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Binding of Zn^{2+} to rat liver fructose-1,6-bisphosphatase and its effect on the catalytic properties

(allosteric inhibitor/chelators/Mg^{2+}/regulation of enzyme activity)

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ABSTRACT **Rat liver fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) contains 12 binding sites for Zn^{2+} per molecule, or 3 per subunit, as determined by gel filtration and by precipitation of an insoluble Zn^{2+}–enzyme complex. The first set of sites binds Zn^{2+} with very high affinity, and the binding constant for these sites could not be determined. The average values of the dissociation constants for the second and third sets of sites were approximately 0.4 and 1.5 μM, respectively. The third set of sites, having lowest affinity, appears to be identical to the binding sites for the activating cation, Mg^{2+}, and the binding of Zn^{2+} to this set of sites is prevented by the addition of Mg^{2+}. Binding of the first 4 equivalents of Zn^{2+} yields an enzyme of intermediate activity, while the binding of 8 equivalents results in almost complete inhibition of catalytic activity. Thus Zn^{2+} appears to function as both an activator and a negative allosteric regulator of fructose-1,6-bisphosphatase activity.**

A number of laboratories have described inhibitory effects of Zn^{2+} on the activity of fructose-1,6-bisphosphatases (Fru-P_2ases; (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) from a variety of sources (1–5), and the activation of Fru-P_2ases by chelating agents has been attributed (5, 6) to the reversal of this inhibition. We have recently identified Zn^{2+} as a powerful and specific inhibitor of rat liver Fru-P_2ase, and also reported that at higher concentrations Zn^{2+} can substitute for Mg^{2+} or Mn^{2+} as the activating cation (6). These observations suggested that the enzyme contained two sets of binding sites for Zn^{2+}, one of which would be identical to the binding sites for the activating cations. Preliminary experiments (6) confirmed the existence of these two sets of binding sites.

We have now carried out more detailed studies of the binding of Zn^{2+} to rat liver Fru-P_2ase, both in the presence and in the absence of the substrate. The results reported here indicate that rat liver Fru-P_2ase possesses *three* sets of binding sites, two of which are related to the inhibitory effects of Zn^{2+}, and the third to its role as an activator.

MATERIALS AND METHODS

Materials. Fructose-1,6-bisphosphatase was purified as previously described (7) from livers of freshly killed Sprague-Dawley rats and the purified enzyme was stored at 4° as a suspension in 60% saturated $(NH_4)_2SO_4$. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and hexose phosphate isomerase (EC 5.3.1.9) were purchased from Boehringer Mannheim Corp., Indianapolis, IN. Fructose 1,6-bisphosphate (Fru-P_2) and NADP were obtained from Sigma Chemical Co., St. Louis, MO. $^{65}ZnCl_2$ and Aquasol were from New England Nuclear Corp., Boston, MA. All other chemicals were the best commercial grade available.

Methods. Fru-P_2ase was assayed spectrophotometrically as previously described (7) or by the release of inorganic phosphate (6). For the latter, the assay mixture (1.0 ml) contained 20 mM triethanolamine/20 mM diethanolamine·HCl buffer at pH 7.5, 0.1 mM Fru-P_2, Fru-P_2ase, $MgCl_2$, $ZnSO_4$, and EDTA or other chelators as indicated. The reaction mixture was incubated at 25° and the reaction was stopped by the addition of 0.5 ml of the color-developing reagent (8). After 15 min at room temperature the absorbance was measured at 650 nm and compared to values obtained with a P_i standard.

Binding studies were carried out by a modification (9) of the method of Hummel and Dreyer (10) as described in the legends to the figures. In the calculations the molecular weight of the enzyme was taken as 140,000 and the subunit molecular weight as 35,000 (7).

