Vol. 80, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1978 Pages 646-652

BINDING OF NUCLEOTIDES AMP AND ATP TO YEAST PHOSPHOFRUCTOKINASE:EVIDENCE FOR DISTINCT CATALYTIC AND REGULATORY SUBUNITS

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Received October 27, 1977

SUMMARY

The binding of ATP to yeast phosphofructokinase, as monitored by flow dialysis, is heterogeneous and is adequately described by assuming two independent classes of three binding sites each per enzyme molecule. Under similar conditions, the binding of 5'AMP is homogeneous and a binding stoichiometry of three 5'AMP molecules per enzyme molecule is evaluated. Displacement experiments show that only the ATP molecules bound to the first class of three tight binding sites are displaced by an excess of 5'AMP. Thus, these tight ATP binding sites can be identified as "regulatory sites" in agreement with kinetic data. Furthermore, molecular weight determinations and electrophoresis results are consistent with an heterologous $\alpha_3\beta_3$ structure of the enzyme oligomer. Therefore the present binding data suggest that yeast phosphofructokinase is constituted by three "catalytic" and three "regulatory" subunits.

INTRODUCTION

Phosphofructokinase (EC 2.7.1.11), a key enzyme of the glycolytic pathway, shows a great variability of structures among prokaryotic and eukaryotic organisms. In the case of yeast phosphofructokinase, although the exact number of subunits (6 or 8) is still a matter of controversy (1,2,3), it is now well established that the native form of this enzyme contains an equal number of two types of subunits α and β differing slightly in their molecular weight (4). These two types of subunits are immunologically distinct (4) and are easily differentiated by their relative susceptibility to proteolytic degradation in the presence of specific substrates (5). These structural features of the yeast enzyme are not unique among eukaryotic phosphofructokinases

0006-291X/78/0803-0646$01.00/0

since the enzyme from human erythrocytes has been recently found (7) to also contain two types of subunits which are distributed into complex, high molecular weight quaternary structures. Until now, however, no particular functional significance has been given to the occurence of two types of subunits in yeast phosphofructokinase. We have previously presented (8) kinetic data showing that yeast phosphofructokinase contains two distinct classes of ATP binding sites; one class of sites binds ATP as phosphate donnor in the enzymatic reaction (i.e "catalytic" sites) and the other class of sites binds ATP as an allosteric inhibitor (i.e "regulatory" sites). The allosteric activator 5'AMP modifies the binding of ATP only to the regulatory sites. In the present work, we report equilibrium binding data for ATP and 5'AMP to yeast phosphofructokinase. These binding data are in agreement with our previous kinetic measurements and can be correlated with the occurence of two equally distributed types of subunits in the enzyme oligomer, leading to the conclusion that yeast phosphofructokinase is likely to be composed of distinct catalytic and regulatory subunits.

MATERIAL AND METHODS

Phosphofructokinase was prepared from baker's yeast according to the slightly modified procedure of Diezel et al. (1). Unlabelled 5'AMP and ATP were purchased from Sigma. Radioactive [U^{14}C] ATP (562 mCi/mmol) and [U^{14}C] 5'AMP (538 mCi/mmol) were obtained from the Radiochemical Center Amersham (England). All other chemicals were of the best available purity.

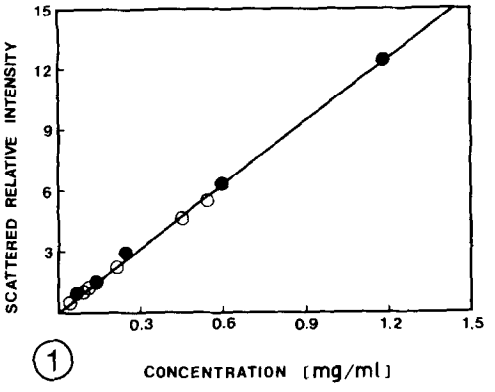
The evaluation of ATP and 5'AMP binding by gel-filtration experiments was performed as previously described (9). Flow dialysis (10) measurements were conducted according to Tenu et al. (11) using Instagel (Packard, France) as scintillation liquid. All binding experiments were carried out at 25°C, pH 6.8, in 50 mM Tris-sulfonate buffer containing 25 mM K_2HPO_4, 1 mM dithiothreitol, 5 mM $MgCl_2$ and 1 mM EDTA. Phosphofructokinase concentrations were determined from refraction index measurements, assuming an increment value dn/dc of 1.85 $(mg/ml)^{-1}$; this value leads to an absorption coefficient of 0.97 A/mg at 280 nm.

