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Ligand-dependent structural changes and limited proteolysis of *Escherichia coli* phosphofructokinase-2☆

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Abstract

Binding of MgATP to the allosteric site of phosphofructokinase-2 promotes a dimer to tetramer conversion. In the presence of Fru-6-P the enzyme remains as a dimer. Limited proteolysis in the presence of MgATP completely protects the enzyme against inactivation and cleavage, while Fru-6-P provides a partial protection. A 28-kDa proteolytic fragment containing the N-terminus of the protein is inactive, but retains the ability to bind Fru-6-P and the allosteric effector MgATP. The fragment remains as a dimer but does not form a tetramer in the presence of MgATP. The results suggest major conformational changes of the enzyme upon ligand binding that confer a higher degree of compactness to the monomers in the dimer and in the tetramer, demonstrate the presence of the active and allosteric sites in this N-terminus fragment, and stress the importance of the C-terminus region of the protein for catalytic activity and ligand-induced oligomerization.

Keywords: Phosphofructokinase; Subunit association; Limited proteolysis; Ligand binding; Intrinsic fluorescence

ATP:fructose-6-phosphate 1-phosphotransferase (fructose-6-phosphate 1-kinase (PFK),[1] EC 2.7.1.11) catalyzes the phosphorylation of Fru-6-P to fructose-1, 6-bisP and is believed to play an important role in the regulation of glycolysis, mainly due to the various physiological effectors that regulate the activity of the enzyme. The wild-type strain of *Escherichia coli* presents two forms of the enzyme. The main isoenzyme, Pfk-1, exhibits sigmoidal kinetics with respect to Fru-6-P, allosteric activation by ADP or GDP, and allosteric inhibition by phosphoenolpyruvate [1,2]. Pfk-2, the minor isoenzyme, presents hyperbolic kinetics with Fru-6-P and inhibition by MgATP when the assay is performed at low Fru-6-P concentration [3]. Previous studies from our laboratory have revealed, through kinetic and intrinsic fluorescence studies, that this inhibition is the result of binding of the nucleotide to an allosteric site of the enzyme rather than to its substrate site [3,4]. The interaction of MgATP cannot take place at the active site in the absence of Fru-6-P, due the ordered Bi–Bi kinetic mechanism [5], with Fru-6-P being the first substrate to bind and fructose-1,6-bisP the last product to be released from the enzyme. Titration of Pfk-2 with MgATP in the absence of Fru-6-P causes a quenching of fluorescence with a cooperative response. On the other hand, addition of compounds that bind to the catalytic site of Pfk-2, such as ATP^{4-} or AMP-PNP, did not produce significant changes in the fluorescence spectrum of the free enzyme [4]. However, addition of ATP^{4-} to the enzyme-Fru-6-P complex shows a hyperbolic increase of fluorescence. This allosteric site has also been linked to a dimer-tetramer association process [6,7].

The primary structure of Pfk-2, as predicted from the nucleotide sequence, shows no significant relationship to the Pfk-1 family, called PfkA. Instead, Pfk-2 belongs to

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[1] *Abbreviations used:* Pfk-1, phosphofructokinase-1; Pfk-2, phosphofructokinase-2; PFK, generic phosphofructokinase; DTT, dithiotreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); HPLC, high-performance liquid chromatography.

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the PfkB family, which includes several kinases such as fructose-1-phosphate kinase, adenosine kinase, tagatose kinase, ribose kinase, and inosine kinase [8,9]. Since no structural information is available for Pfk-2 and in order to develop a more detailed understanding of its structure/function relationship, the conformational changes induced by ligands were evaluated by size-exclusion chromatography, dynamic light-scattering, and limited proteolysis studies. The results show conformational changes induced by binding of the substrate Fru-6-P, and the allosteric effector, which involve changes in the aggregation state of the enzyme and protection of the proteolytic cleavage sites of proteases with different specificities. Although the N-terminal fragment of 28 kDa is inactive, it retains the ability to bind Fru-6-P and the allosteric effector. The results also emphasize the importance of the C-terminal region of the protein for the catalytic activity of the enzyme and for the MgATP-induced oligomerization process.

Experimental procedures

Pfk-2 purification. The strain used for enzyme purification was DF 903, for which construction and growth have been described [10]. Essentially, Pfk-2 was purified as reported before [1].

