However, it is likely that in these situations the effect of substrate is most evident.

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Studies on Heart Phosphofructokinase. Binding of Cyclic Adenosine 3′,5′-Monophosphate, Adenosine Monophosphate, and of Hexose Phosphates to the Enzyme†

Barbara Setlow and Tag E. Mansour*

ABSTRACT: The binding of adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP), adenosine 5′-monophosphate (5′-AMP), and hexose phosphates to purified sheep heart phosphofructokinase were studied using the technique of equilibrium dialysis. The native enzyme binds, at saturation, 1.0 mole each of cyclic 3′,5′-AMP and AMP per 100,000 g of enzyme. Both nucleotides bind to the same site on the enzyme. The total number of sites as well as the dissociation constant for cyclic 3′,5′-AMP is the same for native enzyme and for enzyme which has been desensitized to allosteric control. Binding of the cyclic nucleotide is relatively insensitive to pH. Several effectors of enzyme activity alter the binding of cyclic 3′,5′-AMP by changing its dissociation constant. The enzyme inhibitors, citrate and tripolyphosphate, slightly increase the K_D for cyclic 3′,5′-AMP whereas the activator, fructose 1,6-diphosphate (fructose 1,6-di-P), decreases the K_D about twofold. Studies of the binding of fructose 6-phosphate (fructose-6-P) to the enzyme revealed the presence of two classes of binding sites; high-affinity sites and low-affinity sites. K_D's for the high-affinity sites varied from 0.08 μM in the presence of 5′-AMP to 1.7 μM in the presence of citrate. K_D's for the low-affinity sites ranged from 9.0 to 43 μM under the same conditions. Both the high- and low-affinity sites can also bind fructose-1,6-di-P.

Purified phosphofructokinase (ATP:D-fructose-6-P 1-phosphotransferase, EC 2.7.1.11) from sheep heart displays many of the kinetic properties associated with a typical allosteric enzyme (Mansour, 1963; Mansour and Ahlfors, 1968). At an acidic pH there is a sigmoidal response to one of its substrates, fructose-6-P. At a pH less than 7, a low ionic strength, and a suboptimal concentration of fructose-6-P, phosphofructokinase from the heart as well as from other sources is inhibited by ATP (Lardy and Parks, 1956; Mansour and Mansour, 1962, Mansour, 1963; Passonneau and Lowry, 1962), citrate (Garland *et al.*, 1963; Parmeggiani and Bowman, 1963; Passonneau and Lowry, 1963), and creatine-P[1,2]

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[1] Abbreviations used in this work are: creatine-P, creatine phosphate; β-glycero-P, β-glycerophosphate.

[2] A. S. Otani and T. E. Mansour, unpublished observation.

(Krzanowski and Matschinsky, 1969). It is activated or deinhibited[3] by fructose-6-P, fructose-1,6-di-P, AMP, cyclic 3′,5′-AMP, and P_i. Recently we reported that modification of histidine residues in heart phosphofructokinase by photooxidation (Ahlfors and Mansour, 1969) or ethoxyformylation (Setlow and Mansour, 1970) results in desensitization of the enzyme to inhibition by ATP or citrate and to activation by AMP or cyclic 3′,5′-AMP. Since catalytic activity is not destroyed to a significant degree by either modification procedure, the evidence is consistent with the postulate that the regulatory site (or sites) is distinct from the catalytic site.

Studies on the binding of various ligands to phosphofructokinase have helped to explain some of the kinetic properties of the enzyme. Investigation of the binding of substrates to the heart enzyme showed that a maximum of 3.6 moles of ATP and 1.8 moles of fructose-6-P bind per 100,000 g of enzyme at pH 6.65 (Lorenson and Mansour, 1969). These data suggested that phosphofructokinase contains two catalytic sites each for ATP and fructose-6-P and two regulatory sites for ATP. This assumption is supported by studies on the binding properties of the enzyme under conditions where it does not respond to allosteric control, such as at pH 8.2 or after photooxidation. Under either condition the enzyme binds only 1.8 to 2 moles of both ATP and fructose-6-P per 100,000 g of enzyme (Lorenson and Mansour, 1969). The stoichiometry of citrate binding also fits well with these results. Native heart enzyme binds 2 moles of citrate per 100,000 g at pH 6.65 and no citrate at pH 8.2, whereas the photooxidized enzyme does not bind citrate at either pH. From these findings it is clear that ATP and citrate do not inhibit phosphofructokinase at pH 8.2 or after photooxidation simply because they cannot bind to the enzyme's regulatory sites under these conditions. In contrast, a report on the binding of cyclic 3′,5′-AMP to skeletal muscle phosphofructokinase indicated that pH has little effect on the binding of this activator to the enzyme (Kemp and Krebs, 1967). One purpose of our investigation was to study the binding of AMP and cyclic 3′,5′-AMP to native sheep heart phosphofructokinase as well as to enzyme desensitized to allosteric control by photooxidation in order to understand more fully the mechanism by which they deinhibit the enzyme. In this report we also reexamined the binding of fructose-6-P. The equilibrium dialysis technique used for these studies was less time consuming than the gel filtration method used in a previous investigation (Kemp and Krebs, 1967; Lorenson and Mansour, 1969). In addition, with this technique it was possible to maintain the enzyme at a single concentration during the binding experiment.

Materials and Methods

Equilibrium Dialysis Apparatus. The cells used for the equilibrium dialysis experiments were constructed of Lucite and contained a twin set of chambers. They were essentially identical with those described by Englund *et al.* (1969) except that the diameter and depth of the chambers were enlarged so that their volume was about 200 μl. Either of two types of membranes was used. One was Visking 20/32 dialysis tubing which had been boiled for 5 min in 5% Na_2CO_3–50 mM EDTA and stretched to increase its porosity (Craig and King, 1962). The other was a cellulose Sartorius membrane filter type 115 36, with a mean pore size of 0.005–0.010 μ. Both types of membrane were stored in 50% ethanol at 4°. Before use they were hydrated in distilled water and blotted dry. To assemble the cells, approximately circular pieces of membrane about 1 in. in diameter were placed between the two chambers in each set. The cells were then clamped into metal holders as was described by Englund *et al.* (1969). Using 100-μl Hamilton syringes, 100 μl of protein solution was placed in the chamber on one side of the membrane and 100 μl of ligand solution on the other. This volume of solution was sufficient to wet the entire membrane surface during dialysis. The large air bubble in each chamber ensured adequate mixing. The cells were plugged with short tapered plastic rods and were clamped around the circumference of disks which were mounted parallel to each other on a horizontal rod which revolved at 5 rpm. The entire mixing apparatus was placed inside a double-walled Lucite cylinder. Water maintained at 27° was pumped between the walls of the cylinder by means of a Haake Model FJ constant temperature circulator. In this way the air surrounding the rotating cells was kept at 27°. After equilibration of the contents of the dialysis cells samples were removed from each side of the membrane with a 25-μl Hamilton syringe for determination of radioactivity, protein concentration, and enzyme activity.