RESULTS

Inhibition of the Mg^{2+}-Activated Enzyme by Zn^{2+}. In the absence of Zn^{2+} the activation of Fru-P_2ase by Mg^{2+} showed sigmoid kinetics, with a Hill coefficient of 1.94 and a value of $S_{0.5}$ (substrate concentration at half-maximal velocity) = 0.13 mM (Fig. 1). The addition of 0.25 μM Zn^{2+} reduced the Hill coefficient to 1.14, but the value of $S_{0.5}$ was not significantly altered. The results suggest that inhibition by Zn^{2+} is noncompetitive (or uncompetitive) with respect to Mg^{2+}.

When the activity was tested in the presence of a suboptimal concentration of Mg^{2+}, the inhibition by Zn^{2+}, though pronounced, was incomplete, and appeared to be reversed as the concentration of Zn^{2+} was increased (Fig. 2). The apparent K_m for activation by Zn^{2+} under these conditions was approximately 5 μM, in agreement with the previously reported value for Zn^{2+} as an activating cation in the absence of Mg^{2+} (6). The results suggest that the enzyme possesses at least two distinct sets of sites for Zn^{2+}, one set of inhibitory sites with $K_i \cong 0.1$–0.2 μM, and a second set of activating sites with $K_m \cong 5$ μM.

Binding of Zn^{2+} to Fru-P_2ase. In earlier studies of the binding of Zn^{2+} to rat liver Fru-P_2ase (6) we employed the rate of dialysis procedure of Colowick and Womack (11); this method yielded 1.9–2.2 equivalents of Zn^{2+} bound per subunit in the absence of Mg^{2+}, and 1.6 equivalents per subunit in the presence of excess Mg^{2+}. It was difficult to conclude from these results whether the total number of binding sites per subunit was two or three. In the present work we utilized a gel filtration technique and carried out the binding studies of 4°, which permitted us to study binding in the presence as well as the absence of Fru-P_2.

A typical binding experiment is illustrated in Fig. 3, and the results of a series of experiments carried out at various concentrations of $^{65}Zn^{2+}$ are shown in Fig. 4. The total amount of Zn^{2+} bound approached 12 equivalents per mole, both in the presence and in the absence of substrate, confirming the presence of three binding sites per subunit. The first set of four sites

Abbreviations: Fru-P_2, fructose 1,6-bisphosphate; Fru-P_2ase, fructose-1,6-bisphosphatase.

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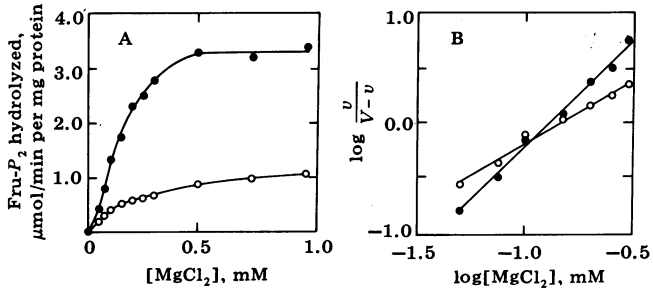

FIG. 1. Effect of Mg^{2+} on the activity of Fru-P_2ase assayed in the presence and absence of an inhibitory concentration of Zn^{2+}. Fru-P_2ase (0.5 μg) was assayed at pH 7.5 by the P_i-release method, as described under *Materials and Methods*, in the presence of 0.1 mM Fru-P_2 and increasing concentrations of Mg^{2+} as indicated. (*A*) In the absence (●) or in the presence (○) of Zn^{2+} (0.25 μM). (*B*) The Hill plot of the data, where V in each case was the maximum velocity obtained experimentally. $S_{0.5}$ for Mg^{2+} was calculated from the intercepts of the Hill plots where $\log[v/(V-v)] = 0$.

was filled at very low concentrations of Zn^{2+}, and binding to the second set occurred at intermediate concentrations. The third set of sites was saturated when the Zn^{2+} concentration reached 40 μM, but the data suggested that the affinity of this site for Zn^{2+} was significantly higher in the presence of Fru-P_2 than in its absence (see Fig. 4). However, in the absence of Fru-P_2 the addition of three equivalents of Zn^{2+} per subunit caused the enzyme to precipitate (see below).