Enzyme activity. Activity was measured spectrophotometrically by coupling the fructose-1,6-bisP formation to the oxidation of NADH with the use of aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase [3].

Reagent concentrations. Fru-6-P concentration was estimated spectrophotometrically using the aldolase-coupling assay. The concentration of NADH was calculated using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\,cm^{-1}$. ATP concentrations were determined spectrophotometrically by the fructose-1,6-bisP formed in the presence of PFK, Fru-6-P, aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase, and NADH. Protein concentration was determined using the Bio-Rad protein assay with the standard curve constructed with bovine serum albumin [11].

Dynamic light-scattering measurements. Studies were carried out at 20 ± 0.1 °C using a DynaPro-MS800 dynamic light-scattering instrument from Protein Solutions, Inc. Samples containing purified Pfk-2 (1 mg/ml) in 25 mM Tris–HCl buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and the indicated concentrations of MgATP were centrifuged for 10 min at 14,000 rpm in an Eppendorf microfuge to remove particulate matter. In dynamic light-scattering experiments, the hydrodynamic radius of gyration of the particle is calculated via the Stokes–Einstein equation from the diffusion coefficient, which is obtained from the measured autocorrelation function [12], using the DYNAMICS Software supplied with the instrument.

Limited proteolysis. To study the kinetics of proteolysis, solutions of Pfk-2 containing 0.2 or 1 mg/ml of protein, 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT, and the indicated concentrations of substrates and effectors were incubated at 37 °C. The proteolytic enzyme was added at zero time in a small volume. The ratio of protease:Pfk-2 was 1:100 in the case of trypsin, 1:150 for proteinase K, and 1:300 for subtilisin. At different times, aliquots (0.1 μg) were removed for activity measurements and diluted 10-fold in the presence of soybean trypsin inhibitor (2:1 with respect to protease) in the case of trypsin, 2 mM phenylmethanesulfonyl fluoride for proteinase K, and directly in the assay mixture for subtilisin. At the same time points, aliquots (2 μg) were removed for electrophoretic analysis and diluted 2-fold in 75 mM Tris–HCl, pH 6.8, 10% glycerol, 2.5% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue. SDS–PAGE was performed essentially as described by Laemmli [13] with running gels containing either 7.5 or 11.5% acrylamide at pH 8.8. Densitometric analyses of the electrophoretic images were performed using a commercial scan and quantified using the Sigma Scan program (Jandel Scientific, U.S.A.).

Purification of the 28-kDa fragment. Pfk-2 (1 mg/ml) was digested with subtilisin as indicated above in the presence of 1 mM Fru-6-P at 37 °C for 60 min. After digestion the sample (4 ml) was separated by HPLC in a Mono Q HR 5/5 column with a linear gradient of 250–550 mM NaCl in 25 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, at a flow rate of 1 ml/min. Under these conditions the elution time of the 28-kDa fragment is 8.9 min. After elution, the fragment was subjected to SDS–polyacrylamide gel electrophoresis using the Laemmli system [13] in order to evaluate the purity of the sample.

Fluorescence measurements. Fluorescence measurements were performed with a Perkin–Elmer LS 50 fluorometer. The excitation wavelength was set to 295 nm to limit fluorescence to tryptophan residues. Emission spectra from 305 to 500 nm were collected and the excitation and emission slits were set to 5 nm. Spectroscopic measurements were collected at room temperature and titration experiments were performed by adding small aliquots of stock solutions of substrate or effector to the enzyme solution. Corrections were made to compensate for protein dilution. The experiments were performed in 20 mM Hepes, pH 8.2, 5 mM $MgCl_2$, and 1 mM DTT and the concentration of protein was approximately 40–50 μg/ml. Data analysis was carried out with the Grams 386 program.

The fractional saturation binding by either ligand was determined from the intensity variation with free ligand concentration by calculating the quantity $(F^0 - F)/(F^0 - F^\infty)$, where F^0 represents the emission intensity in the absence of ligand, F^∞ is the emission intensity at saturating concentration of ligand, and F is the intensity after the addition of a given concentration of ligand.

