Fructose 1,6-diphosphatase from rabbit liver XIII. The number of Mn++ binding sites measured with 54Mn++

ARTICLE *in* BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS · DECEMBER 1969

Impact Factor: 2.3 · DOI: 10.1016/0006-291X(69)90851-1 · Source: PubMed

FRUCTOSE 1,6-DIPHOSPHATASE FROM RABBIT LIVER

XIII. THE NUMBER OF Mn^{++} BINDING SITES MEASURED WITH $^{54}Mn^{++}$ *

S. Pontremoli, E. Grazi and A. Accorsi

Istituto di Chimica Biologica dell'Universita di Ferrara
Ferrara, Italy

Received August 28, 1969

SUMMARY

The requirement of rabbit liver FDPase for a divalent cation for catalytic activity has now been correlated with the binding of $^{54}Mn^{++}$ in gel filtration studies. The enzyme binds four equivalents of $^{54}Mn^{++}$ at neutral pH and four additional equivalents above pH 8.5. Only the binding of the first set of four equivalents is required for catalytic activity, and the pK of binding is approximately 7. The results provide additional support for the 4-subunit model for the enzyme.

We have previously shown that rabbit liver FDPase possesses four binding sites for the substrate, FDP, and an equal number for the allosteric effector, AMP (1-3). We have now used the Sephadex G50 gel filtration technique to determine the binding of $^{54}Mn^{++}$ to this protein. Two sets of binding sites for Mn^{++} appear to be present. In the neutral pH range approximately four equivalents are bound per mole of protein, and four additional equivalents are bound at higher pH (> 9). The binding of the first set of four Mn^{++} ions is associated with the requirement of cations for catalytic activity; the binding of the second set may be related to the inhibition which is observed with high concentrations of Mn^{++}.

MATERIALS AND METHODS

Fructose 1,6-diphosphatase (FDPase) was prepared and assayed as

* Supported by grants from the National Science Foundation (GB 7140) and the Italian Consiglio Nazionale delle Ricerche (U.S.-Italy Cooperative Science Program) and from the National Institutes of Health (GM 12291). The authors are indebted to Dr. B. L. Horecker for helpful discussions.

previously described (4). Specific activity was twenty international units per mg of protein. The molecular weight was assumed to be 130,000 (5). Carrier free $^{54}MnCl_2$ was purchased from the Radiochemical Centre, Amersham, England and was diluted with cold $MnCl_2$ before use. When the concentration of $MnCl_2$ was 10^{-5} M or less, the specific activity was 5 μC/μmole; when higher $MnCl_2$ concentrations were employed, the specific activity was 0.5 μC/μmole.

Sephadex G50 (coarse) was purchased from Pharmacia, Uppsala, Sweden. Measurements of $^{54}Mn^{++}$ binding to FDPase were carried out at 2° with columns of Sephadex G50 (1.2 x 37 cm) which had been equilibrated with 0.02 M Tris-acetate buffer solutions at the desired pH, containing known concentrations of $^{54}Mn^{++}$. The flow rate was 4 ml per min. Fractions of 1 ml were collected. Protein concentration was measured from the absorbance at 280 mμ standardized against a known weight of dialyzed FDPase. Radioactivity determinations were made in a Packard Tri-Carb liquid scintillation counter in 10 ml of Bray's solution (6).

RESULTS

At pH 7.5 the binding of Mn^{++} by FDPase followed the simple mass action law and approximately four equivalents were bound per mole of enzyme, with a dissociation constant of 2.8×10^{-6} M (Fig. 1A). At pH 9.2 (Fig. 1B) a total of approximately eight binding sites were detected, one set of approximately four sites with a dissociation constant of 7.7×10^{-7} M and a second set with a dissociation constant of 7.7×10^{-5} M.

These values may be compared with the apparent dissociation constants for Mn^{++} calculated from the rate of hydrolysis of fructose 1,6-diphosphate, which were found to be approximately 1×10^{-5} M at pH 9.2 and 2×10^{-5} M at pH 7.5 (Fig. 2). These values were approximately ten times larger than the actual dissociation constants calculated from the binding experiments. It is noteworthy, however, that maximum catalytic activity

