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Conformational modification of muscle phosphofructokinase from *Jaculus orientalis* upon ligand binding

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Phosphofructokinase from *Jaculus orientalis* muscle is an allosteric enzyme regulated by substrates and nucleotide effectors. The conformational modifications upon ligand binding were probed by UV difference spectra and reactivities of thiol groups towards dithiobisnitrobenzoate and *N*-ethylmaleimide. The binding of Fru-6-P induced significant perturbations in the environment of the aromatic residues and buried the most reactive on the three accessible cysteines per protomer. The same effect on thiol reactivity was observed upon binding of the activator AMP. Various perturbations of both difference spectra and thiol reactivity were detected in the presence of either Mg-ATP, an allosteric inhibitor, or Mg-ITP which is not an effector.

Phosphofructokinase, 6-; Allostery; Conformational change

1. INTRODUCTION

Phosphofructokinase is a key enzyme of the glycolytic pathway and is regulated by a variety of metabolites depending on the source [1–6]. It is self-regulated through an allosteric control which has been interpreted in terms of conformational equilibria, irrespective of whether a concerted [7] or sequenced [8] model is considered. In some cases, however, the transition has been found to be too complex to be interpreted by one of these models [9,10]. These regulatory properties are pH-dependent [11]. *Jaculus orientalis* is a hibernating rodent from the Moroccan highlands [12,13]. Muscle phosphofructokinase from this rodent has been studied in order to elucidate the role of glycolysis in the phenomenon of hibernation. The enzyme is a tetramer of 340 kDa, comprising four identical protomers. It is an allosteric enzyme showing cooperativity with respect to saturation by fructose 6-phosphate, at neutral pH. AMP is an allosteric activator while the substrate Mg-ATP is an allosteric inhibitor at higher concentrations [14,15]. Here, the effect of ligands on the conformational properties of this enzyme was studied, using variations in the environment of the chromophoric residues and the chemical reactivities of cysteines as conformational probes.

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Abbreviations: PFK, phosphofructokinase (EC 2.7.1.11); Fru-6-P, fructose 6-phosphate; DTT, dithiothreitol; NbS_2, dithiobisnitrobenzoate; NbS, thionitrobenzoate; Mal-NEt, *N*-ethylmaleimide

2. MATERIALS AND METHODS

2.1. *Chemicals*

Nucleotides and Fru-6-P were from Sigma. NbS_2 and non-radioactive Mal-NEt were Aldrich products. *N*-[*ethyl*-2-^{3}H]-Ethylmaleimide (600 mCi/mmol) was purchased from New England Nuclear. Urea (Merck) was recrystallized twice from ethanol. Coupling enzymes, i.e. aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase, were obtained from Boehringer.

2.2. *Enzyme*

Phosphofructokinase from the skeletal muscle of *J. orientalis*

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was purified as described previously (El Hachimi et al., submitted). The enzyme was stored at 0°C in suspension in 4 M ammonium sulfate in the presence of 1 mM Mg-ATP. Before use, the enzyme was desalted and dissolved in 25 mM imidazole buffer (pH 7) containing 1 mM EDTA, 10 mM $MgCl_2$, and 30 mM KCl and kept on ice. Protein concentrations were evaluated according to Bradford [16]. Enzyme activities were determined by coupled assay as in [17].

2.3. *UV difference spectra*

Difference spectra were recorded from 240 to 340 nm using a Cary 219 spectrophotometer. PFK (2.1 μM final concentration) was dissolved in 25 mM Pipes buffer (pH 7), containing 2 mM EDTA, 10 mM KCl and 2 mM 2-mercaptoethanol. Quartz tandem cells (Ellma) with a light path of 0.437 cm were employed. Compartments 1,2 were placed in the sample beam, and 3,4 in the reference beam. Compartments 2,4 contained the enzyme solution in Pipes buffer (pH 7), and 1,3 the reference solutions. Temperature was kept constant at 25°C. 5-μl aliquots of ligands were added sequentially to compartments 2,3, while identical volumes of buffer were added to compartments 1,4.