Size-exclusion chromatography. Isocratic size-exclusion chromatography of native Pfk-2 was performed in a BIO SI SEC 250-5 column (Bio-Rad, U.S.A.). The column was equilibrated and eluted with 20 mM Tris–HCl, pH 7.0, 5 mM $MgCl_2$, 150 mM KCl, and 10 mM β-mercaptoethanol, with or without the indicated concentration of ligands. The samples were preincubated at the ligand concentration present in the equilibrium buffer, prior to injection of a 30-μl protein aliquot of 1 mg/ml. Chromatography of the purified subtilisin-digested Pfk-2 was performed under the same conditions in the absence and in the presence of 0.4 mM MgATP, at room temperature and at a flow rate of 1 ml/min. The absorbance of the column effluent was continuously monitored at 280 nm with an on-line Beckman UV detector. Data were analyzed using the Spectra Calc program (Galactica, U.S.A.). The following proteins were used as molecular weight standards for the calibration of the column: thyroglobuline (670,000); IgG (158,000); ovoalbumin (44,000); myoglobin (17,000); vitamin B_{12} (1350).

Amino acid sequence analysis. Pfk-2 was treated with subtilisin (1:300 protease:Pfk-2 ratio) for 60 min at 37 °C in the presence of 1 mM Fru-6-P. The fragments of this digestion were resolved by SDS–PAGE and blotted on to poly(vinylidene difluoride) (PVDF) membranes (Inmobilon-P; Millipore) for N-terminal sequence analysis. Bands on the blots were identified by staining with Coomassie blue. The 28-kDa fragment was cut off the membrane and subjected to Edman degradation using an automated protein sequencer (Applied Biosystems International).

Results and discussion

Effect of ligands on the aggregation state of Pfk-2. Since binding of MgATP to the allosteric site of Pfk-2 has been previously linked to a dimer-tetramer association process [6,7], we decided to evaluate the effect of different concentrations of this ligand upon the oligomerization transition. In the absence of ligands the elution time of Pfk-2 in size-exclusion chromatography experiments is that corresponding to a dimer; in the presence of 0.4 mM MgATP a tetramer is observed, while in the presence of 1 mM Fru-6-P the enzyme elutes as a dimer (not shown). Also, molecular filtration studies of Pfk-2 at different protein concentrations (between 0.005 and 1 mg/ml), show that the enzyme elutes as a dimer in the absence of ligands. Since no tetramer formation as a function of protein concentration was observed in the absence of MgATP, and in order to obtain some insights about the oligomerization mechanism, we studied the dependence of tetramer formation as a function of protein concentration in the presence of different concentrations of MgATP. However, size-exclusion profiles at different protein concentrations and at different MgATP concentrations show two overlapping peaks, which were unsuccessfully subjected to deconvolution techniques. For this reason we decided to use dynamic light-scattering in order to evaluate the effect of the MgATP concentration on the oligomerization process. Pfk-2 shows an increase in the hydrodynamic radius of gyration in the presence of different nucleotide concentrations (Fig. 1), in agreement with a dimer to a tetramer protein transition. The transition is cooperative with a half-saturation value for MgATP of 70 μM, similar to the K_d of 80 μM obtained through intrinsic fluorescent measurements for the binding of MgATP to the allosteric site of Pfk-2 [4].

Associations of subunits are widely observed in phosphofructokinases from mammals and plants, and these processes might play an important role in the regulation of enzymatic activity. However, the only case reported for prokaryote phosphofructokinases, besides the *E. coli* Pfk-2 enzyme, is the tetramer–dimer conversion of the enzyme from *Thermus thermophilus* induced by its allosteric effector, phosphoenolpyruvate [14].

Limited proteolysis of Pfk-2 with different proteases. In order to evaluate the changes in tertiary structure of the monomers that occur in the oligomerization process brought about by MgATP binding, we decided to use different proteases for detecting the subunit-exposed surface sites in different enzyme–ligand complexes. This technique is based on the hypothesis that the segments of the polypeptide chain that are likely to be accessible to proteinases are exposed loops within domains or the

