Fructose-6-phosphate modifies the pathway of the urea-induced dissociation of the allosteric phosphofructokinase from *Escherichia coli*

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Phosphofructokinase from *Escherichia coli* binds fructose-6-phosphate with the sugar moiety of the substrate interacting with one subunit and the phosphate group with another one, so that bound fructose-6-phosphate lies across the interface between the subunits [(1988) J. Mol. Biol. 204, 973–994]. When this interface is 'cross-linked' by fructose-6-phosphate, it becomes more stable because of the extra interactions between subunits. Inactivation upon dissociation occurs only above 5 M urea, instead of 1 M urea for the free protein. At saturation in fructose-6-phosphate, this interface is no longer the first to dissociate as in the free protein [(1989) Biochemistry 28, 6836–6841]; instead, the addition of urea to phosphofructokinase in the presence of fructose-6-phosphate induces a conformational change within the tetramer which alters the environment of Trp-311 and distorts the regulatory site.

Subunit interaction; Ligand-induced stabilization; Urea; Phosphofructokinase; *Escherichia coli*

1. INTRODUCTION

Phosphofructokinase (PFK) from *Escherichia coli* is a tetrameric enzyme composed of four identical chains [1] arranged as a dimer of dimers with a D_2 symmetry [2] in which the subunits are paired along two different interfaces: the binding site for allosteric effectors belongs to one interface, the R(egulatory) interface, and the binding site for the substrate fructose-6-phosphate (Fru-6-P) belongs to the other, the A(ctive) interface [3,4]. It has recently been shown that dissociation of PFK by various agents is an ordered process in which the A interface is broken before the R interface [5–7]. In tetrameric PFK, the A interface is therefore less stable than the R interface. This lower stability is in agreement with a smaller area of contact between subunits, about 1200 $Å^2$ for the A interface vs 1800 $Å^2$ for the R interface [4].

The substrate Fru-6-P markedly protects PFK against dissociation by guanidine hydrochloride [7], KSCN [5], urea [6], or against thermal inactivation [8,9]. X-Ray crystallography has shown that Fru-6-P is bound across the A interface with the sugar moiety held by one subunit and the phosphate group interacting with two positively charged residues, Arg-162 and -243, of the other subunit [4]. These two subunits are thus non-covalently cross-linked by Fru-6-P, and these extra-ionic interactions increase the stability of subunit interactions. The present work shows that upon binding of Fru-6-P, the A interface becomes more stable than the R interface against the urea-induced dissociation of PFK.

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Abbreviations: PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11)); HPLC, high-performance liquid chromatography; Fru-6-P, D-fructose-6-phosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane

2. MATERIALS AND METHODS

The origin of the chemicals, the preparation of PFK, the measurements of enzymatic activity, fluorescence intensities, and the HPLC gel filtration methods have been described previously [5–7]. Experimental conditions are given in the legend to Fig. 1.

3. RESULTS AND DISCUSSION

The dissociation and/or denaturation of PFK by urea can be measured by either the loss of activity or the change in the fluorescence of the unique tryptophan residue, Trp-311 [7]. Reactivation of PFK after dissociation is a slow process [5–7,10]. No reactivation of PFK occurs during the time needed for the enzymatic assay, so that the measured activity corresponds to the conformational state of PFK in urea. Under the conditions used here, both the activity and native fluorescence can be fully recovered after 24 h in urea upon dilution of the denaturant [6], which shows that PFK has not been damaged by reaction with cyanate ions. Fig. 1 shows the transition curve obtained

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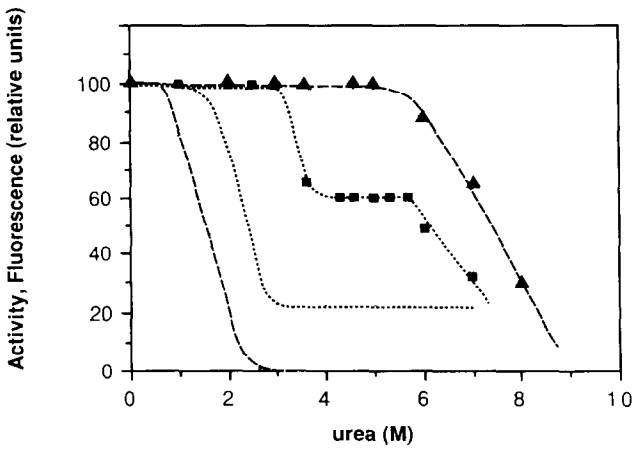

