S (B).

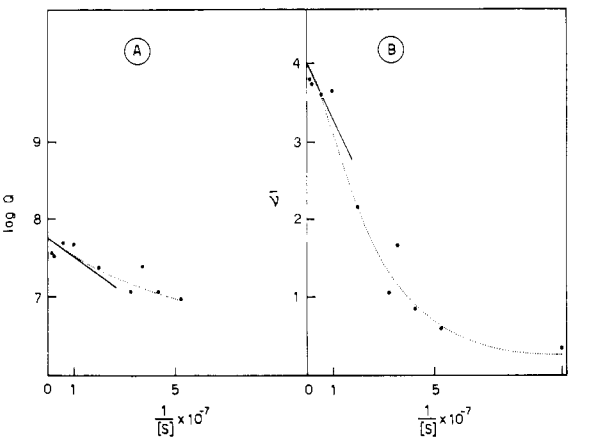

FIGURE 7: Plot of log Q *vs.* $1/S$ (A) and plot of $\bar{\nu}$ *vs.* $1/S$ (B).

eq 1. As shown in Figure 4, the theoretical curve

$$\bar{\nu} = \frac{4[k_1S + 3k_1k_2S^2 + 3k_1k_2k_3S^3 + k_1k_2k_3k_4S^4]}{1 + 4k_1S + 6k_1k_2S^2 + 4k_1k_2k_3S^3 + k_1k_2k_3k_4S^4} \quad (1)$$

(dashed line) fits quite satisfactorily the experimental data.

Equilibrium Constant of the Formation Reaction of the Enzyme–Substrate Complex at pH 9.2. The formation reaction of the enzyme–substrate complex at pH 9.2 has been studied with the same technique as at pH 7.5. The data of formation of the enzyme–substrate complex are reported in Table VI. A plot of $1/\bar{\nu}$ against $1/S$ (Figure 8) shows that at pH 9.2, as at pH 7.5, $\bar{\nu}$ approaches a limiting value of four at high substrate concentration. The data follow simple Michaelis–Menten kinetics. This makes very unlikely the existence of an interaction at pH 9.2 and provides good evidence for the equivalence of the four binding sites. From the data of Figure 8 an association constant of 0.5×10^7 M can be calculated.

Discussion

The results of the experiments presented in this paper show that the fructose diphosphatase reaction can be formulated in reactions a and b. The binding of the sub-

enzyme + fructose 1,6-diphosphate $\rightleftarrows$ enzyme–fructose 1,6-diphosphate (a)

TABLE V: Microscopic Association Constants of the Fructose 1,6-Diphosphate–Enzyme Complex.

pH	Constant	M
7.5	k_1	0.77×10^7
	k_2	1.34×10^7
	k_3	3.75×10^7
	k_4	5.80×10^7
9.2	k	0.5×10^7

TABLE VI: Fructose 1,6-Diphosphate–Enzyme Complex Formation at pH 9.2 as a Function of Substrate Concentration.[d]

Expt	Total[c] FDPase (mμmoles)	[32P]FD Added to the Enzyme before the Filtration cpm	[32P]FD Added to the Enzyme before the Filtration mμmoles	[32P]FD Equil Concn (× 10⁻⁷ M)	[32P]FD Bound[a] to the Enzyme cpm	[32P]FD Bound[a] to the Enzyme mμmoles	$\bar{\nu}$[b]
1	2.8	5,750	2.50	0.43	5,090	2.21	0.79
2	2.8	11,500	5.00	0.80	8,330	3.84	1.37
3	2.8	17,250	7.50	1.50	14,100	6.13	2.19
4	2.8	23,000	10.00	1.60	14,680	6.38	2.28
5	2.8	28,980	12.60	2.20	19,000	8.26	2.95
6	2.8	34,500	15.00	4.00	17,780	7.73	2.76
7	2.8	34,500	15.00	5.90	19,830	8.62	3.08
8	2.8	34,500	15.00	9.20	20,610	8.98	3.20
9	9.0	103,500	45.00	100.00	80,730	35.10	3.90