Cross-linked hemoglobin and bovin serumalbumin prepared according to the procedure of Payne (12) were a generous gift of Dr. A. d'Albis. The electrophoresis technique was adapted from that of Taucher et al. (6) using a 4% polyacrylamide gel. Light scattering measurements were performed as described by Dessen and Pantaloni (13).

RESULTS

QUATERNARY STRUCTURE OF YEAST PHOSPHOFRUCTOKINASE.

The molecular weight of native phosphofructokinase has been consistently evaluated by light scattering and gel filtration on Utrogel AcA 22 (LKB) to 730 000 $\pm$ 30 000 daltons (fig. 1). This value is slightly smaller than that reported by Diezel et al. (1) and agrees very well with that reported by Tamaki and Hess (2) in the case of the brewer yeast enzyme. As illustrated in fig. 2, sodium dodecylsulfate polyacrylamide gel electrophoresis performed on freshly



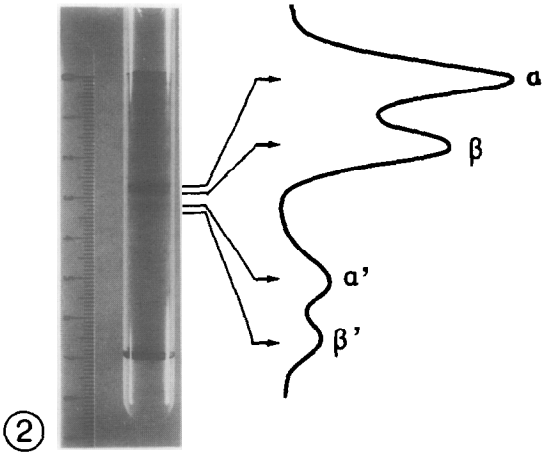


Fig. 1 : Light scattering data for yeast phosphofructokinase.
Plot of the relative scattered intensity at 436 nm, 20°C, versus protein concentration as obtained for two distinct enzyme preparations (○ and ●). Scattered intensities are extrapolated to 0° angle and are normalized with respect to the scattered intensity of the reference glass of the FICA 50 photogoniometer. The least square fitted straight line has a slope of 10.84 ± 0.39 intensity units/mg of protein. From this value, a molecular weight of 730 000 ± 30 000 daltons can be calculated for yeast phosphofructo kinase, using a calibration factor of 67 000 ± 400 daltons per unit of scattered intensity/mg of protein (13). Note that no significant departure from linearity is observed, as an indication of a negligible contribution of the second virial coefficient.

Fig. 2 : Polyacrylamide gel electrophoresis of yeast phosphofructokinase in 0.1% sodium dodecyl sulfate. Experimental conditions : 4% acrylamide gels , 5 hrs. of migration at 8 mA/gel tube, 20 µg of protein were applied. Staining of the bands was achieved with Coomassie brilliant blue (6). According to the densitometric analysis shown on the right, the α, β, α' and β' bands are in the ratio 1 : 0.87 : 0.22 : 0.18 respectively. The amount of proteolysed material (α' and β' chains) was particularly high in this one week old enzyme preparation. For most enzyme preparations used in the present work, when electrophoresis was carried out immediately after the last purification step, the partially proteolysed material amounted to 5-10% of the total protein material.

prepared enzyme shows the typical migration pattern previously reported by Diezel et al. (1). A strong doublet corresponding to the native α and β chains is followed by a rather faint doublet which is formed by the partially proteolysed α' and β' chains (about 5-10% of the total protein). Evaluation of the molecular weight of these subunits with a series of cross-linked proteins as markers gives the values of 124 000, 113 000, 95 000 and 88 000 daltons for the α, β, α' and β' chains, respectively. These values were confirmed by electrophoresis

with uncrosslinked molecular weight markers in sodium dodecylsulfate and by gel filtration experiments on Sepharose CL 6B in 6 M guanidine.Thus, from these results, native yeast phosphofructokinase appears as an hexameric enzyme formed by the association of three α and three β chains (i.e $\alpha_3\beta_3$ structure, A. Chaffotte, F. Seydoux and J. Yon, manuscript in preparation).