Procedure. Phosphofructokinase was purified and crystallized from sheep heart (Mansour *et al.*, 1966) with the modification described previously (Lorenson and Mansour, 1969). Crystals of enzyme were washed, solubilized at 60°, and treated with charcoal as described previously with the following exception: the concentration of K-PO_4 buffer in the solubilizing solution for the crystalline suspension was decreased by tenfold to 5 mM in order to minimize the final concentration of P_i in the dialysis experiments. This was done because P_i has been shown to influence the binding of several ligands to phosphofructokinase (Kemp and Krebs, 1967; Lorenson and Mansour, 1969). In addition, the solubilizing solution for the enzyme crystals contained 5 μM fructose-1,6-di-P (unless fructose-1,6-di-P binding was being studied), 1 mM EDTA, and 1 mM dithioerythritol. The fructose-1,6-di-P was necessary to prevent it from precipitating during experiments conducted at pH 6.65. The solubilized enzyme was then diluted 10- to 20-fold into the buffer used for the dialysis experiment. The buffers used were either 25 mM imidazole or 25 mM β-glycero-P, pH 6.65, or 25 mM glycylglycine, pH 8.2, containing 1 mM EDTA and 1 mM mercaptoethanol. Equilibrium was attained after about 2.5 hr of dialysis, but 3 hr were allowed for each experiment. Samples of 25 μl were withdrawn from each side of the membranes and expelled into counting vials containing 1 ml of distilled water. Dioxane scintillation solution (9 ml) (Lorenson and Mansour, 1969) was added to each vial, and the radioactivity of the samples was counted. An additional 25 μl of the protein solutions was removed for determination of protein concentration by the method of Lowry *et al.* (1951). This step was necessary because phosphofructokinase showed a tendency to precipitate during dialysis under some conditions. Protein concentrations after dialysis were usually 95–100% of those obtained before dialysis, although in a few experiments as much as 15% of the protein precipitated. In some cases, 5 μl of protein solution was removed from the chambers to determine enzyme activity at pH 8.2 as described previously (Mansour, 1966). Usually, no decrease in specific activity was observed. Occasionally aliquots from the chambers were chromatographed to measure whether any alteration of the ligand present had occurred during dialysis. Nucleotides were separated on thin-layer DEAE plates.

[3] The term "deinhibited" is used here to mean the reversal of enzyme inhibition without implying a mechanism of action.

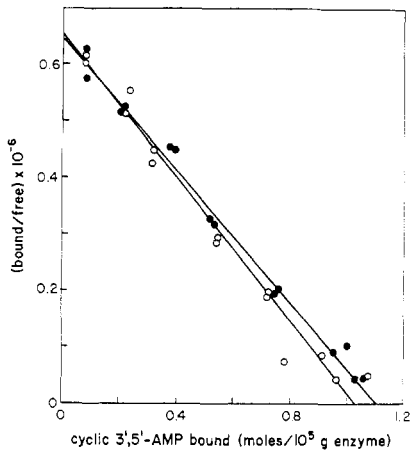

FIGURE 1: Effect of photooxidation on cyclic 3′,5′-AMP binding to phosphofructokinase. Cyclic 3′,5′-AMP binding was studied at pH 6.65 in 25 mM β-glycero-P buffer to the native (○) and photooxidized (●) enzymes. Protein concentrations varied from 0.37 to 0.45 mg/ml. Initial cyclic 3′,5′-AMP concentrations varied from 0.6 to 50 μM.

5′-AMP was separated from adenosine using 0.02 N HCl as solvent (Grippo *et al.*, 1965) and cyclic 3′,5′-AMP was separated from 5′-AMP using 1 M ammonium acetate saturated with sodium tetraborate–95% ethanol, 1:1, v:v.[4] Fructose-1,6-di-P, fructose-6-P, and fructose were separated by chromatography on DEAE paper in 0.1 M triethylamine bicarbonate, pH 7.5. Fructose-6-P and glucose-6-P were separated by chromatography in 95% ethanol–0.1 M potassium borate, pH 10, 67:33, v:v on Whatman No. 1 paper previously washed in 0.1 M potassium borate, pH 10 (Bieleski and Young, 1963). No breakdown of the two nucleotides was ever observed. On some occasions at the lowest concentrations used in the binding experiments, up to 20% of the fructose-1,6-di-P was converted to fructose-6-P, and 15% of the fructose-6-P was converted to fructose. In these cases the data were discarded since it was not clear that equilibrium had been attained.

The concentration of ligands in solutions used for binding experiments was determined from optical density measurements in the case of the nucleotides, or from enzymatic assay. Phosphoglucose isomerase and glucose-6-P dehydrogenase were used to determine fructose-6-P. Aldolase, triosephosphate isomerase, and α-glycero-P dehydrogenase were used to determine fructose-1,6-di-P (Klotzsch and Bergmeyer, 1963).

Calculation of Data. Data were plotted according to the rearrangement of the equation for binding of a ligand to a protein described by Scatchard (1949).

$$\frac{\bar{n}}{c} = -\frac{1}{K_D}(\bar{n} - n) \qquad (1)$$

where c is the free ligand concentration and $\bar{n}$ is the average moles of ligand bound per mole of protein. Plots of $\bar{n}/c$ *vs.* $\bar{n}$ should yield straight lines which extrapolate to the abscissa at n, the number of identical, independent binding sites on the protein. The dissociation constant for the ligand (K_D) is obtained from the slope of the line. The quantity of ligand bound to phosphofructokinase was determined from the difference in radioactivity between the two sides of the dialysis chamber. For simplicity, a value of 100,000 was used as an approximate molecular weight of a phosphofructokinase protomer (Paetkau and Lardy, 1967). The radioactivity on the side lacking enzyme was used to calculate the concentration of free ligand. No corrections were made for Donnan effects since the addition of 0.1 N NaCl to a few of the binding experiments had no noticable effect on the results.