[a] Calculated from the specific activity of fructose 1,6-diphosphate-^{32}P. [b] $\bar{\nu}$ = moles of fructose 1,6-diphosphate-^{32}P bound per mole of enzyme. [c] Calculated on the basis of a molecular weight of 130,000 (Pontremoli *et al.*, 1965d). [d] The experiments have been performed as described under Table IV except that the incubation mixtures contain 0.02 M glycine buffer (pH 9.2) and the columns are equilibrated with the same buffer.

$$\text{enzyme–fructose 1,6-diphosphate} \xrightarrow{Mn^{2+}} \text{enzyme} + \text{fructose 6-phosphate} + \text{orthophosphate} \quad \text{(b)}$$

strate to the enzyme is therefore independent of Mn^{2+}; the cation, however, is needed for the hydrolysis of the substrate.

The formation reaction of the fructose diphosphate–enzyme complex has been studied both at pH 7.5 and at 9.2, because most of the kinetic data reported in the literature are obtained at these pH values. It has been found that four binding sites for fructose 1,6-diphosphate are available on the enzyme molecule both at pH 7.5 and at 9.2. At pH 9.2 the formation reaction of the enzyme–substrate complex follows the Michaelis–Menten law with an association constant of 0.5 × 10⁷ M. Since no difference is detected in the affinity of the substrate for the four binding sites it seems safe to conclude that the four sites are functionally equivalent.

At pH 7.5 the situation is completely different. The four binding sites are interacting and the interaction is positive. The values of the microscopic association constants between the substrate and the enzyme have been estimated on the assumption that also at pH 7.5 there is intrinsic equivalence between the four binding sites. The values of the microscopic association constants obtained increase from 0.77 × 10⁷ M for the first molecule of substrate to 5.8 × 10⁷ M for the fourth molecule of substrate.

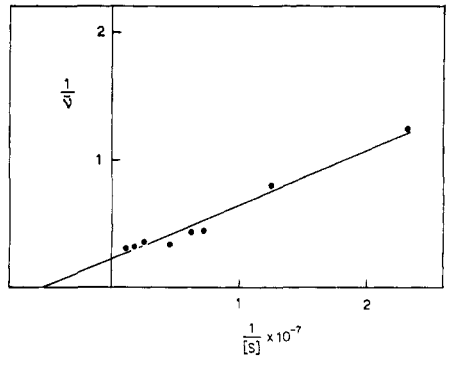

FIGURE 8: Plot of $1/\bar{\nu}$ *vs.* $1/S$ (pH 9.2).

These results are clear indication of the extremely high affinity of the enzyme for the substrate, in accordance with the indications derived from ordinary kinetic studies. The cooperative effect present at pH 7.5 and absent at pH 9.2 represents a further evidence of the different enzyme properties at the two pH values previously postulated on the basis of the results obtained by chemical modification of the protein and on the different sensitivity to AMP inhibition.

The existence of four substrate binding sites per molecule of enzyme is in perfect agreement with the number of four tyrosine residues shown by us to be involved in the catalytic activity (Pontremoli *et al.*, 1966a).

Evidence so far indicates that the enzyme is composed of a minimum of two different subunits (Pontremoli *et al.*, 1966b). More recent experiments performed in our laboratory tend to indicate that the protein is made up of four peptide chains; this result however needs further confirmation.

Experiments are now in progress to correlate the number of substrate binding sites to the quaternary structure of the enzyme and to investigate the extent of participation of the four sites to the catalytic activity.