BINDING OF 5'AMP AND ATP TO YEAST PHOSPHOFRUCTOKINASE.

The binding isotherm of 5'AMP, as shown in fig. 3a, is clearly monophasic, and a stoichiometry of three binding sites per oligomer can be determined with a dissociation constant of about 16 μM.

In contrast to the binding of 5'AMP, the binding isotherm of ATP to yeast phosphofructokinase is distinctly biphasic, as shown in fig. 3b. Accordingly, the binding data can be fitted to the following equation :

$$\text{ATP bound per enzyme (mol/mol)} = \bar{\nu} = \frac{n(ATP)_{free}}{K_1 + (ATP)_{free}} + \frac{n(ATP)_{free}}{K_2 + (ATP)_{free}}$$

which accounts for the binding of ATP to two distinct classes of equal number of sites. The number of sites in each class (n) was found close to three and the dissociation constants K_1 and K_2 which correspond respectively to the tight and loose binding sites, are in the ratio of 15 (i.e 2.3 and 36.5 μM respectively). Thus, yeast phosphofructokinase appears to contain two distinct classes of three binding sites each for ATP.

As illustrated in Table 1, these peculiar binding stoichiometries obtained by flow dialysis with 5'AMP and ATP were confirmed by gel filtration experiments using several different enzyme preparations. From these binding stoichiometries, it can be deduced that yeast phosphofructokinase contains only three regulatory sites per enzyme oligomer. In view of our previous kinetic results, only the ATP molecules bound to the regulatory sites should be significantly displaced by 5'AMP. This has been tested in a flow dialysis experiment where a small substoichiometric amount of bound ATP was displaced by an excess of unlabelled 5'AMP. As depicted in fig. 4, this experiment shows that 5'AMP rapidly displaces tightly bound ATP but that an appreciable fraction of ATP remains boundto the enzyme even at high 5'AMP concentrations. This behavior can be quantitatively described by assuming that 5'AMP displaces more efficiently the ATP molecules bound to the three tight sites of the enzyme hexamer. Thus, this experiment allows us to identify the first class of ATP binding sites as regulatory sites and the second class of sites as the catalytic sites. In agreement with this interpretation, the dissociation constant for the second class of sites (36.5 μM) compares well with the K_m value for ATP (31 μM) as determined kinetically for the catalytic reaction (8) under similar conditions.

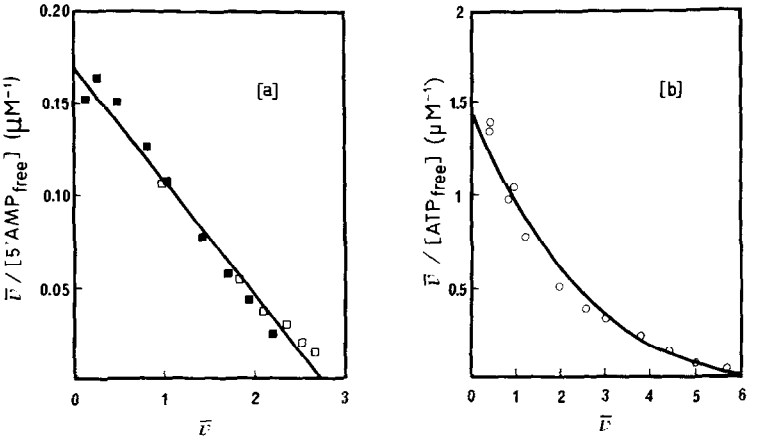


Fig.3 : Binding isotherm of 5'AMP and ATP to yeast phosphofructokinase as determined in flow dialysis experiments. Scatchard plot of the data $\bar{\nu}$ corresponds to the apparent number of bound ligand molecules per enzyme oligomer.
(a) Binding isotherm of 5'AMP. □ : 5.2 µM enzyme. ■ : 12.6 µM enzyme The solid line is calculated for a single class of 2.75 sites per enzyme oligomer with a dissociation constant of 16.2 µM.
(b) Binding isotherm of ATP : 3.9 µM enzyme. Solid line is calculated from equation 1 with K_1 = 2.35 µM, K_2 = 36.6 µM and n = 3.07.