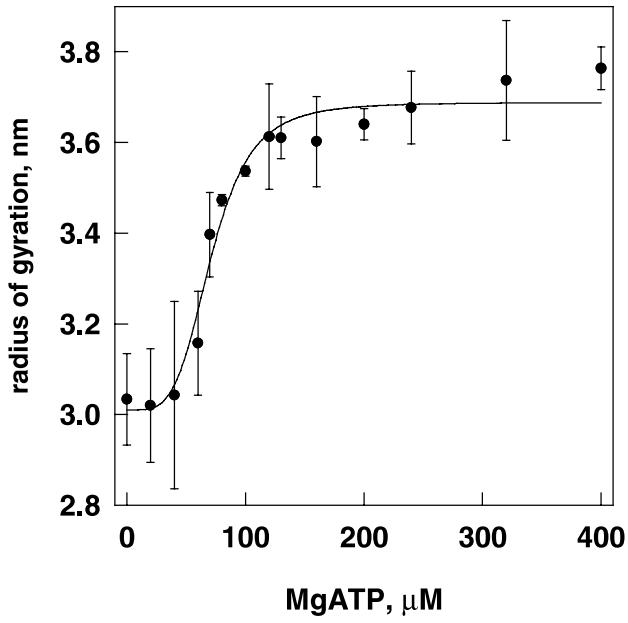

Fig. 1. Effect of MgATP on the aggregation state of Pfk-2. Dynamic light-scattering measurements of Pfk-2 at different MgATP concentrations. Experiments were performed as described under Experimental procedures. The curve is fitted to the Hill equation.

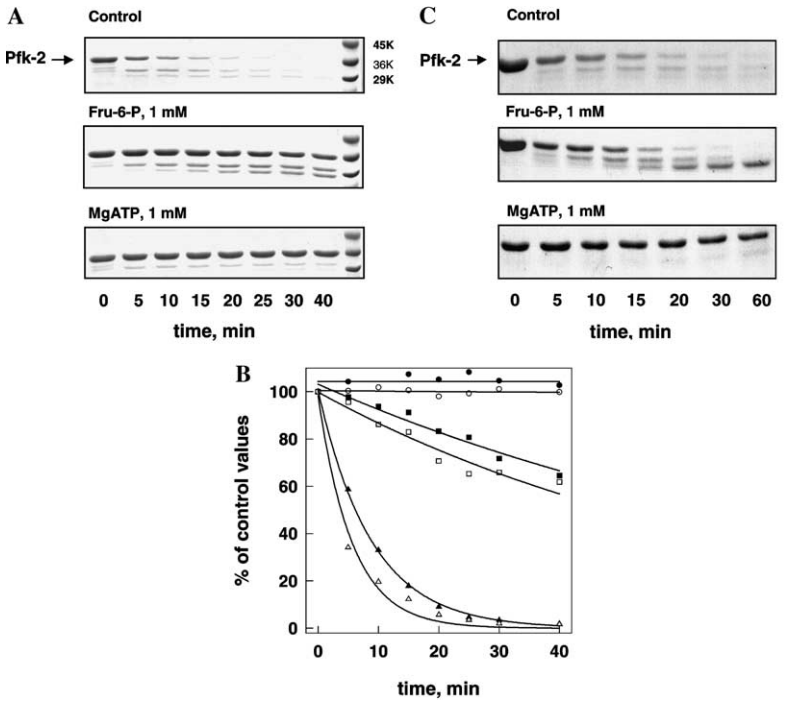

Fig. 2. Limited proteolysis of Pfk-2 with trypsin and proteinase K in the presence and absence of ligands. (A) Time course of the cleavage reaction of Pfk-2 by trypsin (1/100 molar ratio protease/Pfk-2). Samples were withdrawn from the proteolysis mixture at the indicated times, diluted in the presence of soybean trypsin inhibitor, then denatured by boiling in 1% SDS and analyzed by PAGE under denaturing conditions as described under Experimantal procedures. The arrow shows the position of the nonproteolyzed Pfk-2. Molecular weights of standard proteins are indicated in the right lane of A. (B) Densitometric analysis of the intact Pfk-2 polypeptide (35 kDa) from the electrophoretic pattern shown in (A) and changes in enzymatic activity. Densitometric traces for proteolysis in the absence of ligands (▲), in the presence of 1 mM Fru-6-P (■), and in the presence of 1 mM MgATP (●). Results are expressed as percentage of the nonproteolyzed Pfk-2 band present at zero time. For activity measurements the samples were withdrawn from the proteolysis mixture and assayed immediately: control (△), in the presence of 1 mM Fru-6-P (□), in the presence of 1 mM MgATP (○). The curves are fitted to single exponential decays. (C) Time course of digestion of Pfk-2 with proteinase K (molar ratio of 1/150 protease/Pfk-2) as described under Experimental procedures. The arrow indicates the position of the 35-kDa polypeptide.

