BINDING OF FRUCTOSE-6-PHOSPHATE TO PHOSPHOFRUCTOKINASE FROM YEAST

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SUMMARY: Yeast phosphofructokinase binds one molecule of fructose-6-phosphate per subunit. The binding curve exhibits sigmoidality and yields a good fit to an equation derived from the kinetic model as developed previously for this enzyme. The results show that the allosteric kinetic response of the enzyme to fructose-6-phosphate is due to cooperativity of the binding process.

INTRODUCTION: Phosphofructokinase (EC 2.7.1.11) from yeast (Saccharomyces cerevisiae) is an octameric enzyme with a molecular weight of 835 000 (1, 2). Owing to its location in the glycolytic chain, as well as the irreversibility of the catalyzed reaction and its allosteric properties phosphofructokinase is regarded dominating in the regulation of the glycolytic flux (3).

The enzyme exhibits sigmoidal dependence in its activity on the fructose-6-phosphate concentration, is inhibited by the second substrate ATP and is subjected to a variety of positive and negative allosteric effectors (4). In contrast to the mammalian phosphofructokinase, the yeast enzyme does not show an association-dissociation behavior over a wide range of protein concentration (1).

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For the interpretation of the complex kinetic properties of yeast phosphofructokinase an allosteric transition model has been developed by Freyer et al. (4) based on the model of Monod, Wyman and Changeux (5). This model suggests that a) the basic conformations of the enzyme R and T are degenerated into subconformations R_1, R_2 and T_1, T_2, b) fructose-6-phosphate binds with different affinities only to R_1 und T_1, c) ATP binds to two different types of binding sites, a catalytic and a regulatory one, the latter mediating the inhibitory action of ATP. Binding studies performed with MgATP yielded a good fit to the proposed model and showed that two molecules of MgATP are bound per subunit of yeast phosphofructokinase, one to a high affinity and the other to a low affinity site (6). According to the model the binding of fructose-6-phosphate should cause a shift in the allosteric equilibrium between the R and T conformations, which should be expressed in a sigmoidal shape of the fructose-6-phosphate binding curve.

In this communication the stoichiometry and affinity of fructose-6-phosphate binding to yeast phosphofructokinase will be reported and compared with the binding functions of the kinetic model (4).

MATERIALS AND METHODS: Phosphofructokinase from baker's yeast (Stellhefe I, VEB Backhefe, Leipzig) was isolated in homogeneous form (7) and the binding experiments were performed with the ultrafiltration method (8). 0.5 ml of dialyzed phosphofructokinase (composition of the medium: imidazole buffer, 50 mM, pH 7.2, 5 mM $MgSO_4$, 2 mM 2-mercaptoethanol, 1 mM $(NH_4)_2SO_4$, containing 0.5 - 7.0 mg of enzyme) were mixed with 0.2 ml buffer with the respective concentrations of [^{32}P] fructose-6-phosphate. The mixture was placed into the water-jacketed ultrafiltration teflon cell equipped with a Diaflow XM 50 membrane. The temperature was held constant at 20 °C. Nitrogen with a pressure of 55 psi was applied for 5 - 10 min until 20 - 30 µl of the free fructose-6-phosphate solution were forced through the membrane. Radioactivity was

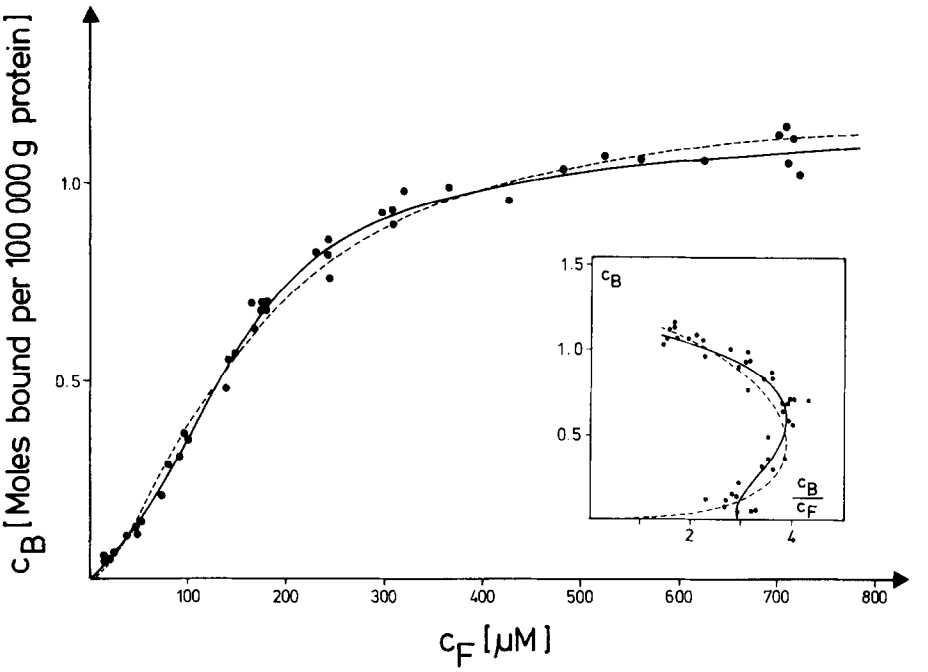

Fig. 1. Binding of fructose-6-phosphate to yeast phosphofructokinase

The dependence of the amount of fructose-6-phosphate bound per subunit of phosphofructokinase (c_B) on the free concentration of fructose-6-phosphate (c_F) is shown.
The insertion demonstrates the Scatchard-plot (14) of the data.
The curves were obtained by a comparison of the experimental data with eqn. [1] (full line) and eqn. [4] (dotted line). The calculations were based on a subunit molecular weight of 100 000 (1).