The data for Zn^{2+} binding in the presence of Fru-P_2 were plotted according to Scatchard (Fig. 5). The average dissociation constants for the second and third sets of sites in the presence of Fru-P_2 were $K_{d_2} = 0.34$ μM and $K_{d_3} = 1.5$ μM, respectively. Binding of Zn^{2+} to the first set of four sites was so tight that a dissociation constant could not be calculated, but it was estimated to be less than 0.01 μM. Tight binding to the first set of sites was confirmed by an experiment similar to that described in Fig. 3, in which the enzyme was equilibrated with 3 equivalents of Zn^{2+} per subunit in the presence of 0.1 mM Fru-P_2 and then passed through the column in the absence of Zn^{2+}. The enzyme that emerged still contained 0.7 equivalents of Zn^{2+} per subunit.

Binding to the third set of sites was eliminated by the addition of 2 mM Mg^{2+} (Fig. 4).

Comparison of the Kinetic Constants and Binding Constants. In order to compare the binding constants measured at 4° to the kinetic constants, it was necessary to measure the effects of Zn^{2+} at the same temperature. The concentration of Zn^{2+} required for half-maximal activation, in the absence of Mg^{2+} or Mn^{2+}, was 4.5 μM (data not shown), not significantly different from the value previously obtained at 25° (6). When

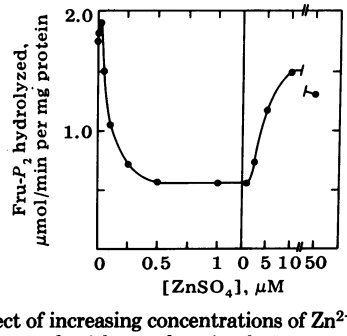

FIG. 2. Effect of increasing concentrations of Zn^{2+} on the activity of Fru-P_2ase assayed with a suboptimal concentration of Mg^{2+}. Fru-P_2ase (0.5 μg) was assayed at pH 7.5 by the P_i-release method as described under *Materials and Methods*, in the presence of 0.1 mM Fru-P_2 and 0.1 mM $MgCl_2$.

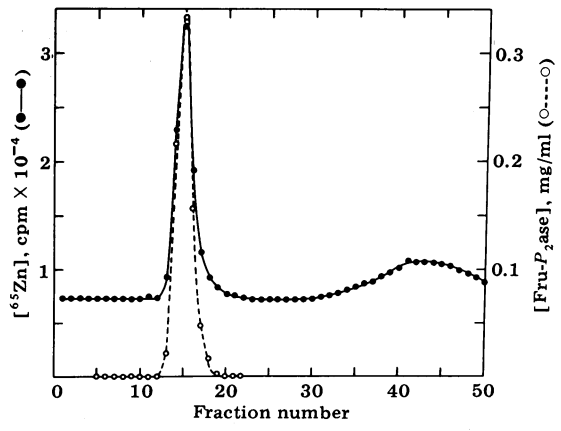

FIG. 3. Binding of $^{65}Zn^{2+}$ to Fru-P_2ase measured by gel filtration. A solution [250 μl containing 1.04 mg of Fru-P_2ase (7.40 nmol), 30 nmol of Fru-P_2, and 125 nmol of $^{65}ZnSO_4$ (specific activity = 5.33 cpm/pmol) in 20 mM triethanolamine/20 mM diethanolamine Cl buffer, pH 7.5] was incubated for 10 min at 4°, and applied to a column (38 × 1.16 cm) of Sephadex G-50 (coarse grade) equilibrated with 20 mM triethanolamine/20 mM diethanolamine·HCl buffer, pH 7.5, containing 0.1 mM Fru-P_2 and 6.9 μM $^{65}ZnSO_4$ (specific activity = 5.33 cpm/pmol). The column was developed with the same equilibrating solution. Fractions of 1.1 ml were collected at a flow rate of 0.4 ml/min. Aliquots of 20 μl of 100 mM EDTA were added to each fraction before determining protein (○) by measurement of A_{280} and radioactivity (●) by liquid scintillation. The amount of Zn^{2+} bound was calculated from the excess radioactivity in the protein peak, after subtracting the radioactivity in the same volume of equilibrating solution. Protein was determined from the values of A_{280}, using the value of 0.63 for absorbance of a solution containing 1 mg/ml (7).