Values for K_D and n were calculated from what we estimate by eye to be the best line through the data. Duplicate values for binding obtained in a single experiment usually agreed well. Repetition of experiments has shown the estimated errors in K_D and n are ±5% in β-glycero-P buffer and ±10–20% in imidazole or glycylglycine buffer. Occasional precipitation of the enzyme was probably the largest source of error in these experiments. β-Glycerophosphate buffer appeared to completely prevent such precipitation. A change in the buffer used caused a considerable change in the values for K_D but no significant change in the values for n.

Purification of Radioactive Ligands. The purity of the labeled adenine nucleotides was checked by thin-layer DEAE chromatography as described above. Both the [^{14}C]- and [^{3}H]cyclic 3′,5′-AMP contained less than 0.5% of 5′-nucleotides and were used without further purification. The [^{14}C]AMP contained about 8% adenosine and was chromatographed on DEAE plates (see "Methods"). The 5′-AMP spot was scraped and eluted with 0.05 M KCl. The [^{14}C]fructose-6-P was free of nonphosphorylated and diphosphorylated sugars, but contained an estimated 5% adenine nucleotides. These were largely removed by treating the sample with a small amount of acid-washed charcoal. The [^{14}C]fructose-1,6-di-P contained about 12% fructose-6-P, 3% fructose, and 8% other impurities which were probably adenine nucleotides. The purchased hexose diphosphate (about 0.3 μmole) was absorbed on a 1 × 9 cm Dowex-1-Cl column at 2°. Fructose and fructose-6-P were eluted with 200 ml of 0.01 N HCl. The adenine nucleotides, followed by fructose-1,6-di-P, were eluted with 250 ml of 0.02 N HCl + 0.04 N KCl. Fractions containing fructose-1,6-di-P were taken to dryness in a rotary evaporator. The dried material was suspended in water and again taken to dryness. This material was dissolved in 3 ml of water and stored frozen at pH 6. It contained < 0.1% impurities.

Materials. The materials used in this study were obtained from the following sources: sodium salts of 5′-AMP and cyclic 3′,5′-AMP, P-L Biochemicals; sodium tripolyphosphate, Alfa; naphthalene and scintillators, Packard; and DEAE-TLC, Gallard-Schlesinger. All radioactive compounds were purchased from Schwarz BioResearch except [^{14}C]-fructose-1,6-di-P which was purchased from New England Nuclear. Other reagents were obtained from sources mentioned previously (Lorenson and Mansour, 1969).

Results

Binding of Cyclic 3′,5′-AMP to Phosphofructokinase. EFFECT OF PHOTOOXIDATION ON CYCLIC 3′,5′-AMP BINDING. The effect of desensitizing the enzyme to allosteric control by photooxidation (Ahlfors and Mansour, 1969) on cyclic 3′,5′-AMP binding was tested. Figure 1 shows Scatchard plots for the binding of cyclic 3′,5′-AMP to both native and photooxidized enzyme samples in β-glycero-P buffer at pH 6.65. The native enzyme binds 1.03 moles of cyclic 3′,5′-AMP per 100,000 g with a K_D of 1.6 μM. After photooxidation the enzyme binds 1.1 moles of the nucleotide with a K_D of 1.8 μM. In these experiments β-glycero-P was used as the buffer. When

[4] T. E. Mansour, M. Bergami, and E. Scarano, unpublished observation.

TABLE I: Effects of Various Ligands on Cyclic 3′,5′-AMP Binding.[a]

Buffer	Additions	Cyclic 3′,5′-AMP Binding K_D (μM)	Cyclic 3′,5′-AMP Binding n (moles/10^5 g)
Glycylglycine, pH 8.2	None	4.6	0.9
Imidazole, pH 6.65	None	3.5	0.9
Imidazole, pH 6.65	Citrate	5.4	0.9
Imidazole, pH 6.65	Tripolyphosphate	5.4	0.9
Imidazole, pH 6.65	Fructose-1,6-di-P	1.7	0.9

[a] Cyclic 3′,5′-AMP was studied in the presence of 25 mM of the indicated buffer and 100 μM of the additions listed. Other conditions are described under "Methods."

imidazole buffer was used the only major effect was a twofold increase in the dissociation constant of native enzyme for cyclic 3′,5′-AMP (Table I). The results summarized in Table I also show that the binding of cyclic 3′,5′-AMP is only slightly affected by pH. At pH 8.2 phosphofructokinase binds a maximum of 0.9 mole of the cyclic nucleotide per 100,000 g with a K_D of 4.6 μM. The fact that neither photooxidation nor an alkaline pH affected significantly the binding of cyclic 3′,5′-AMP is in contrast to the binding of ATP which is reduced from a maximum of 3.6 to 1.8 moles per 100,000 g after photooxidation and at an alkaline pH (Lorenson and Mansour, 1969).

EFFECT OF OTHER LIGANDS ON CYCLIC 3′,5′-AMP BINDING. Kinetic studies of phosphofructokinase showed that activation of the enzyme by cyclic 3′,5′-AMP is affected by other allosteric modifiers of the enzyme. For example, both citrate and tripolyphosphate inhibit activation by cyclic 3′,5′-AMP. Tripolyphosphate functions as an ATP analog for phosphofructokinase. It acts as an allosteric inhibitor of the enzyme with a K_i similar to that for ATP.[2] These ligands also alter the binding of the cyclic nucleotide by changing its affinity for the enzyme. Table I shows that the presence of citrate or tripolyphosphate increases the K_D for cyclic 3′,5′-AMP from 3.5 to 5.4 μM, but does not change the number of sites. Fructose-1,6-di-P decreases the K_D for cyclic 3′,5′-AMP from 3.5 to 1.7 μM. The enhancing effect of fructose-1,6-di-P on cyclic 3′,5′-AMP binding can also be observed if the experiments are carried out using β-glycero-P instead of imidazole as the buffer.