2.4. *Thiol titration with NbS_2*

The titration with NbS_2 of the accessible cysteine residues of PFK in the presence and absence of ligands was according to Ellman [18] under conditions of pseudo-first-order kinetics (NbS_2, 200 μM; PFK, 1 μM; final concentrations). The reagent was freshly prepared daily by dissolution in imidazole buffer and kept on ice. Its concentration was determined by titration with excess DTT. The increase in absorbance was measured at 412 nm using a Varian 634 spectrophotometer at 25°C (ϵ_{NbS} = 13600 $M^{-1} \cdot cm^{-1}$). The progress curves were analyzed by one or two exponential terms using a non-linear regression program adapted to a Hewlett-Packard 9816. For enzyme activity

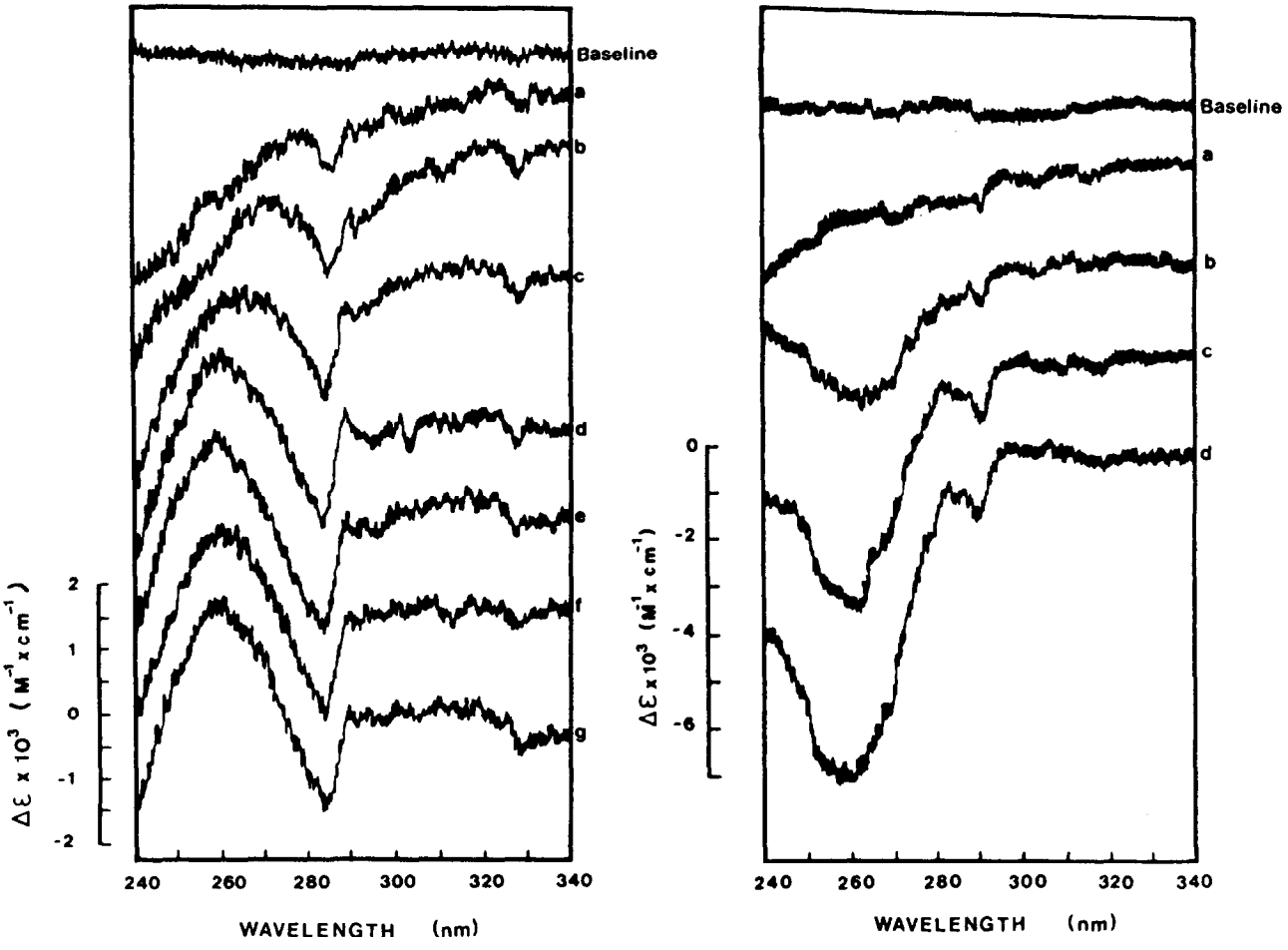

Fig.1. Difference spectra of PFK from *J. orientalis* induced by ligands at various concentrations; at pH 7 and 25°C (see text). (Left) Fru-6-P at: (a) 13, (b) 27, (c) 55, (d) 110, (e) 220, (f) 486, (g) 2065 μM. (Right) Mg-ATP at: (a) 6, (b) 11, (c) 44, (d) 75 μM. To clarify the presentation, all difference spectra are arbitrarily shifted on the ordinate axis. The scale is given on the (g) spectrum (left) and on the (d) spectrum (right).

measurements, the reaction was previously stopped by dilution of the reaction mixture in 10 vols ice-cold imidazole buffer [19].