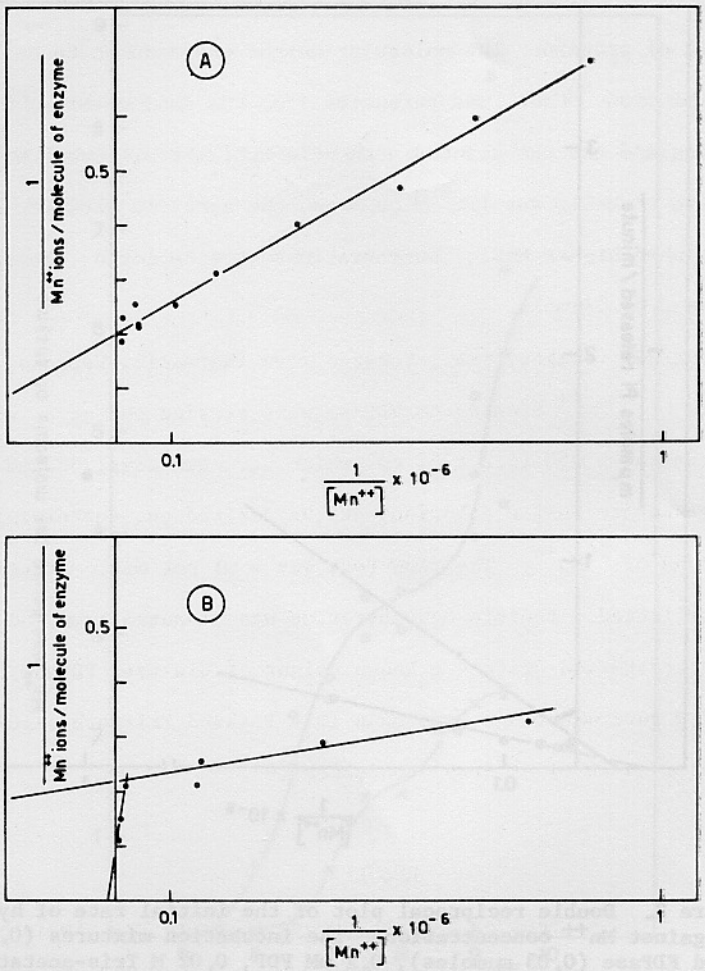

Figure 1. Binding of $^{54}Mn^{++}$ to FDPase as a function of Mn^{++} concentration. Binding was measured by filtration of FDPase (10 mμmole), dissolved in 0.5 ml of 0.02 M Tris-acetate buffer, through Sephadex G50 columns equilibrated with the same buffer containing Mn^{++} at each of the concentrations indicated. The temperature was 2°. (A) pH = 7.5; (B) pH = 9.2.

is observed when the concentration of Mn^{++} is approximately 2 x 10^{-6} M, where the binding studies show that four equivalents of Mn^{++} are bound (compare Figs. 1 and 2). Higher concentrations of Mn^{++} were inhibitory (Fig. 2).

When the effect of pH on the binding of $^{54}Mn^{++}$ to FDPase was studied, little or no binding was observed below pH 6, even at concentrations

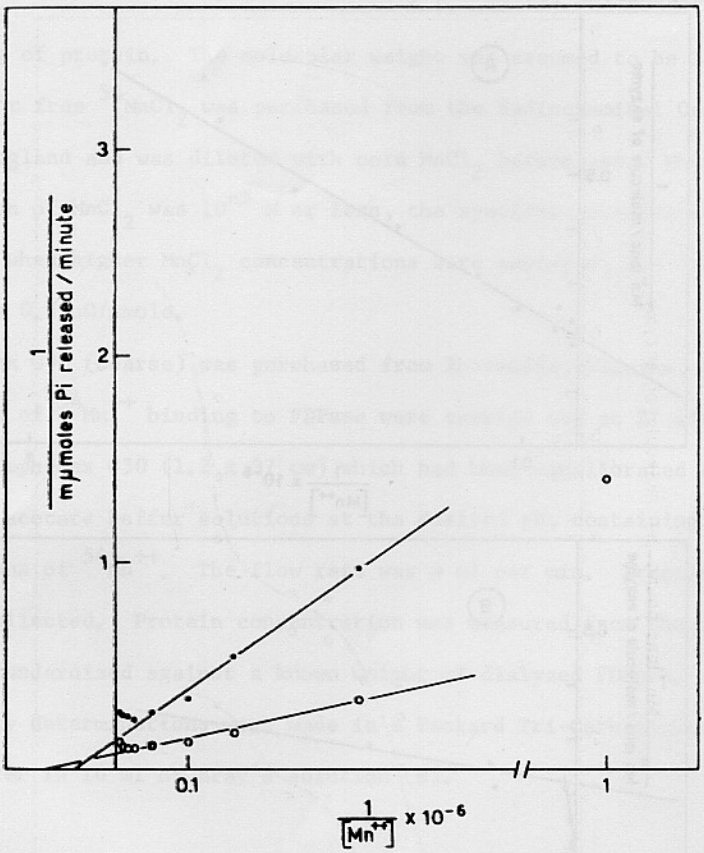

Figure 2. Double reciprocal plot of the initial rate of hydrolysis of FDP against Mn^{++} concentration. The incubation mixtures (0.5 ml) contained FDPase (0.03 mμmoles), 0.2 mM FDP, 0.02 M Tris-acetate buffer and Mn^{++} as indicated. Closed circles, pH = 7.5; open circles, pH = 9.2.

The mixtures were incubated at 2° for 10 min. The reaction was stopped by the addition of 0.1 ml of 5 N sulfuric acid and the orthophosphate formed was determined by the method of Fiske and SubbaRow (7).

