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# BINDING OF MANGANESE TO PHOSPHOFRUCTOKINASE FROM YEAST

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SUMMARY: The binding of manganese to yeast phosphofructokinase has been studied using the equilibrium dialysis technique. Three independent binding sites per enzyme subunit have been found with identical affinities. The dissociation constant for $Mn^{2+}$ binding is 2,26 mM.

INTRODUCTION: Phosphofructokinase (EC 2.7.1.11) from baker's yeast is an octameric enzyme possessing a molecular weight of about 835 000. Its structural and kinetic properties have been thoroughly investigated (1,2). Equilibrium binding studies of substrates to this phosphofructokinase provided evidence that the enzyme binds $MgATP^{2-}$ and fructose 6-phosphate in stoichiometric manner and independently of each other (3,4). Per subunit, one molecule of Fru 6-P and two molecules of $MgATP^{2-}$, one to a high affinity site and one to a low affinity site, are bound.

Phosphofructokinase is activated by the monovalent cations $NH_4^+$ and $K^+$. In addition, the enzyme requires magnesium ions, the $MgATP^{2-}$ complex being considered the virtual substrate in the transphosphorylation process. Concerning the inhibitory action of ATP for the yeast enzyme $MgATP^{2-}$ rather than free $ATP^{4-}$ seems to be the proper inhibitory species (5). The role of free $Mg^{2+}$ itself in the catalytic reaction is uncertain. Both the catalytic

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and allosteric regulatory properties of phosphofructokinase are retained when $Mg^{2+}$ is replaced by $Mn^{2+}$ (6).

In order to elucidate further the role of bivalent cations in the enzymic reaction, a direct metal binding study appeared to be necessary. Due to the short half time of magnesium isotopes $^{54}Mn^{2+}$ as bivalent metal ligand was employed in equilibrium dialysis experiments.

MATERIALS AND METHODS: Imidazole was purchased from SERVA (Heidelberg, FRG), $MnCl_2$ from VEB Berlin-Chemie (GDR), labelled $^{54}MnCl_2$ ($\geq$ 100 mCi/g Mn) was obtained from Isocommerz (Berlin, GDR). All other chemicals were of reagent grade quality.

Phosphofructokinase from baker's yeast was purified according to the procedure of Diezel et al.(7). Enzyme preparations were characterized by specific activity (about 60 U/mg) and SDS polyacrylamide gel electrophoresis. Prior to the binding studies phosphofructokinase was dialyzed exhaustively at 4 °C against Mg-free imidazole buffer, 50 mM, pH 7,2, 1 mM 2-mercaptoethanol, 3 mM $(NH_4)_2SO_4$. $Mg^{2+}$-contents of buffers and enzyme preparations were checked by atomic absorption spectrophotometry (Model AAS 1, VEB Carl Zeiss Jena, GDR). The residual $Mg^{2+}$-content of the dialyzed enzyme was $<$ 0,02 Moles $Mg^{2+}$/subunit and can therefore be neglected.

Binding experiments were performed using equilibrium dialysis cells with a chamber volume of 100 µl (8). One half-cell was loaded with the respective concentration of labelled $MnCl_2$ and the other with enzyme in the same buffer. The device was rotated for two hours at 20-22 °C. Radioactivity was determined in 30 µl aliquots in a gamma counter (Type 20026, VEB Messelektronik Dresden, GDR). The amount of protein bound ligand was calculated from the difference of the radioactivities in the two half-cells after complete equilibration. Protein was assayed with the microbiuret method of Janatova et al.(9) using bovine serum albumin as standard. The statistical treatment of experimental data was performed by a non-linear regression analysis (10).

Donnan effects, which may affect binding results, were controled by a direct method (11) using $Cs^+$-distribution after equi-