2.5. *Titration of thiols with Mal-NEt*

The reaction of PFK with Mal-NEt was monitored at 25°C in imidazole buffer (pH 7). The kinetics of incorporation of radioactive Mal-NEt (900 μM final concentration) in PFK (1 μM) were measured routinely as in [20]. For enzyme activity measurements, the reaction was stopped by addition of excess DTT (90 mM).

3. RESULTS

3.1. *Difference spectra of PFK in the presence of ligands*

The binding of Fru-6-P to *J. orientalis* PFK induced significant difference spectra as shown in fig.1 (left), with a negative peak centered at 288 nm and a positive one at 275 nm. Their amplitudes increased with Fru-6-P concentration. These spectral variations are characteristic of perturbations in the environment of the aromatic groups resulting from conformational changes upon substrate binding. The difference spectra in the presence of Mg-ATP were clearly distinct from those induced by Fru-6-P with a negative peak at 290 nm corresponding to a perturbation in the environment of tryptophans (fig.2, right). However, the absorption of the adenine prevented further information being obtained. Nevertheless, these data indicated a conformational change of the enzyme induced by Fru-6-P and a different one by Mg-ATP.

3.2. *Effect of ligands on the accessibility of cysteine residues*

In PFK from *J. orientalis*, previously denatured in 8 M urea, 32 ± 2 thiol groups per oligomer were titrated with NbS_2. In the native enzyme 4 ± 1 cysteines (class 1) reacted very rapidly with both NbS_2 and Mal-NEt, as deduced from the progress curves (figs 2,3). These residues become inaccessible in the presence of either Fru-6-P or AMP which favor the R state of the enzyme [14,15]. They remained titratable in the presence of Mg-ATP or Mg-ITP. Another class of cysteine residues (class II) reacting at a slower rate were also titrated, 6 ± 0.5 being found to be accessible to NbS_2 and 8 ± 1 to Mal-NEt. In the presence of either Fru-6-P or AMP, four of them became buried as probed by both reagents, and in the presence of Mg-ATP or Mg-ITP no cysteine of class II was titratable. A third class of very slowly reacting cysteines was not significantly affected by the presence of Fru-6-P or AMP, as summarized in table 1, however their rate constant was decreased by a factor of 10 in the presence of Mg-ATP or Mg-ITP. Enzyme activity rapidly decreased with chemical modification as shown in fig.4. Fru-6-P protected the enzyme against inactivation by the chemical reagents; this protection was only partial in the presence of AMP. Mg-ATP and Mg-ITP did not significantly prevent the inactivation of the enzyme.

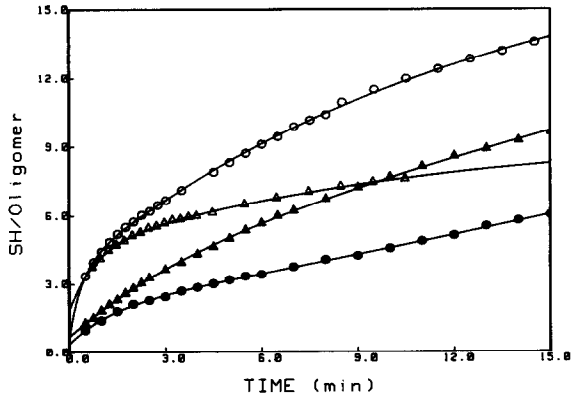

Fig.2. Chemical modification of cysteine residues in PFK by NbS_2 at pH 7 and 25°C (see text). Progress curves: (○) in the absence of ligands, (●) in the presence of 2 mM Fru-6-P, (▲) 2 mM AMP, (△) 2 mM Mg-ATP. Solid lines were fitted according to the equation: $n_{SH(t)} = n_1 e^{-k_1 t} + n_2 e^{-k_2 t} + kt$.

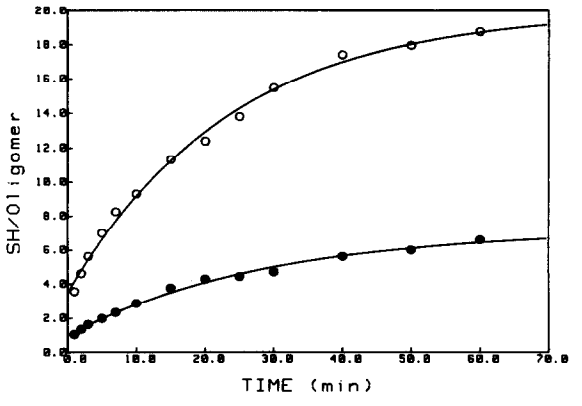

Fig.3. Chemical modification of cysteine residues in PFK by Mal-NEt at pH 7 and 25°C (see text). Progress curves: (○) in the absence of ligands; (●) in the presence of 2 mM Fru-6-P. Solid lines were fitted as in fig.2.