determined in a 20 μl aliquot with Bray's solution (9) (Tricarb model 3375). The amount of fructose-6-phosphate bound per subunit of phosphofructokinase was calculated from the difference between the total and free ligand concentration. [^{32}P]fructose-6-phosphate was enzymatically synthesized with hexokinase using [γ-^{32}P]ATP and purified (10). [γ-^{32}P]ATP was prepared according to Gibbs et al. (11). The determination of fructose-6-phosphate was carried out according to Hohorst (12). Protein was assayed as described by Janatova et al. (13).

RESULTS AND DISCUSSION: Binding of fructose-6-phosphate to the enzyme was investigated in a concentration range of

1 - 700 μM at pH 7.2 (Fig. 1). The experimental data suggest sigmoidal binding characteristics and indicate that at saturation the enzyme binds one molecule of fructose-6-phosphate per subunit. The binding data were interpreted in terms of the model of Freyer et al. (4). The amount of fructose-6-phosphate bound per subunit of phosphofructokinase (c_B) in dependence on the concentration of the free substrate is expressed by a mathematical relationship being isomorphous to that of the model of Monod, Wyman and Changeux (5):

$$c_B = c_B \left(\frac{K^R[F\text{-}6\text{-}P]}{1+K^R[F\text{-}6\text{-}P]}[R] + \frac{K^T[F\text{-}6\text{-}P]}{1+K^T[F\text{-}6\text{-}P]}\cdot[T] \right) \qquad [1]$$

[F-6-P] is the concentration of the free fructose-6-phosphate, K^R and K^T designate the actual affinities of the binding sites to fructose-6-phosphate in the R and T conformations. [R] and [T] mean the concentrations of the R and T states and are determined by equations [2] and [3]:

$$L = \frac{[T]}{[R]} = \left(M_o \left(\frac{1+K^T[F\text{-}6\text{-}P]}{1+K^R[F\text{-}6\text{-}P]} \right) \right)^n \qquad [2]$$

$$[R] = \frac{1}{1+L} \quad ; \quad [T] = \frac{1}{1+L} \qquad [3]$$

M_o is the allosteric constant referred to one phosphofructokinase subunit; n denotes the number of subunits per enzyme molecule (for yeast phosphofructokinase n is equal to 8) and c_B represents the amount of fructose-6-phosphate bound per subunit of phosphofructokinase at saturating conditions. This parameter has been introduced to recognize deviations from stoichiometry. The binding function of fructose-6-phosphate to the enzyme is described by the parameters K^R, K^T, c_B and M_o.

Table 1. Calculated parameters of yeast phosphofructokinase

Equation [1] (according to (4))		Equation [4] (according to (16))	
K^R	$1.20 \pm 0.2 \times 10^{-2}\ \mu M^{-1}$	K	$6.17 \pm 0.25 \times 10^{-3}\ \mu M^{-1}$
K^T	$2.36 \pm 0.6 \times 10^{-3}\ \mu M^{-1}$		
M_o	1.98 ± 0.2	n_H	1.58 ± 0.2
C_B	1.21 ± 0.1	C_B	1.22 ± 0.1

The experimental data were compared with eqn. [1] by applying a non-linear regression analysis (15) and using minimum χ^2 error distribution, which turned out to be the most reliable approach for error evaluation.

In addition, the data were compared with the Hill-equation (16):

$$c_B = C_B \frac{([F\text{-}6\text{-}P]K)^{n_H}}{([F\text{-}6\text{-}P]K)^{n_H+1}} \qquad [4]$$

The fitted curves are included into Fig. 1 and the calculated parameters are presented in Table 1. Obviously, the experimental data fit to the model of Freyer et al. (4). The mean relative deviation of the experimental data from the calculated curves is less than 2 %.

The computed association constants (Table 1) are within the same order of magnitude of those as determined from the kinetic experiments ($K^R = 4.4 \times 10^{-3}\ \mu M^{-1}$, $K^T = 6.6 \times 10^{-4}\ \mu M^{-1}$ (4)). In addition, the M_o value and the Hill-coefficient derived from the binding data are approximately in accordance to those as obtained kinetically ($M_o = 1.55$, $n_H = 1.8$). The observed differences may arise from the complexity of the kinetically determined constants (17).

The results suggest, that the sigmoidality in the kinetic response with respect to fructose-6-phosphate is due to cooperative binding of this ligand to phosphofructokinase from yeast. The binding of two molecules of MgATP (6) and of one molecule of fructose-6-phosphate per subunit in mutual absence of each other corroborates the basic assumptions of the model of Freyer et al. (4) about the stoichiometry of substrate binding and the existence of a non-ordered reaction mechanism.

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