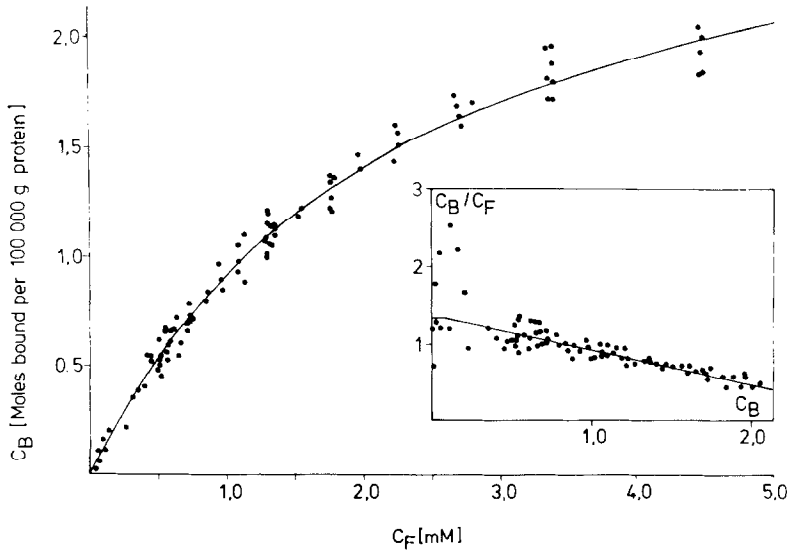


Fig. 1: Binding of $^{54}Mn^{2+}$ to yeast phosphofructokinase at pH 7,2. The solid line is calculated for three independent binding sites ($C_B^o$= 3,02 Moles $Mn^{2+}$/subunit) with identical dissociation constants of 2,26 mM. A subunit molecular weight of 100 000 was used.
The insertion shows the Scatchard plot of the data.

librium dialysis. Significant binding of $Cs^+$ to phosphofructokinase was excluded by means of gel filtration equilibrium dialysis. Protein concentration ranged between 2,5-18 mg/ml. At 1 mM free $Cs^+$ a Donnan distribution on dependence of the protein concentration could not be detected.

RESULTS AND DISCUSSION: The binding of $Mn^{2+}$ to the yeast enzyme was investigated at pH 7,2 in a concentration range of $6 \cdot 10^{-3}$ to 4,5 mM $Mn^{2+}$. The data were obtained in individual experiments using several enzyme preparations. Protein concentration was 8-10 mg/ml. The results are presented in Fig. 1. For description of the data three mathematical functions have been compared, the Hill equation, a straight-forward hyperbolic function and the Klotz equation, reflecting the binding of $Mn^{2+}$ to independent binding sites. Application of the Hill equation revealed non-coopera-

tive binding ($n_H$=1). Analysis by the Klotz equation indicated equivalent binding sites. The Scatchard plot shows a straight line giving no indication of a non-hyperbolic binding of the ligand (Fig.1, insertion). Therefore, the following equation has been taken for the description : $C_B = C_B^o \cdot \frac{C_F}{K + C_F}$ ,

where $C_B^o$ denotes the number of binding sites per subunit of phosphofructokinase, while K designates the affinity constants of these binding sites to $Mn^{2+}$. Different approaches for evaluating the error distribution were applied: constant absolute and constant relative error as well as minimum $\chi^2$ distribution. Most compatible results were obtained using the minimum $\chi^2$ distribution. As a result, three independent binding sites ($C_B^o$= 3,02 ± 0,01 Moles $Mn^{2+}$/subunit) with a dissociation constant of K= 2,26 ± 0,02 mM have been calculated. From this result, the question arises whether two of the $Mn^{2+}$-binding sites can be coordinated to the $MgATP^{2-}$-binding sites of phosphofructokinase which became evident from the kinetic characterization (2) and the $MgATP^{2-}$-binding study, allowing the conclusion that the catalytic activity of the yeast enzyme and also its allosteric inhibition are dependent on the interaction with $MgATP^{2-}$-complexes (3).

While Jones et al.(6) have shown only small enhancement in the water proton relaxation rate in $Mn^{2+}$ solutions of the enzyme, Cottam and Uyeda (12) provided evidence for the formation of binary complexes between rabbit muscle phosphofructokinase and $Mn^{2+}$. Also, ternary and quarternary complexes including ATP and Fru 6-P could be detected by the employed magnetic resonance technique. X-ray data of B. stearothermophilus phosphofructokinase are consistent with the conception of bivalent cation binding to the active site and to the effector site (13). In respect to the high stability of $Mg/MnATP^{2-}$-complexes (14), the metal must form a com-

plex with the nucleotide before interacting directly to the enzyme. But once bound in this manner, the available data are quite consistent with the formation of a $E<^{M}_{S}$ coordination scheme for the phosphofructokinase-metal-ATP complex (15).

Interestingly, manganese occupies a third binding site with the same affinity. If this can be coordinated to a distinct activating nucleotide binding site or to a separate bivalent cation site essential for correct spatial structure and/or catalytic activity remains to be established.

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