Table 1

Effect of ligands on the accessibility of cysteine residues in *Jaculus orientalis* phosphofructokinase

Ligand	No. of cysteine residues							
	Class I (very fast) (n_1)		Class II (slower) (n_2)		Class III (very slow) (k^a)		% residual activity	
	NbS_2	Mal-NEt	NbS_2	Mal-NEt	NbS_2	Mal-NEt	NbS_2	Mal-NEt
None	4 ± 1	4 ± 0.5	6 ± 0.5 k_2 = 0.08	8 ± 0.5 k_2 = 0.06	0.34	0.06	0	0
Fru-6-P (2 mM)	0	0	2 ± 0.5 k_2 = 0.5	4 ± 0.5 k_2 = 0.06	0.29	0.013	90	50
AMP (2 mM)	0	0	4 ± 0.5 k_2 = 0.24		0.37		14	
Mg-ATP (2 mM)	4 ± 1		0		0.03		7	
Mg-ITP (2 mM)	4 ± 1		0		0.03		2	

The numbers of cysteines in the different classes were evaluated from the progress curves according to the equation:

$$n_{SH(t)} = n_1 e^{-k_1 t} + n_2 e^{-k_2 t} + kt,$$

n_1 being the number of very rapid cysteines (class I), and n_2 the number of cysteines from class II; k_1 was too fast to be evaluated; k_2, the pseudo-first-order constant, is given in min^{-1}; [a] k the rate constant of the cysteines from class III is given in modified cysteines per min

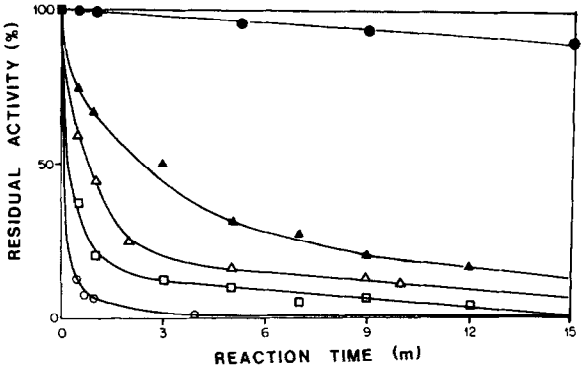

Fig.4. Percentage of residual activity of PFK during modification by NbS_2: (○) in the absence of ligands, (●) in the presence of 2 mM Fru-6-P, (▲) 2 mM AMP, (△) 2 mM Mg-ATP, (□) 2 mM Mg-ITP.

4. DISCUSSION

Both conformational intrinsic probes, difference spectra and reactivity of cysteine residues, indicated that PFK from *J. orientalis* underwent conformational changes upon substrate and effector binding. The binding of Fru-6-P produced significant perturbations in the environment of the aromatic residues. Furthermore, one rapidly reacting cysteine per protomer and another with a lower rate constant became buried in the reagents NbS_2 and Mal-NEt in the presence of either Fru-6-P or AMP which shift the allosteric equilibrium to the 'R' state. Kemp and co-workers [21,22] have reported the existence, in rabbit muscle PFK, of one highly reactive cysteine which acts as a label of the conformational state of the enzyme; however, in contrast with the present results, the reactivity of this group was enhanced by Fru-6-P and decreased by Mg-ATP. Furthermore, with *J. orientalis* PFK, a quite complete protection against the chemical reaction was produced by the substrate Fru-6-P; this protection was only partial in the presence of AMP. In the presence of Mg-ATP, the allosteric inhibitor, or of Mg-ITP which does not play any regulatory role [23], the rapid cysteine residues remained accessible to NbS_2 as in the unliganded enzyme, but the slower groups (class II) became buried. Moreover, Mg-ATP induced a small but significant difference spectrum

in the tryptophan region. A possible explanation might be that in the absence of ligands the enzyme is close to the 'T' state which has a higher affinity for Mg-ATP, the 2 ± 0.5 cysteine residues per protomer protected by Mg-ATP being located near its binding site. An alternative and more plausible model could be assumed by analogy with the allosteric regulation of yeast PFK [10]. This model involves concerted conformational changes upon binding of Fru-6-P and sequential ones induced by the adenylates. Such a model could account for the partial protection of the *J. orientalis* enzyme by AMP and the conformational effects of Mg-ATP; indeed, in a fully concerted model, the same effects are expected for Fru-6-P and AMP.

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