---

of Mn^{++} as high as 27 μM (Fig. 3). Above pH 6, binding became apparent and reached a plateau between pH 8-8.5. Even when the concentration of Mn^{++} was 100 μM, only four equivalents were bound at pH 7.5. Above pH 9, the second set of four binding sites was detected. This second set was completely filled at pH 9.2 when the concentration of Mn^{++} was 100 μM.

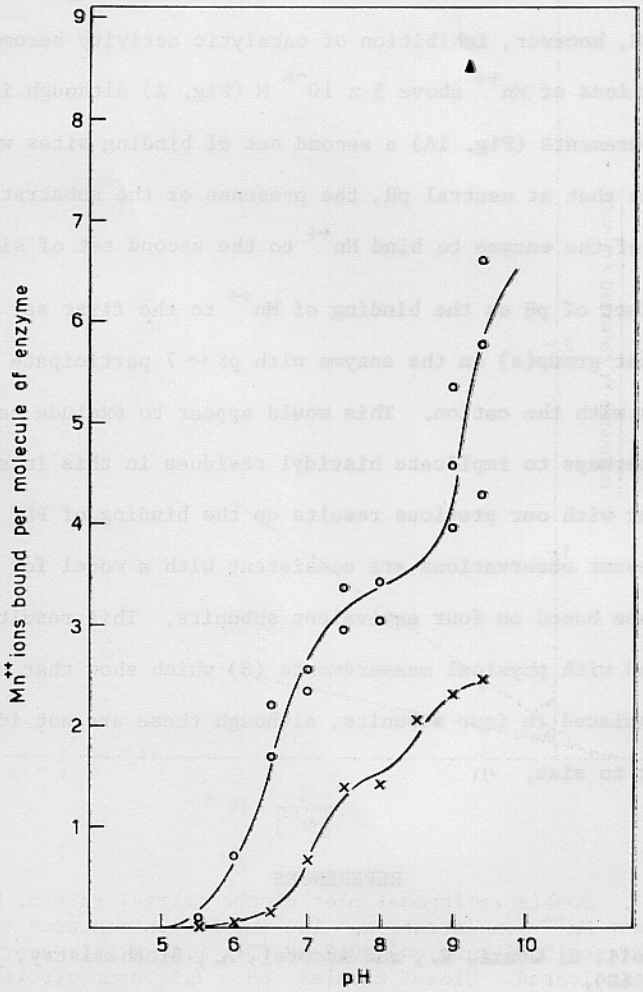

Figure 3. Binding of $^{54}Mn^{++}$ to FDPase as a function of pH. Binding was measured by filtration of FDPase (12 mμmoles) dissolved in 0.5 ml of 0.02 M Tris-acetate buffer at each pH indicated in the Figure, through Sephadex G50 columns equilibrated with the same buffer containing 1.53 μM (X), 27 μM (O) and 100 μM (▲) $MnCl_2$. The temperature was 2°.

DISCUSSION

Rabbit liver FDPase contains two sets of approximately four binding sites each for Mn^{++}. The binding of Mn^{++} to the first set appears to be correlated with the requirement of Mn^{++} for catalytic activity. Binding to the second set may be associated with the inhibition which is observed at high concentrations of Mn^{++}. At alkaline pH, binding and inhibition

are detected in the same range of Mn^{++} concentration (Figs. 1B and 2). At neutral pH, however, inhibition of catalytic activity becomes evident at concentrations of Mn^{++} above 5×10^{-6} M (Fig. 2) although in the direct binding measurements (Fig. 1A) a second set of binding sites was not detected. This suggests that at neutral pH, the presence of the substrate alters the ability of the enzyme to bind Mn^{++} to the second set of sites.

The effect of pH on the binding of Mn^{++} to the first set of sites indicates that group(s) on the enzyme with pK ∽ 7 participate in the interactions with the cation. This would appear to exclude carboxyl groups and perhaps to implicate histidyl residues in this interaction.

Together with our previous results on the binding of FDP and AMP, the present observations are consistent with a model for the structure of FDPase based on four equivalent subunits. This result must be reconciled with physical measurements (8) which show that the protein can be dissociated in four subunits, although these are not identical with respect to size.

REFERENCES

1. Pontremoli, S. Grazi, E., and Accorsi, A., Biochemistry, 7, 1655 (1968).
2. Pontremoli, S., Grazi, E., and Accorsi, A., Biochemistry, 7, 3628 (1968).
3. Pontremoli, S., Grazi, E., and Accorsi, A., Biochem. Biophys. Res. Commun., 33, 335 (1968).
4. Pontremoli, S., Traniello, S., Luppis, B., and Wood, W. A., J. Biol. Chem., 240, 3459 (1965).
5. Pontremoli, S., Luppis, B., Traniello, S., Rippa, M., and Horecker, B. L., Arch. Biochem. Biophys., 112, 7 (1965).
6. Bray, G. A., Anal. Biochem., 1, 279 (1960).
7. Fiske, C. H., and SubbaRow, Y., J. Biol. Chem., 66, 375 (1925).
8. Sia, C. L., Traniello, S., Pontremoli, S., and Horecker, B. L., Arch. Biochem. Biophys. 132, 325 (1969).