Binding of AMP to Phosphofructokinase. A Scatchard plot of the binding of AMP to phosphofructokinase with β-glycero-P as a buffer at pH 6.65 indicates that the enzyme binds a maximum of 1 mole per 100,000 g of this nucleotide with a K_D of 4.3 μM (Figure 2). In order to determine whether cyclic 3′,5′-AMP and AMP bind to the same site on the enzyme, an experiment was conducted in which the simultaneous binding of [^{3}H]cyclic 3′,5′-AMP and [^{14}C]AMP was measured. In the case of competition for a single binding site, the binding of each nucleotide would be governed by the equation

$$\frac{\bar{n}_A}{c_A} = -\frac{1}{K_A}(\bar{n}_A + \bar{n}_B - 1) \qquad (2)$$

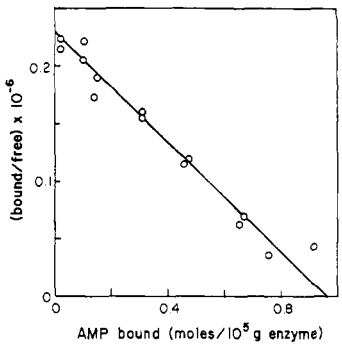

FIGURE 2: Binding of AMP to phosphofructokinase. AMP binding was studied at pH 6.65 in 25 mM β-glycero-P buffer. Initial AMP concentrations varied from 2.4 to 48 μM. Protein concentration was 0.49 mg/ml.

In this case plots of $\bar{n}_A/c_A$ *vs.* $(\bar{n}_A + \bar{n}_B)$ and of $\bar{n}_B/c_B$ *vs.* $(\bar{n}_A + \bar{n}_B)$ should yield straight lines with common intercepts of 1 on the abscissa (Englund *et al.*). Figure 3 shows that cyclic 3′,5′-AMP and AMP compete for the same site on the enzyme. The K_D of 1.5 μM obtained for cyclic 3′,5′-AMP in this experiment is in excellent agreement with the value of 1.6 μM obtained under the same conditions but in the absence of AMP (Figure 1). However, the K_D for AMP in this experiment (5.4 μM) is somewhat higher than the value of 4.3 μM shown in Figure 2. These results indicate that the enzyme binding unit for the two nucleotides is 100,000 g.

Binding of Hexose Phosphates to Phosphofructokinase. BINDING OF FRUCTOSE-6-P. Figure 4 shows a Scatchard plot of the binding of fructose-6-P to phosphofructokinase at pH 6.65 in the presence of 5 mM P_i. This concentration of P_i has been shown to greatly reduce the dependence of fructose-6-P binding on enzyme concentration, but has a minimum effect on the K_D and n for such binding (Lorenson and Mansour, 1969). The data summarized in Figure 4 shows a marked deviation from linearity indicating more than one class of sites for fructose-6-P. At low concentrations, the hexose phosphate binds very tightly to the enzyme. If an apparent K_D for this binding is estimated from the slope of the line, a value of 0.2 μM is obtained. When the equilibrium concentration of

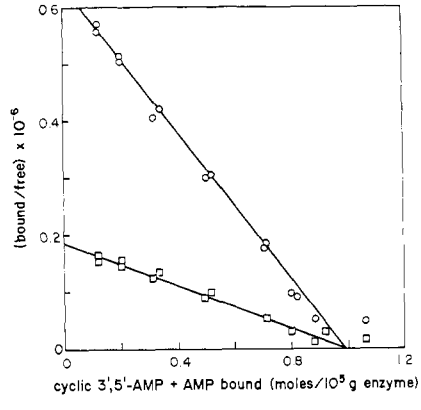

FIGURE 3: Simultaneous binding of AMP (□) and cyclic 3′,5′-AMP (○) to phosphofructokinase. Binding of [^{3}H]cyclic 3′,5′-AMP and [^{14}C]AMP was studied at pH 6.65 in 25 mM β-glycero-P buffer. Initial cyclic 3′,5′-AMP concentrations varied from 1.2 to 59 μM. Initial AMP concentrations varied from 1.7 to 99 μM. Protein concentration was 0.49 mg/ml. ^{3}H counts were corrected for 18% spillover from the ^{14}C present.

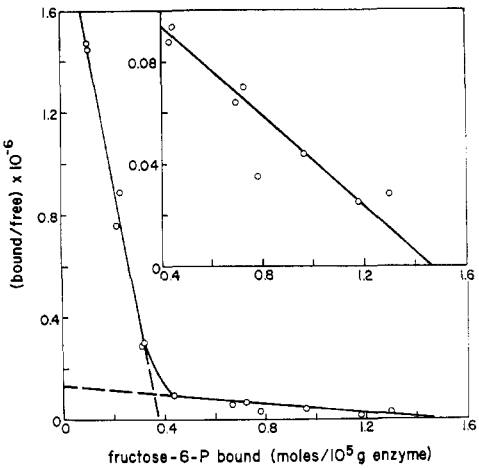

FIGURE 4: Binding of fructose-6-P to phosphofructokinase. Fructose-6-P binding was studied at pH 6.65 in 25 mM imidazole buffer in the presence of 5 mM K-PO_4. Other conditions are described in "Methods." Initial fructose-6-P concentrations varied from 0.86 to 103 μM. Protein concentrations varied from 0.56 to 0.61 mg/ml. The inset shows an enlargement of that portion of the plot near the bottom of the figure.

fructose-6-P is above about 2 μM, binding occurs with a high K_D. Under these conditions fructose-6-P binds in a normal hyperbolic fashion with a K_D of 11 μM and a maximum binding of 1.46 moles per 100,000 g of enzyme. The fact that the two apparent binding constants differ by a factor of 50 made it unnecessary to use other methods of estimating values of K_D and n. In a recent paper Klotz and Hunston (1971) reported an extensive mathematical treatment for multiple classes of binding sites but found it not essential for sites whose K_D's differ by at least a factor of 10.

In an attempt to see whether the apparent presence of two different sites for fructose-6-P may be related to a change in the degree of aggregation of the enzyme, the binding of fructose-6-P was tested under conditions which reduce enzyme aggregation. This includes alkaline pH or in the presence of high concentrations of Na_2SO_4. Figure 5 shows that fructose-6-P binding curves obtained at pH 8.2 are similar in shape to those obtained at pH 6.65. At pH 8.2 maximal binding is about the same although the K_D's are changed to 0.4 μM for

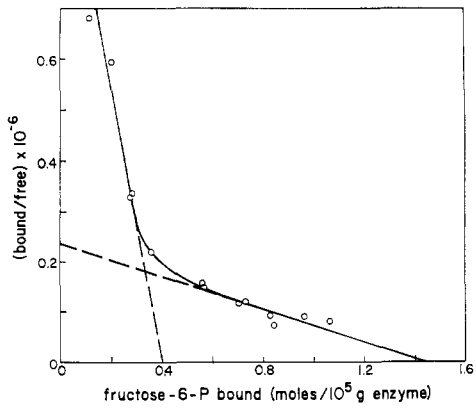

FIGURE 5: Binding of fructose-6-P to phosphofructokinase. Fructose-6-P binding was studied at pH 8.2 in 25 mM glycylglycine buffer in the presence of 5 mM K-PO_4. Initial fructose-6-P concentrations varied from 1.1 to 103 μM. Protein concentrations varied from 0.55 to 0.78 mg/ml.