Zn^{2+} was tested as an inhibitor in the presence of 2.0 mM Mg^{2+}, the value of K_i at 4° was 0.1 μM, compared to the value of 0.3 μM previously reported at 25° (6). Thus, the second set of Zn^{2+}-binding sites, with $K_{d_2} = 0.34$ μM, appears to be related to the inhibitory effects, while the low-affinity sites, with $K_{d_3} = 1.5$ μM, appear to be the sites associated with activation.

Binding of Zn^{2+} to the Precipitated Enzyme. During the

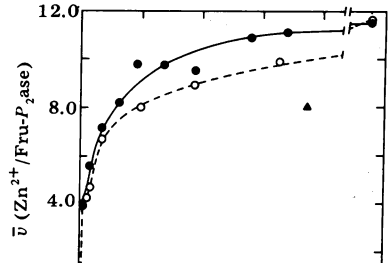

FIG. 4. Binding of $^{65}Zn^{2+}$ to rat liver Fru-P_2ase. The experiments were carried out as described in the legend to Fig. 3, except that the specific activity of $^{65}ZnSO_4$ was 1.44 cpm/pmol for the experiment with 40 μM ^{65}Zn; 9.04 cpm/pmol for the experiments with 0.05, 0.1, and00.25 μM ^{65}Zn; and 34.2 cpm/pmol for the experiment with 0.025 μM ^{65}Zn. In all other experiments the specific activity was 5.33 cpm/pmol. ●, Experiments carried out in the presence of 0.1 mM Fru-P_2; ○, experiments carried out in the absence of Fru-P_2. The ▲ represents a single experiment carried out in the presence of 0.1 mM Fru-P_2 and 2 mM $MgCl_2$. In each case the solution applied to the column contained one equivalent of Fru-P_2 and four equivalents of $^{65}Zn^{2+}$, respectively, per Fru-P_2ase subunit. At the highest concentration of Zn^{2+} used in the absence of Fru-P_2 the effluent was turbid and only one-third of the protein emerged from the column. For the calculation of $\bar{v}$, moles of Zn^{2+} bound per mole of Fru-P_2ase, the molecular weight was taken to be 140,000 (7).

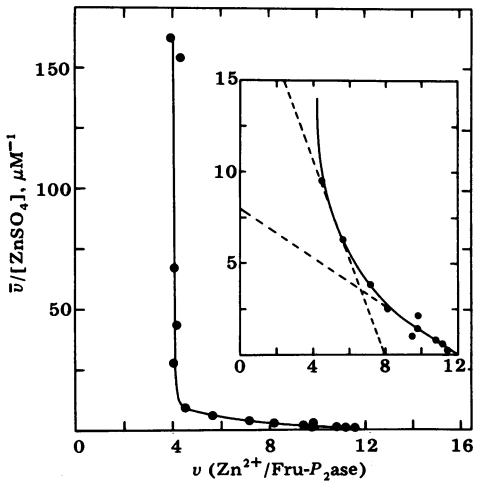

FIG. 5. Scatchard (12) plot of the data shown in Fig. 4 for Zn^{2+} binding to Fru-P_2ase in the presence of substrate. The *inset* shows the data for the second and third sets of sites on an expanded scale.