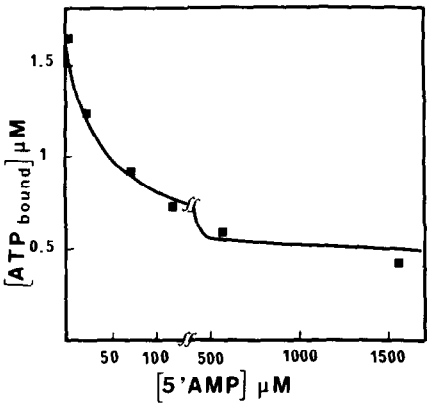


Fig. 4 : Displacement of ATP by 5'AMP from yeast phosphofructokinase as measured by flow dialysis. Experimental conditions : 4 µM enzyme, 1.93 µ M (U-^{14}C)ATP. The solid curve is calculated by assuming a competitive displacement of ATP by 5'AMP from the regulatory tight binding sites as discussed in the text. The corresponding equation used in that case is obtained by multiplying the dissociation constant K_1 in equation 1 by (1 + AMP/K), K (=9 µM) being the dissociation constant for 5'AMP ; other parameters values as given in fig. 3b.

TABLE 1

Comparison of the data obtained by gel filtration and flow dialysis for the binding of ATP and 5'AMP to yeast phosphofructokinase. Apparent numbers of bound ligand molecules per enzyme oligomer ($\bar{\nu}$) measured in gel filtration experiments were obtained with 3 distinct enzyme preparations.

Ligand	Concentration[a] (µM)	$\bar{\nu}$	
		gel filtration	flow dialysis (b)
ATP	9.30	3.02 ± 0.37	3.05
ATP	47.5	4.95 ± 0.40	4.62
5'AMP	69.3	2.45 ± 0.20	2.23

[a] free ligand concentration.

[b] These values were interpolated from the data of fig. 3.

DISCUSSION

Although the present data are consistent with an hexameric $\alpha_3\beta_3$ structure, the essential qualitative conclusion of the present work is that yeast phosphofructokinase contains a number of regulatory ATP and 5'AMP binding sites which is equal to only half the number of subunits constituting the enzyme oligomer. From the present data, we cannot exclude the possibility that one type of subunits contains both regulatory and catalytic sites, but the most plausible interpretation in view of the $\alpha_3\beta_3$ structure of yeast phosphofructokinase is to assume that the enzyme oligomer is composed of three regulatory and three catalytic subunits. Therefore, each regulatory subunit should contain one regulatory site for ATP or 5'AMP and each catalytic subunit contains only one binding site for ATP as phosphate donnor. In this sense, the enzyme oligomer should be considered as a trimer of asymmetrical dimeric α β units. This interpretation is supported by our previous kinetic results showing that the cooperativity of yeast phosphofructokinase with respect to fructose 6-phosphate was best explained on the basis of three interacting protomers per enzyme oligomer (8). Although this tentative interpretation should be substantiated by additional evidence at both structural and functional levels, yeast phosphofructokinase may constitute a new example

of a highly specialized allosteric enzyme with structurally and functionally distinct subunits, as already described for the enzyme aspartate transcarbamylase from E. coli (14).

ACKNOWLEDGMENTS

We are indebted to Professor J. Yon for her interest to this work. We are very grateful to Dr. A. d'Albis for her generous gift of cross-linked proteins and to Dr. Pantaloni for his assistance and advices concerning light scattering measurements. We acknowledge Dr. Thusius and M.J. Lavorel for careful reading of this manuscript.

This work was supported by grants from the Centre National de la Recherche Scientifique (G.R. n° 13).

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