Fig. 1. Influence of urea on the activity and fluorescence of PFK in the presence of Fru-6-P. PFK at a concentration of 520 nM in subunits has been incubated for 24 h at 20°C in a buffer composed of 100 mM sodium phosphate, 2 mM dithiothreitol, 1 mM magnesium acetate, at pH 7.6, together with urea at the indicated concentration and 2 mM Fru-6-P. The fluorescence intensity (■···■) is measured in the same buffer. Activity (▲---▲) is determined from the change in absorption at 340 nm immediately after diluting PFK and urea at least 50-fold in the standard assay buffer composed of 100 mM Tris-HCl, 10 mM magnesium acetate, 1 mM ATP, 1 mM Fru-6-P, 0.2 mM NADH, and the auxiliary enzymes aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase. The concentration of residual urea was always below 0.2 M, and it was checked not to interfere with the assay. Activity is expressed relative to native PFK; the fluorescence change is normalized using a value of 100 for native PFK and of 0 for PFK in 6 M urea. Also shown are the changes in fluorescence (···) and in activity (– – –) reported previously for free PFK in urea [6].

after incubating native PFK for 24 h at 20°C in the presence of various urea concentrations and 2 mM Fru-6-P, as measured by changes in protein fluorescence and activity. For concentrations up to 3 M urea, the properties of PFK are not modified indicating that the protein remains native. A conformational change takes place around 3.5 M urea as seen from the 40% decrease in protein fluorescence, but this conformational change does not alter the activity of PFK. Above 5.5 M urea, the parallel decreases in activity and fluorescence suggest that the protein dissociates into monomers. The changes in activity and fluorescence previously obtained with free PFK are also shown in Fig. 1. In the absence of Fru-6-P, the loss of activity of PFK begins at a lower concentration of urea, around 1 M, than the decrease of its fluorescence which occurs at about 2 M urea [6]. This order is reversed in the presence of Fru-6-P, with the loss of activity occurring at a higher urea concentration than the change in fluorescence (Fig. 1).

After 24 h in urea, between 3.5 and 5.5 M, and in the presence of 2 mM Fru-6-P, the fluorescence of PFK shows that the protein is in a conformational state (called state I) in which the environment of Trp-311 is modified. In this state I, PFK has retained a tetrameric structure because its elution volume from a calibrated size-exclusion HPLC column is the same as that of the native protein [6]. No lag phase is observed when the enzymatic activity of state I is measured immediately after dilution of the denaturant. This shows that state I does not need to reassociate to be active and is thus tetrameric. State I possesses an active site in the A interface, which seems to retain a functional conformation. The fluorescence change observed for PFK upon raising the urea concentration from 0 to 5 M is complete within minutes, indicating that state I is formed rather rapidly from native PFK.

The activity measured for the I state after removal of urea is still influenced by the presence of allosteric effectors: it is activated by GDP (Fig. 2A) and inhibited by PEP (Fig. 2B). This confirms that the quaternary structure of state I is still that of native PFK, because only a tetrameric species can be both active and allosterically regulated [3]. When activity is measured immediately after diluting urea out of state I, slightly altered regulatory properties are observed (Fig. 2). The finding that state I is less sensitive to activation or inhibition than the native state of PFK (Fig. 2) suggests that the effector binding site could be somewhat distorted. The conversion from native PFK into state I modifies the conformation of the R interface which contains both Trp-311 and the effector binding site [4].

In the presence of Fru-6-P, the first change in PFK observed upon raising the concentration of urea above 3 M is no longer the dissociation of the A interface, as it is for the free protein [6]. Instead, a conformational change within the tetrameric structure leads to state I, in which a distortion of the R interface affects both the environment of Trp-311 and the effector site. Above 5.5 M urea, the tetrameric structure preserved in state I is no longer stable and 'explodes': an all-or-none transition leads to the monomeric and largely unfolded species described earlier [6].

Between 2 M and 3 M urea, unliganded PFK is not stable for 24 h in the absence of Fru-6-P, while it becomes stable when saturated by Fru-6-P (Fig. 1). It is therefore expected that the addition of an excess Fru-6-P to PFK in the presence of urea will reverse (at least in part) the structural changes leading to inactivation and cause some renaturation, such as was observed in the case of KSCN-inactivated PFK [5]. PFK was first incubated in various concentrations of urea for 24 h, then 2.5 mM Fru-6-P was added without removing the denaturant. Reactivation was allowed to proceed for another 24 h before the activity is assayed. Reappearance of activity is indeed induced by the addition of Fru-6-P to PFK in urea: complete reactivation occurs between 0.8 M and 1.8 M urea, partial reactivation between 1.8 M and 2.8 M urea, and no reactivation above 3 M urea (results not shown). In addition, HPLC measurements show that the extent of reactivation of PFK upon addition of 2.5 mM Fru-6-P