References

Ashwell, G. (1957), *Methods Enzymol. 3*, 78.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Edsall, J. T., Felsenfeld, G., Goodrian, D. S., and Gurd, F. R. N. (1954), *J. Am. Chem. Soc. 76*, 3054.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. I, New York, N. Y., Academic, p 626.
Gancedo, C., Salas, M. C., Giner, A., and Sols, A. (1965), *Biochem. Biophys. Res. Commun. 20*, 15.
Gomori, G. (1943), *J. Biol. Chem. 148*, 139.
Hanes, C. S., and Isherwood, F. A. (1949), *Nature 164*, 1107.
Khym, J. X., and Cohn, W. E. (1953), *J. Am. Chem. Soc. 75*, 1153.
Leloir, L. F., and Cardini, C. E. (1957), *Methods Enzymol. 3*, 847.
Mandl, I., and Neuberg, C. (1957), *Methods Enzymol. 3*, 162.
McGilvery, R. W. (1961), *in* Fructose Diphosphatase and Its Role in Gluconeogenesis, McGilvery, R. W., and Pogell, B. M., Ed., Washington, D. C., American Institute of Biological Sciences, p 3.
Mortimer, D. C. (1952), *Can. J. Chem. 30*, 653.
Opie, L. A., and Newsholme, E. A. (1967), *Biochem. J. 104*, 353.
Pontremoli, S., Grazi, E., and Accorsi, A. (1966a), *Biochemistry 5*, 3072.
Pontremoli, S., Luppis, B., Traniello, S., and Bargellesi, A. (1966b), *Arch. Biochem. Biophys. 114*, 24.
Pontremoli, S., Luppis, B., Traniello, S., Rippa, M., and Horecker, B. L. (1965a), *Arch. Biochem. Biophys. 112*, 7.
Pontremoli, S., Luppis, B., Traniello, S., Wood, W. A., and Horecker, B. L. (1965b), *J. Mol. Chem. 240*, 3469.
Pontremoli, S., Luppis, B., Wood, W. A., Traniello, S., and Horecker, B. L. (1965c), *J. Biol. Chem. 240*, 3464.
Pontremoli, S., Traniello, S., Luppis, B., and Wood, W. A. (1965d), *J. Biol. Chem. 240*, 3459.
Taketa, K., and Pogell, B. M. (1963), *Biochem. Biophys. Res. Commun. 12*, 229.
Taketa, K., and Pogell, B. M. (1965), *J. Biol. Chem. 240*, 651.

Two Aspartokinases from *Escherichia coli*. Nature of the Inhibition and Molecular Changes Accompanying Reversible Inactivation*

D. Eugene Wampler† and Edward W. Westhead

ABSTRACT: Two aspartokinases (adenosine triphosphate: 1-aspartate-4-phosphotransferase, EC 2.7.2.4) have been isolated from *Escherichia coli* K_{12} and some kinetic properties have been studied. Both enzymes are activated by potassium and to a lesser extent by ammonium, but not by sodium or tetramethylammonium ions. Both feedback inhibitors, L-lysine and L-threonine, exhibit cooperative binding to their respective enzymes while aspartate, adenosine triphosphate, and potassium (in the absence of inhibitors) display classical saturation kinetics. In the presence of inhibitor, aspartate binding to the lysine-sensitive enzyme remains hyperbolic, while aspartate binding to the threonine-sensitive enzyme becomes sigmoid. This deviation from classical saturation kinetics is related to the degree of competition between substrate and inhibitor. Aspartate, adenosine triphosphate, and potassium offer little protection to the lysine-sensitive enzyme against lysine inhibition while aspartate and potassium (but not adenosine triphosphate) strongly protect the threonine-sensitive enzyme against threonine inhibition. Both enzymes gradually lose activity when kept in phosphate buffer (pH 7.0), the threonine-sensitive enzyme more rapidly than the lysine-sensitive enzyme. Lost activity can be recovered by adding the feedback inhibitors to the partially inactivated enzyme solution. In the case of the threonine-sensitive enzyme, activity can also be restored by adding potassium chloride. The loss of threonine-sensitive activity is accompanied by a reduction in molecular size as measured by gel filtration.

There are, in *Escherichia coli*, three enzymes which catalyze the conversion of aspartic acid into aspartyl phosphate, the first reaction in the formation of lysine, threonine, methionine, and several other cellular components. For a more detailed discussion of the role of aspartokinase in metabolic control, see Patte *et al.* (1967). Two of these three aspartokinases were originally distinguished by their sensitivity to the feedback inhibitors, lysine and threonine (Stadtmann *et al.*, 1961). The third enzyme is not inhibited by any of the "aspartic acid family" of amino acids but is repressed by methionine (Patte *et al.*, 1967). Both the threonine- and lysine-sensitive aspartokinases are effectively stabilized by

* From the Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received November 20, 1967.* Supported by Grants GM 14945 and AM 11157 from the National Institutes of Health.
† Postdoctoral fellow of the National Institutes of Heath.