NEGATIVE COOPERATIVITY IN THE INTERACTION OF FRUCTOSE 1,6-DIPHOSPHATE WITH PYRUVATE KINASE OF *NEUROSPORA CRASSA*

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1. Introduction

Pyruvate kinase (ATP:pyruvate-2-*o*-phosphotransferase, EC 2.7.1.40) of *Neurospora* is an allosteric, tetrameric enzyme, regulated by metabolites exerting both positive and negative controls [1,2]. Fructose 1,6-diphosphate (FDP) is a potent activator that interacts with the enzyme eliciting marked conformational changes, as demonstrated by UV difference spectroscopy, susceptibility towards proteolytic enzymes, accessibility to sulphydryl group reagents and cross-linking by bifunctional reagents [2–5]. Each of the ligands, the substrates and FDP, appear to be implicated in propagation of distinct conformations, on binding to the enzyme.

In view of the significance of ligand-induced effects in regulation, the interaction of *Neurospora* pyruvate kinase and FDP was investigated by direct equilibrium binding studies, employing the ultrafiltration procedure in [6]. Our results show that FDP binds to pyruvate kinase in the absence of any other ligands, exhibiting a pronounced negative homotropic cooperativity.

2. Materials and methods

Pyruvate kinase was isolated from a wild-type strain of *N. crassa* (FGSC no. 262) and purified to apparent homogeneity as in [7]. In the ultrafiltration experiments the enzyme and ligands were mixed in 200 μl total vol. in 0.1 M phosphate buffer (pH 7.5) equilibrated at room temperature for 20 min and filtered at 40 lb/in^2 through PM 10 membranes, held in an ultrafiltration apparatus (MRA Corp., Boston). Following the passage of the solution the underside of the membranes was washed with 5 ml ethylene glycol. The filters were then transferred to scintillation vials containing 0.5 ml of dimethylformamide and the radioactivity determined in a scintillation cocktail containing 4 g 2,5-diphenyloxazole (PPO), 0.2 g *p*-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) and 30% ethanol in 1 l toluene, employing a Beckman model LS 8000 liquid scintillation spectrometer. [^{14}C]FDP (239 mCI/mmol) was obtained from Amersham and PEP, ADP and unlabeled FDP were from Sigma.

3. Results

The binding of FDP to pyruvate kinase was examined in a system containing 1.1 μM enzyme with the ligand varying from 0.86–94.6 μM (with ~0.05 μCi [^{14}C]FDP). As shown in fig.1 the Scatchard plot for FDP was non-linear and concave upwards, characteristic of negative cooperativity. The binding curve could be resolved into two classes of sites: a high affinity (site 1); and a low affinity (site 2) class. By extrapolation of the initial and the final parts of the curve, corresponding to the low and high free ligand concentration regions, to the abscissa, an estimate of minimal n, representing the number of FDP molecules bound to the high affinity site and the total per tetrameter, respectively, was obtained. The approximate dissociation constants, K_{d1} and K_{d2}, given by the slopes of the low and high free ligand concentration segments of the curve, were calculated to be 4×10^{-4} M and 9.1×10^{-6} M, respectively; n for the two sites was 0.7 and 1.48 (per tetramer). These data suggest the occurrence of strong negative cooperativity in the binding of FDP to pyruvate kinase.

On repeating the above experiment in the presence

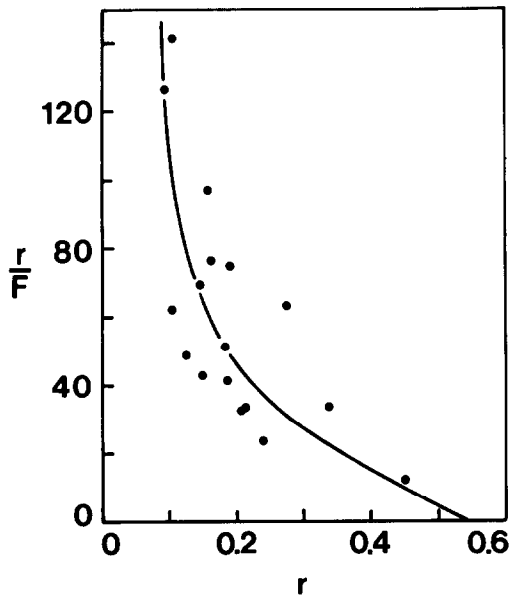

Fig.1. Scatchard plot showing binding of fructose 1,6-diphosphate by *Neurospora* pyruvate kinase in the absence of other ligands. r is the no. mol FDP bound/mol monomer and F is free FDP (μM); r/F (× 10^3) is plotted vs r. Concentration of PK monomer was 1.1 μM (monomer M_r 60 000).

of 1.5 mM ADP the Scatchard plot, again, exhibited negative cooperativity, the dissociation constants for sites 1 and 2 being 5.5 × 10^{-7} M and 5 × 10^{-6} M, respectively (fig.2A). The value of n was almost equal for the two sites indicating that ADP promoted an increased binding of FDP to site 1 without altering