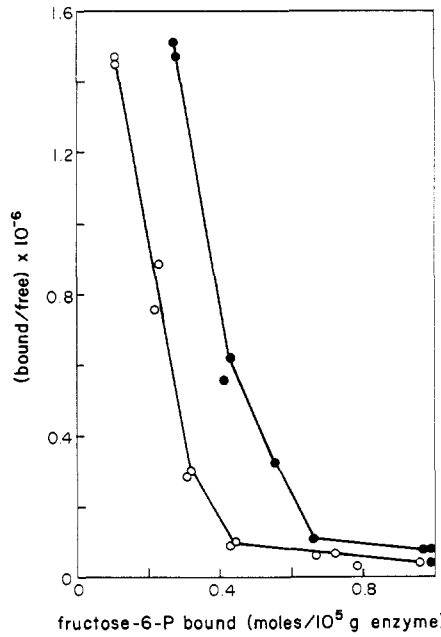

FIGURE 6: Effect of protein concentration on fructose-6-P binding to phosphofructokinase. Fructose-6-P binding was studied at pH 6.65 in 25 mM imidazole buffer in the presence of 5 mM K-PO_4. Initial fructose-6-P concentrations varied from 0.86 to 50 μM. Protein concentrations were either 0.60 (○) or 0.15 (●) mg/ml.

the high-affinity binding and 6.2 μM for the low-affinity binding. When the binding of fructose-6-P was measured at pH 6.65 in the presence of 50 mM Na_2SO_4 (not shown here) binding was greatly inhibited, but the general shape of the Scatchard plot remained unchanged. K_D's estimated from the slopes of the two limbs of the curves for the high- and low-affinity binding were 2.6 and 61 μM, respectively. Phosphofructokinase undergoes a concentration-dependent aggregation. High concentrations of enzyme favor large aggregates, whereas low concentrations of enzyme favor a more dissociated form (Ling *et al.*, 1965; Mansour, 1965; Mansour *et al.*, 1966; Parmeggiani *et al.*, 1966). In order to further test the effect of the state of aggregation of the enzyme on fructose-6-P binding, we compared binding of the hexose phosphate at two different protein concentrations. Figure 6 shows the results of such an experiment conducted at pH 6.65 in the presence of 5 mM P_i. The general shape of the curves is the same at the two protein concentrations, but at a given fructose-6-P concentration phosphofructokinase binds proportionately more hexose phosphate at the lower enzyme concentration. These results demonstrate that the biphasic nature of the fructose-6-P binding curves is not due to changes in the state of aggregation of the enzyme.

HIGH- AND LOW-AFFINITY BINDING FOR HEXOSE PHOSPHATES. The data for fructose-6-P binding in Figure 4 show that extrapolation to the abscissa of the steeper portion of the Scatchard plot yields an apparent value of 0.38 site per 100,000 g of enzyme. Since phosphofructokinase possesses roughly integral numbers of binding sites for all other ligands tested, we investigated the possibility that under our conditions this particular site was not completely accessible to fructose-6-P. As mentioned above under "Methods" we found that it was necessary to solubilize the enzyme crystals in a small amount of fructose-1,6-di-P to study fructose-6-P binding at pH 6.65. The possibility existed that some fructose-1,6-di-P remained bound to the enzyme used for these fructose-6-P

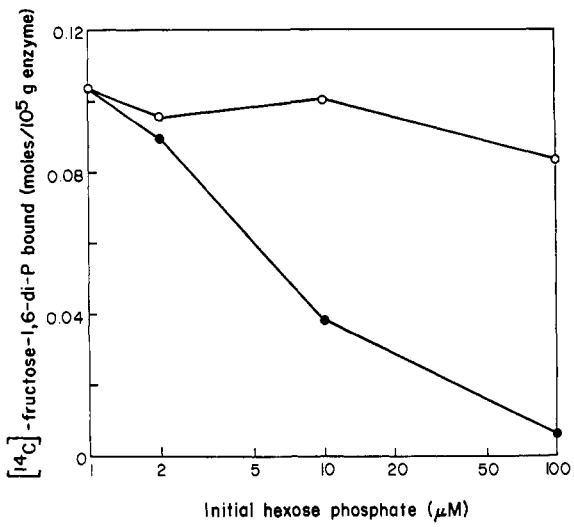

FIGURE 7: Exchange between enzyme-bound fructose-1,6-di-P and free fructose-6-P or fructose-1,6-di-P. Enzyme crystals were solubilized (5.0 mg/ml) in the presence of 5 μM [^{14}C]fructose-1,6-di-P and treated with charcoal as described in "Methods," 0.13 mole of [^{14}C]fructose-1,6-di-P per 10^5 g remained bound to the enzyme. The enzyme was diluted as described in "Methods" for an equilibrium dialysis experiment and dialyzed against several concentrations of unlabelled fructose-6-P (○) or fructose-1,6-di-P (●). After 3 hr the radioactivity remaining bound to the enzyme was determined. The dialysis was conducted at pH 6.65 in 25 mM imidazole buffer in the presence of 5 mM K-PO_4.

binding experiments and could interfere with fructose-6-P binding. To test such a possibility the enzyme was solubilized in [^{14}C]fructose-1,6-di-P instead of the unlabeled hexose diphosphate. About 85% of the counts remained bound after routine charcoal treatment of the enzyme (see "Methods"). Figure 7 shows that the bound fructose-1,6-di-P did not exchange with up to 50 μM unlabeled fructose-6-P after 3 hr of dialysis under conditions identical with those used for the equilibrium dialysis experiment shown in Figure 4. The hexose diphosphate bound to the enzyme, however, did exchange with free fructose-1,6-di-P (Figure 7). The failure of fructose-6-P to exchange with bound fructose-1,6-di-P was not due to failure of fructose-6-P to equilibrate between the two sides of the dialysis chamber (see "Methods"). In a second experiment, the enzyme was solubilized in the presence of enough fructose-1,6-di-P to saturate the high-affinity sites. Figure 8 shows the binding of fructose-6-P to this enzyme at pH 6.65 in the presence of 5 mM P_i and 5 μM fructose-1,6-di-P. It is evident from the plot in this figure that only the low-affinity site is seen in this case (K_D = 7.6 μM) presumably because the high-affinity site was previously saturated with fructose-1,6-di-P. The value of n for the low-affinity site in Figure 8 is nearly 1.4 per 100,000 g. We are uncertain whether this number actually represents 1 or 2 sites per 100,000 g. The fact that we have never observed binding of greater than 2 moles of fructose-6-P per 100,000 g makes it likely that there is actually only 1 low-affinity site. In order to ascertain the actual number of high-affinity sites on the enzyme, the binding of fructose-1,6-di-P was measured using enzyme crystals which had been solubilized as described in "Methods" except that fructose-1,6-di-P was omitted from the solubilizing solution. The binding experiment was conducted at pH 8.2 since enzyme solubilized in this way precipitated at pH 6.65. Figure 9 shows the results of such an experiment. Under these conditions 1.1 high affinity sites per 100,000 g of enzyme are evident