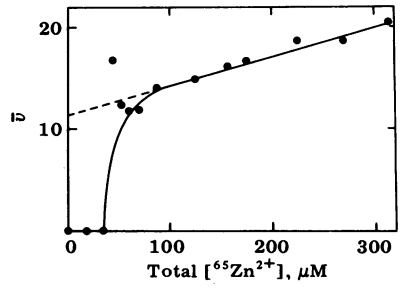

FIG. 6. Precipitation of rat liver Fru-P_2ase by $ZnSO_4$. Solutions (1.0 ml) containing 5.0 nmol of Fru-P_2ase, 20 mM triethanolamine/20 mM diethanolamine·HCl buffer at pH 7.5 and increasing amounts of $^{65}ZnSO_4$ (specific activity = 5.33 cpm/pmol) as indicated were incubated at 3° for 45 min. Aliquots of 50 μl were taken to determine the total concentration of $^{65}Zn^{2+}$ in the solution (to correct for binding to the walls of the vessel). The tubes were then centrifuged for 15 min at 23,000 × g. The supernatant solutions were removed and the inner walls of the tubes were carefully blotted. The protein pellets were solubilized in 0.95 ml of 20 mM triethanolamine/20 mM diethanolamine·HCl buffer, pH 7.5, containing 1 mM EDTA. $^{65}Zn^{2+}$ radioactivity was measured in 50-μl aliquots by liquid scintillation and the protein content was determined spectrophotometrically. The precipitate first appeared when the amount of Zn^{2+} added exceeded 8 equivalents per mole of enzyme. The graph shows the plot of $\bar{v}$ (moles of ^{65}Zn bound per mole of precipitated Fru-P_2ase) as a function of the total $^{65}ZnSO_4$ concentration. The final slope represents nonspecific binding and the broken line extrapolated to the ordinate provides an estimate of the number of equivalents of Zn^{2+} required to precipitate the enzyme. The precipitated enzyme, solubilized by the addition of EDTA, was fully active.

binding studies we observed that a precipitate formed when the amount of Zn^{2+} added in the absence of substrate exceeded 8 equivalents per mole of enzyme. In the presence of Fru-P_2, the Zn^{2+}–enzyme complex remained soluble. Because of the formation of this complex it was not possible to measure K_d for the third set of binding sites in the absence of Fru-P_2 by the gel filtration method (see Fig. 4). However, we took advantage of the formation of this insoluble complex to determine the stoichiometry of Zn^{2+} binding (Fig. 6). When the results were corrected for nonspecific binding, it was found that the precipitated enzyme contained 11.5 equivalents of Zn^{2+} per mole of enzyme. This result confirms the presence of the third binding site.

DISCUSSION

The evidence presented here suggests that rat liver Fru-P_2ase possesses three binding sites for Zn^{2+} per subunit, or a total of 12 sites for the tetrameric protein, and that the binding of Zn^{2+} to the first two sets of sites may play a role in the modulation of its catalytic activity. It has long been known that the addition of a chelator such as EDTA is required for maximum activity in the neutral pH range (13, 14); in the absence of chelating agents the pH optimum is shifted to the more alkaline range (15–18). Nimmo and Tipton were able to eliminate the requirement for EDTA by treating the assay mixture and enzyme solutions with Chelex-100, suggesting that the partial inhibition was related to the presence of traces of heavy metal, and indeed Frey *et al.* (19) reported that the enzyme isolated in the absence of EDTA contained significant quantities of bound Zn^{2+}. We have shown (ref. 6, and the present work) that Zn^{2+} is a powerful and specific inhibitor of Fru-P_2ase activity, but that at higher concentrations Zn^{2+} can also substitute for Mg^{2+} or Mn^{2+} as the activating cation.