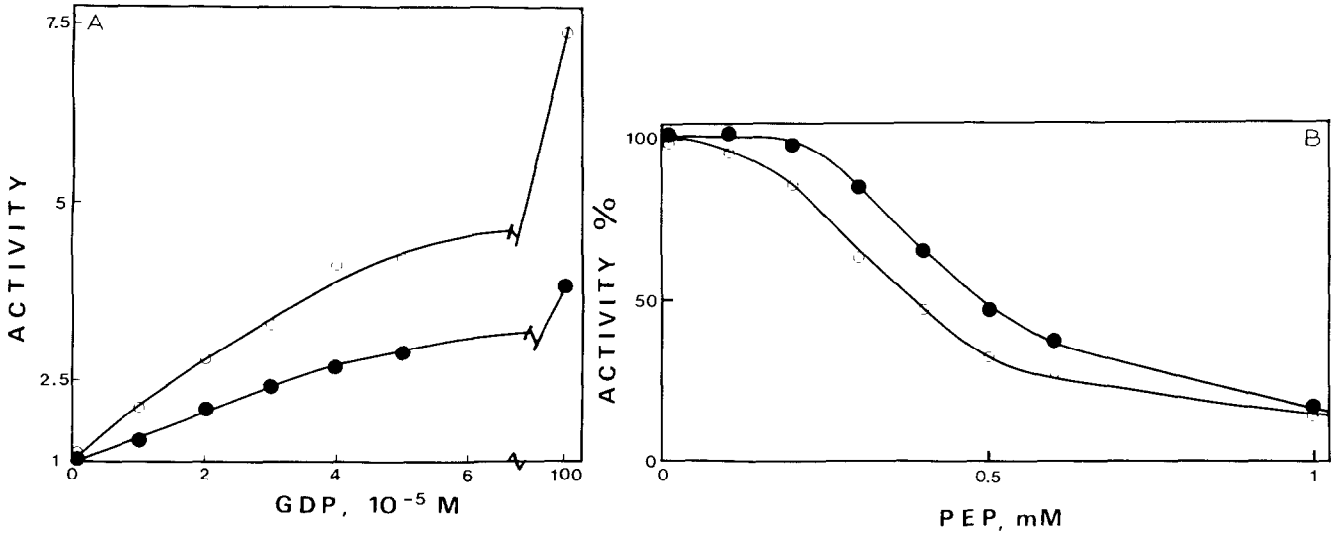

Fig. 2. The I state of PFK has altered regulatory properties. The activity of PFK is measured immediately after dilution as described in the legend to Fig. 1, using substrate concentrations of 1 mM ATP and of either 0.3 mM Fru-6-P for measuring the activation by GDP (Fig. 2A), or 1 mM Fru-6-P for measuring the inhibition by PEP (Fig. 2B). (●) PFK incubated for 24 h in 5 M urea under the conditions given in the legend to Fig. 1; (○) native PFK. Activities are expressed relative to the values in absence of effector.

in urea is related to the amount of tetrameric species formed. These results indicate that the complex between PFK and Fru-6-P is thermodynamically more stable than free PFK.

In the absence of Fru-6-P, the urea-induced denaturation of PFK is an ordered two-step process, where dissociation of the A interface produces a dimeric intermediate which is inactive and still has a native fluorescence [6]. In the presence of an excess of Fru-6-P, the A interface becomes markedly more resistant to urea and does not dissociate; instead, state I is formed first, with the R interface distorted, albeit not dissociated. No dimeric intermediate can be detected, showing that, when Fru-6-P is bound, the dissociation of PFK obeys an all-or-none mechanism, from tetramer to monomer. When non-covalently cross-linked by Fru-6-P, the A interface is at least as stable as the R interface, and the urea concentration needed for its half-dissociation increases from below 1 M urea [6] to 6 M or above (Fig. 1). This effect of Fru-6-P on the dissociation of PFK therefore suggests that large changes in the strength of the interaction between two proteins and/or in the stability of an oligomeric protein could be achieved by only a few amino acid substitutions. This could be of interest for further studies of thermostability or halophily, either natural or engineered, of multichain proteins.

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REFERENCES

[1] Hellinga, H.W. and Evans, P.R. (1985) Eur. J. Biochem. 149, 363–373.
[2] Evans, P.R. and Hudson, P.J. (1979) Nature 279, 500–504.
[3] Evans, P.R., Farrants, W.G. and Hudson, P.J. (1981) Phil. Trans. Roy. Soc. Lond. B293, 53–62.
[4] Shirakihara, Y. and Evans, P.R. (1988) J. Mol. Biol. 204, 973–994.
[5] Deville-Bonne, D., Le Bras, G., Teschner, W. and Garel, J.-R. (1989) Biochemistry 28, 1917–1922.
[6] Le Bras, G., Teschner, W., Deville-Bonne, D. and Garel, J.-R. (1989) Biochemistry 28, 6836–6841.
[7] Teschner, W. and Garel, J.-R. (1989) Biochemistry 28, 1912–1916.
[8] Blangy, D. (1971) Biochimie 53, 139–149.
[9] Le Bras, G. and Garel, J.-R. (1982) Biochemistry 21, 6656–6660.
[10] Martel, A. and Garel, J.-R. (1984) J. Biol. Chem. 259, 4917–4921.