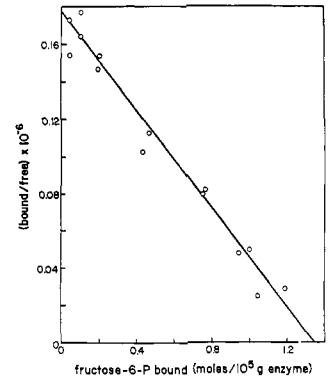

FIGURE 8: Effect of enzyme-bound fructose-1,6-di-P on fructose-6-P binding to phosphofructokinase. Fructose-6-P binding was studied at pH 6.65 in 25 mM imidazole buffer in the presence of 5 μM fructose-1,6-di-P. Enzyme crystals were solubilized as described in "Methods" except that the fructose-1,6-di-P concentration in the solubilizing solution was 100 μM instead of 5 μM. Other conditions are described in Figure 4.

with a K_D of 0.23 μM. Binding to a low-affinity site is also evident. The K_D for this site is 56 μM and the total number of sites for fructose-1,6-di-P appears to be about 1.9 per 100,000 g of enzyme. Attempts to carry on the same type of experiment with fructose-6-P were not successful because of the instability of the enzyme and its tendency to precipitate under these conditions. A conclusion can be drawn from these experiments that the apparent fractional number of high-affinity sites for fructose-6-P (0.4) seen in Figures 4–6 is probably due to partial saturation of this site with fructose-1,6-di-P which occurs during solubilization of the enzyme crystals. Presumably fructose-6-P is unable to exchange with fructose-1,6-di-P prebound to the high-affinity sites under the conditions of the equilibrium dialysis experiments, but can exchange with hexose diphosphate bound to the low-affinity sites. The possibility also exists that fructose-1,6-di-P binds at sites different from those for fructose-6-P and induces a conformational change that results in the inaccessibility of the high-affinity sites to fructose-6-P.

EFFECT OF OTHER LIGANDS ON FRUCTOSE-6-P BINDING. Previous studies have indicated that the presence of other ligands

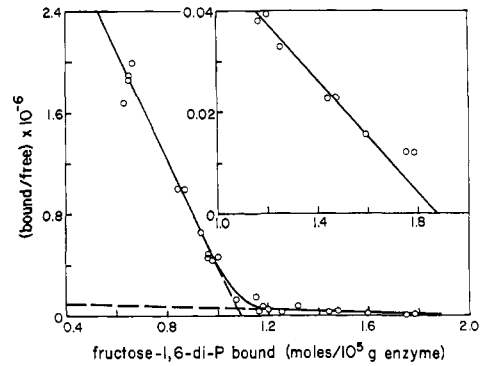

FIGURE 9: Binding of fructose-1,6-di-P to phosphofructokinase. Fructose-1,6-di-P binding was studied at pH 8.2 in 25 mM glycylglycine buffer. Enzyme crystals were solubilized as described in "Methods" except that fructose-1,6-di-P was omitted from the solubilizing solution. Initial fructose-1,6-di-P concentrations varied from 0.90 to 81 μM. Protein concentration varied from 0.45 mg/ml at low fructose-1,6-di-P concentrations to 1.6 mg/ml at high fructose-1,6-di-P concentrations. The inset shows an enlargement of that portion of the plot near the bottom of the figure.

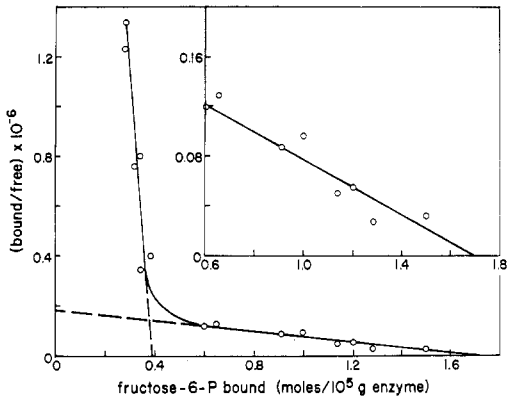

FIGURE 10: Effect of AMP on fructose-6-P binding to phosphofructokinase. Fructose-6-P binding was studied at pH 6.65 in 25 mM imidazole buffer in the presence of 100 μM AMP. Initial fructose-6-P concentrations varied from 0.87 to 102 μM. Protein concentrations varied from 0.48 to 0.57 mg/ml. The inset shows an enlargement of that portion of the plot near the bottom of the figure.

affects the binding of fructose-6-P to phosphofructokinase (Kemp and Krebs, 1967; Lorenson and Mansour, 1969). In general, activators of the enzyme tended to enhance fructose-6-P binding whereas inhibitors decreased the binding. Since we had observed two types of fructose-6-P binding to the enzyme, it was of interest to determine whether other ligands would affect the two types differently. Fructose-6-P binding could not be studied at pH 6.65 in the absence of all ligands since the enzyme precipitated under these conditions. Therefore results obtained in the presence of various effectors are compared to those obtained in the presence of 5 mM P_i. Figure 10 shows the binding of fructose-6-P in the presence of 100 μM AMP. The K_D's of the high- and low-affinity sites were 0.08 and 9.0 μM, respectively, as compared to 0.19 and 11.3 μM in the control. The extrapolated number of sites was not significantly changed. The results show that the effect of AMP as an allosteric activator, could be due to an increase in the affinity of the high-affinity site for fructose-6-P. Figure 11 illustrates the effect of the allosteric inhibitor, citrate, on the binding of fructose-6-P. The results show that citrate decreases fructose-6-P binding by raising the K_D's of both the high- and low-affinity sites to 1.7 and 43 μM, respectively. The