The information pertaining to the effects of Zn^{2+} and chelating agents on the catalytic activity of Fru-P_2ase and the

Table 1. Summary of binding and kinetic constants for Zn^{2+} as related to the filling of the three binding sites

Addition	V_{max}*	Zn^{2+} bound, eq/mol	K_i or K_m, μM	K_d, μM	Sites occupied
EDTA	9	0	—	—	Mg^{2+} a b c
None	3	1–4†	—‡	<0.01‡	Zn^{2+} Mg^{2+} a b c
Zn^{2+} <1.0 μM	0.3	5–8	0.10§	0.4¶	Zn^{2+} Zn^{2+} Mg^{2+} a b c
Zn^{2+} >1.0 μM	1.5	9–12	4§	1.5¶	Zn^{2+} Zn^{2+} Zn^{2+} a b c

* μmol of Fru-P_2 hydrolyzed per min/mg of protein at pH 7.5. Data are of Tejwani *et al.* (Table 2 and Fig. 1 of ref. 6), and from Fig. 2 of this paper. The concentration of Mg^{2+} was 2.0 mM, except for the experiments with added Zn^{2+}, where it was 0.1 mM.

† It is not known whether the degree of inhibition depends on the number of sites occupied.

‡ These sites appear to bind Zn^{2+} stoichiometrically and values for the constants could not be determined. The K_d for $^{65}Zn^{2+}$ for this site must be less than 0.01 μM (see Fig. 4).

§ Values for low and high Zn^{2+} estimated from the left-hand and right-hand portions of Fig. 2, respectively.

¶ Calculated from the data in Fig. 5.

binding of Zn^{2+} to the enzyme protein may be summarized as illustrated in Table 1.

In the presence of EDTA the enzyme is presumed to be free of Zn^{2+} and the full activity is expressed, provided that sufficient free Mg^{2+} or Mn^{2+} is present (16). In the absence of EDTA the activity of Fru-P_2ase at pH 7.5 is approximately one-third of the activity observed in its presence. This value is relatively independent of the amount of endogenous Zn^{2+} present, and it may be assumed that the first set of binding sites (the *a* set) is partially filled under the usual assay conditions. Because of the high affinity of this set of sites for Zn^{2+}, they would tend to be filled by traces of Zn^{2+} derived from the glassware and buffer solutions.

Filling of the second set of sites (*b* set) reduces the activity of the enzyme to very low values, but complete inhibition is difficult to achieve, because as the concentration of Zn^{2+} is increased it begins to fill the activator sites (*c* set) and the inhibition is reversed. Activation by Zn^{2+} appears to be competitive with Mg^{2+} and Mn^{2+}, and these cations will displace Zn^{2+} from the third binding site (see Fig. 4). The evidence suggests that when sites *a* and *b* are occupied by Zn^{2+}, Zn^{2+} can activate more effectively than Mg^{2+} by binding to site *c*.

It is significant that the inhibition by Zn^{2+} is completely reversed by histidine at concentrations found in rabbit liver under gluconeogenic conditions (17). Thus Zn^{2+} and histidine together may act to modulate the levels of Fru-P_2ase activity. In the presence of histidine the maximum activity of Fru-P_2ase would be expressed, while in its absence the level of Fru-P_2ase activity would depend on whether the concentration of free Zn^{2+} was low, filling only the sites with high affinity (set *a*), or high (above 0.1 μM), which would begin to fill the sites with intermediate affinity (set *b*).

Under physiological conditions it is unlikely that the ratio of Zn^{2+} to Mg^{2+} would ever be sufficiently high for Zn^{2+} to displace Mg^{2+} from the catalytic sites (set *c*). The content of free intracellular Mg^{2+} has been estimated to be approximately 1 mM (20), and the total concentration of Zn^{2+} in the cytosol as 0.1 mM (21). The concentration of free Zn^{2+} is not known, but if we assume it to be 10% of the total, as is the case for Mg^{2+} (20), then it is unlikely that Zn^{2+} would effectively compete for the activator sites. However, as indicated above, it could function as a negative allosteric effector that would allow the enzyme to function at high activity (no sites filled), intermediate activity (only the *a* sites filled), or low activity (*a* and *b* sites filled).

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