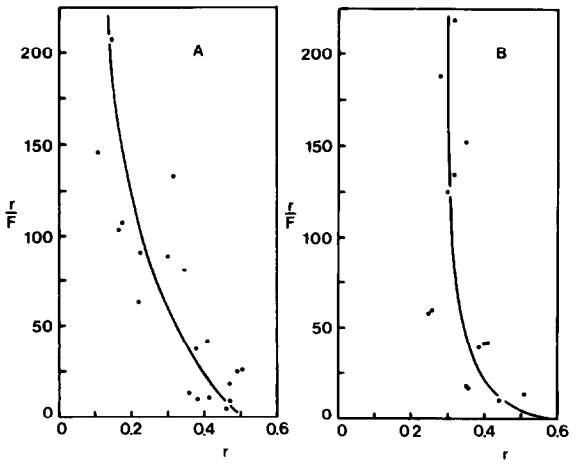

Fig.2. Binding of FDP to pyruvate kinase in the presence of 1.0 mM adenosine 5'-diphosphate (A) and 1.0 mM phosphoenolpyruvate (B). PK monomer was 1.1 μM.

Table 1
The binding parameters for the interaction of FDP with *Neurospora* pyruvate kinase

Additions	Site 1		Site 2		K_{d2}/K_{d1}
(mM)	n	K_{d1} (M)	n	K_{d2} (M)	
None	0.7	4.0 × 10^{-7}	1.48	9.1 × 10^{-6}	23
ADP, 1.5	1.04	5.5 × 10^{-7}	1.0	5.0 × 10^{-6}	9
PEP, 1.0	1.3	1.6 × 10^{-7}	1.0	2.0 × 10^{-5}	125

the total no. molecules bound/mol enzyme. In the presence of PEP, the binding of FDP to site 1 was enhanced still further so that the value of n for the two sites was 1.3 and 1.0, with K_{d1} and K_{d2} of 1.6 × 10^{-7} M and 2 × 10^{-5} M, respectively (fig.2B). Therefore, PEP enhanced the binding of FDP to site 1 without exerting a pronounced effect on its binding affinity. The major consequence of the presence of PEP was to alter the distribution of n for the two sites such that the first one bound more FDP relative to site 2, whereas in its absence the reverse relationship prevailed (table 1). Concomitant addition of $MgCl_2$ and PEP to the system did not result in a marked change relative to that observed with PEP alone. On examination of the ratios of the dissociation constants, K_{d2}/K_{d1}, a value of 23 was obtained for the binding of FDP to unliganded pyruvate kinase. In contrast, this ratio was 124 for its binding to [E-PEP]. Furthermore, ADP tended to diminish the difference between the binding affinity of the two sites for FDP.

4. Discussion

The interaction of the allosteric activator, FDP with *Neurospora* pyruvate kinase revealed a very interesting and novel feature: extreme negative cooperativity approaching half-of-the-sites reactivity. The presence of the substrates, ADP and PEP, led to the propagation of conformational changes in the enzyme resulting in an alteration in the binding parameters for FDP. However, the two substrates did not cause identical structural changes in the enzyme as demonstrated by the marked difference in the K_{d2}/K_{d1} ratios recorded in the presence of each of these ligands. Both substrates led to an increased binding of FDP to site 1 compared to site 2 PEP eliciting an increase of 2-fold in n for the former. The heterotropic

effect of ADP was less pronounced. These observations are consistent with our previous data showing that [E–ADP] and [E–PEP] are characterized by distinct conformations.

Negative cooperativity has not been reported with respect to the binding of FDP to any other pyruvate kinase. While the PK of rabbit liver and human erythrocytes displays a non-cooperative interaction with FDP [8,9], with the yeast and *Escherichia coli* enzymes positive cooperativity has been encountered [10,11]. Our observations, in conjunction with those with PK from other organisms, illustrate the principle that the regulation of a given enzyme, in different organisms, may be governed by distinct mechanisms.

Pyruvate kinase of mammalian system and yeast shows kinetics and binding properties consistent with the concerted model in [12] based on the assumption of two alternative conformational states. The demonstration of negative cooperativity in ligand binding tends to exclude the two-state, concerted, symmetrical model, the behaviour of *Neurospora* pyruvate kinase providing a strong support for the multi-state, sequential model proposed in [13]. As FDP is an activator of PK the question arises as to why binding of this effector should be attended by negative cooperativity. A plausible explanation is that it might be advantageous for *Neurospora* to insulate the system against development of inordinately high PK activity when the intracellular level of FDP exceeds a critical value. The binding of the allosteric activator, GTP, to CTP synthetase has been reported to be negatively cooperative at high temperatures while at 4°C it is positively cooperative [14,15]. The metabolic significance of such observations can only be assessed by quantitative studies correlating the levels of the target enzymes and their regulators in vivo, thereby furnishing a rationale for the evolution of positively or negatively cooperative responses towards effectors for the enzyme, in different organisms.

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