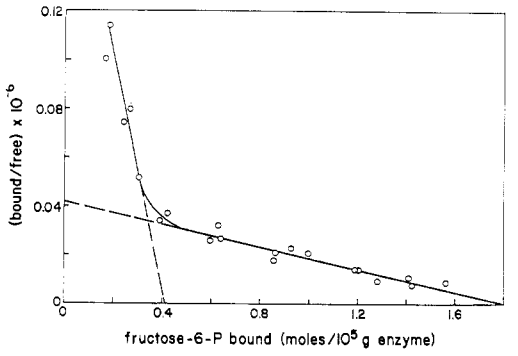

FIGURE 11: Effect of citrate on fructose-6-P binding to phosphofructokinase. Fructose-6-P binding was studied at pH 6.65 in 25 mM imidazole buffer in the presence of 20 μM citrate. Initial fructose-6-P concentrations varied from 0.86 to 380 μM. Protein concentrations varied from 0.44 mg/ml at low fructose-6-P concentrations to 1.3 mg/ml at high fructose-6-P concentrations.

TABLE II: Effect of Various Compounds on Fructose-6-P Binding.[a]

Additions	High-Affinity Sites K_D (μM)	High-Affinity Sites n (moles/10^5 g)	Low-Affinity Sites K_D (μM)	Low-Affinity Sites n (moles/10^5 g)
5 mM P_i	0.19	0.38	11.3	1.46
50 mM Na_2SO_4	2.9	0.20	>60	*b*
0.1 mM AMP	0.08	0.39	9.0	1.69
0.1 mM ADP	0.25	0.39	13.5	1.72
0.02 mM citrate	1.7	0.42	43.0	1.80
0.1 mM glucose-6-P	0.75	0.31	13.1	1.16
0.1 mM glucose-1,6-di-P	0.49	0.27	24.9	1.62
25 mM β-glycero-P	0.34	0.30	11.1	1.14

[a] The binding of fructose-6-P at concentrations from 0.3 to 100 μM was studied at pH 6.65 in the presence of the additions indicated in the table. K_D's were calculated from the slopes of the two limbs of Scatchard plots of the binding data. "*n*" refers to the value on the abscissa to which each limb of the plots extrapolated. [b] Could not be estimated.

effects of several other ligands on fructose-6-P binding were tested. The results summarized in Table II show that the total number of sites was not changed except in the presence of glucose-6-P or β-glycero-P. On the other hand the K_D's for the two classes of binding sites were significantly changed by the different ligands. For example, both glucose-6-P and β-glycero-P increased the K_D for the high-affinity binding, but had little or no effect on the low-affinity binding. Glucose-1,6-di-P is a potent activator of the enzyme[2] (Krzanowski and Matschinsky, 1969), but its effect on fructose-6-P binding is similar to that of the inhibitor, citrate. The K_D's of both the high- and low-affinity binding are increased. The K_D and maximum binding for the low-affinity binding obtained in the presence of 25 mM β-glycero-P are in good agreement with those obtained for the muscle enzyme under the same conditions except that the pH was 6.95 (K_D = 17 μM, n = 1.11) (Kemp and Krebs, 1967). It should be noted here that the values for n for the high-affinity binding listed in Table I represent only a fraction of this site since in all these experiments the enzyme contained some tightly bound fructose-1,6-di-P (see above).

Discussion

The results reported above on the binding of cyclic 3′,5′-AMP to phosphofructokinase show that the enzyme can bind a maximum of 0.9–1.1 moles of either nucleotide per 100,000 g under all the conditions tested. This would be equivalent to 4 sites on the active enzyme which has a molecular weight of 360,000 to 400,000 (Mansour and Ahlfors, 1968; Paetkau and Lardy, 1967). The competitive nature of the binding of both the cyclic nucleotide and of AMP indicate that the activation by these nucleotides occurs as a result of binding at the same site.

In a previous report Lorenson and Mansour (1969) showed that ATP does not bind to its regulatory sites on the heart enzyme under conditions which render the enzyme insensitive

to ATP inhibition, *e.g.*, at pH 8.2 or after photooxidation. Competition between the binding of ATP and of cyclic 3′,5′-AMP to muscle phosphofructokinase indicated that the site for the cyclic nucleotide was identical with one of the ATP sites (Kamp and Krebs, 1967). It was therefore unexpected to find that the number of binding sites for cyclic 3′,5′-AMP was not changed after photooxidation nor at pH 8.2. This finding could argue for a separate site for cyclic 3′,5′-AMP. This is supported by the stoichiometry of binding of the two nucleotides. There are apparently 2 regulatory sites for ATP per 100,000 g of enzyme (Lorenson and Mansour, 1969) and only 1 site for cyclic 3′,5′-AMP. An alternative explanation for this data is the possibility that the cyclic 3′,5′-AMP site is identical with one of the ATP regulatory sites. However, in this case photooxidizing the enzyme or simply raising the pH to 8.2 must alter this binding site in such a way that the binding of cyclic 3′,5′-AMP is completely unaffected, whereas ATP can no longer bind at all. If the sites for ATP and cyclic 3′,5′-AMP are different, then the kinetic activation by cyclic 3′,5′-AMP must be due to an effect secondary to its binding and not to competitive displacement of ATP from the inhibitory sites. Unfortunately an experiment to test directly the question of competition between cyclic 3′,5′-AMP and ATP has not been possible because ATP is hydrolyzed in our system at a rate which makes measurement of its binding inaccurate. ADP is the first product of the hydrolysis, but it is also hydrolyzed to form the final product, AMP. The hydrolysis is not catalyzed by phosphofructokinase, but could be caused by contaminants in the membrane or possibly bacterial growth during equilibrium dialysis. Attempts to stop ATP hydrolysis during dialysis were unsuccessful.

The results on the binding of fructose-6-P indicate that the enzyme can bind a maximum of about 1.8 moles of the hexose phosphate per 100,000 g, a value in agreement with that published previously (Lorenson and Mansour, 1969). This would be equivalent to 8 sites on the active enzyme. However, in this study we have found that the affinity of these sites to the hexose phosphate is not identical. The low-affinity class of binding apparently represents those sites which were reported previously for the heart (Lorenson and Mansour, 1969) and skeletal muscle (Kemp and Krebs, 1967) enzymes since the observed dissociation constants are all of the same magnitude (7–40 μM). The high-affinity binding which is described here was probably not observed earlier chiefly because the binding of fructose-6-P was not studied at concentrations below 2 μM. The nature of the high-affinity binding is somewhat unusual. Apparently the binding of fructose-1,6-di-P interferes with the binding of fructose-6-P at these sites. Fructose-1,6-di-P like fructose-6-P exhibits two classes of binding to the enzyme. Moreover, there is no significant difference between the apparent K_D's for fructose-6-P and fructose-1,6-di-P binding to these sites. In view of this it is rather unexpected to find that once fructose-1,6-di-P is bound it will not exchange with fructose-6-P. A contributing factor to this observation is the fact that the fructose-1,6-di-P was bound to the enzyme at 60° during solubilization of the enzyme crystals (see Methods). These conditions are quite different from those used for the equilibrium dialysis experiments.

The nature of binding sites for fructose-6-P is still a matter of speculation. In our previous report based on the fact that two out of four ATP sites per 100,000 g were not affected following desensitization of the enzyme to allosteric control by photooxidation, we concluded that the enzyme has 2 catalytic sites per 100,000 g or 8 per mole of fully active enzyme. The major evidence we have so far on the subunit structure points to identical subunits for this enzyme. It is, therefore, difficult to conclude from the above binding data for fructose-6-P that there are two different types of catalytic sites. Kinetic data show cooperative interaction between fructose-6-P sites. We were not able to show any evidence of positive cooperative binding for fructose-6-P. One reason for this conflicting evidence is probably the fact that the binding experiments were carried out in the absence of ATP. Kinetic studies on this enzyme have shown repeatedly that the demonstration of positive homotropic interaction between fructose-6-P sites is dependent on the presence of high ATP concentration. An interpretation of the results on the high- and low-affinity binding constants for fructose-6-P could be done on the basis of Koshland's idea of negative cooperativity (Koshland, 1969). That is, binding of fructose-6-P to the high-affinity sites would reduce the affinity of the other sites to this ligand as a result of conformational change. Accordingly, the enzyme shows positive cooperativity in the presence of high ATP concentrations as was shown kinetically and negative cooperativity in the absence of ATP as the above binding studies would show. Negative cooperativity has as yet not been demonstrated kinetically with phosphofructokinase. An analogous example for an enzyme which can show the two types of cooperativity is cytidine triphosphate synthetase. Levitzki and Koshland (1969) have shown that this enzyme exhibits negative cooperativity for GTP (an effector) and glutamine (a substrate) and positive cooperativity for ATP and UTP (both substrates). If such an interpretation is correct it could argue for the validity of the sequential model of Koshland (1969) to explain the allosteric control of phosphofructokinase.

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Catalytic Steps during the Single-Turnover Reduction of Aldehydes by Alcohol Dehydrogenase†

James T. McFarland‡ and Sidney A. Bernhard*

ABSTRACT: The reaction of pyrazole with liver alcohol dehydrogenase·nicotinamide–adenine dinucleotide complex has been studied kinetically. The pseudo-first-order rate constant for reaction in 0.02 M pyrazole at 25° and pH 8.75 is 105 sec^{-1}, a specific rate far in excess of that for the slowest step in the transient reaction of aromatic aldehydes with the enzyme–reduced coenzyme (NADH) complex at this temperature and pH. The reverse dissociation of ternary complex to E·NAD^+ complex has a first-order specific rate too slow to be of significance to the transient course of conversion of free aldehyde to free alcohol. Pyrazole does not inhibit any reaction or interaction involving NADH or aldehyde. Therefore it can be utilized as a rapid and specific poison of enzyme sites once NAD^+ has been produced by reduction of aldehyde. This "catalytic-site suicide" can be utilized to follow a single transient reduction of aldehyde substrate in an amount equivalent to the total number of *sites*, under conditions of excessive substrate and coenzyme. The biphasic rate behavior for the formation of products without any detectable intermediates noted previously under conditions of excessive enzyme sites (Bernhard, S. A., Dunn, M. F., Luisi, P.-L., and Shack, P. (1970), *Biochemistry 9*, 185) persists under these newly accessible conditions. Particularly at high-substrate concentrations, the fractional reaction associated with each of the two steps are not necessarily equal, although the biphasic character is always apparent by casual inspection of the kinetic profiles for reduction of aromatic aldehydes. The faster of the two steps is 2.5-fold slower for the transfer of deuterium from NAD-D to substrate, independent of the nature or concentration of substrate. The slow step shows no deuterium isotope effect. These results substantiate previous conclusions that the rate of the slow transient (but not the fast) is regulated by desorption rather than chemical transformation. In correspondence, no fast rate of formation of the ternary complex, E·NAD^+·pyrazole, is observable. The pseudo-first-order rate constant for this complex formation under aromatic aldehyde reaction conditions is the same as that for the slow step of substrate disappearance. This result suggests that the rate of the slow transient, and the turnover number, is limited by alcohol desorption from the ternary product complex. The present results strongly affirm previous hypotheses (Bernhard, S. A., Dunn, M. F., Luisi, P.-L., and Schack, P. (1970), *Biochemistry 9*, 185; Harada, K., and Wolfe, R. G. (1968), *J. Biol. Chem. 243*, 4123, 4131) that the state of liganding at one subunit in oligomeric dehydrogenases regulates the activity at the other(s).

Horse liver alcohol dehydrogenase (EC 1.1.1.1) catalyzes the reduction of aldehyde by NADH (eq 1). The enzyme is a dimer (mol wt 84,000) with two apparently independent coenzyme binding sites (Ehrenberg and Dalziel, 1958; Theorell and Bonnischen, 1951; Theorell and Winer, 1959). The amino acid sequence of the ethanol-active isozyme indicates that the two polypeptide chains making up the dimer are identical (Jörnvall, 1970). The steady-state mechanism of ethanol–acetaldehyde catalysis is apparently compulsory ordered (eq 2) with coenzyme dissociation rate limiting (Theorell and Chance, 1951; Wratten and Cleland, 1963).

$$RCHO + NADH + H^+ \rightleftharpoons RCH_2OH + NAD^+ \quad (1)$$

$$E + NAD^+ \rightleftharpoons E(NAD^+) \rightleftharpoons E(NAD^+)(C_2H_5OH) \rightleftharpoons E(NADH)(CH_3CHO) \rightleftharpoons E(NADH) \quad (2)$$

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