linking segments between domains. These accessible regions could be present in the native structure of the protein or can arise or be protected due to ligand-induced conformational changes [15,16]. The susceptibility of Pfk-2 to the proteolytic attack by trypsin was analyzed through the electrophoretic pattern produced in the presence of Fru-6-P and MgATP (Fig. 2A). MgATP completely protects the polypeptide integrity, while Fru-6-P presents an intermediate effect, compared to the enzyme in the absence of ligands. In the absence of ligands two fragments of 30 and 28 kDa were initially observed. The presence of Fru-6-P does not produce changes in the proteolytic pattern, but stabilizes the two fragments formed. A similar protection was observed when the effect of ligands on the enzymatic activity was tested (Fig. 2B). After 30 min of incubation with the proteolytic enzyme, no enzymatic activity was left in the control, whereas 100 and 65% were retained in the presence of MgATP and Fru-6-P, respectively. In order to establish if the protection effect afforded by the ligands was due to changes in the accessibility of specific trypsin cleavage sites or to a global structural change of the enzyme, we used an unspecific protease such as proteinase K. The protection effect of the ligands against this protease, in terms of the integrity of the polypeptide chain (Fig. 2C) as well as the enzymatic activity (not shown), is similar to that observed with trypsin. MgATP provides almost complete protection of the polypeptide chain and enzymatic activity, while in the presence of Fru-6-P a partial protection of both parameters was observed, although less pronounced than that observed with trypsin. In the presence of Fru-6-P the major species observed after 20 min of incubation with this protease is a 28-kDa fragment (Fig. 2C). The results presented above suggest a precursor-product relationship between the 30- and the 28-kDa fragments, and since these fragments correspond to the N-terminal region of the protein (see below) it is possible that they are produced by a stepwise cleavage from the C-terminal end. If this were the case it would reflect some structural compartmentation related to domain structure of the enzyme, in the C-terminal 8-kDa segment. Removal of a segment at the C-terminus of the polypeptide chain has been demonstrated to be important for subunit interactions in *E. coli* Pfk-1 [17,18], and for stabilization of the octameric structure as was reported for the yeast

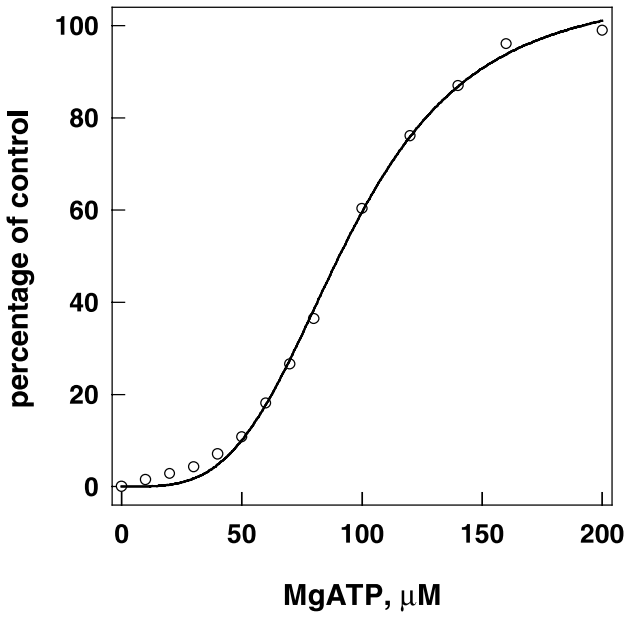

Fig. 3. Effect of MgATP concentration in the protection against trypsin digestion of Pfk-2. Enzymatic activity was determined for aliquots removed from the proteolysis mixture after 30 min of incubation at the indicated MgATP concentrations. Values are expressed as percentage of the control in the absence of trypsin. The curve is fitted to the Hill equation.

PFK [19]. Also, removal of a C-terminal heptadecapeptide from muscle PFK produces the desensitization of the enzyme to ATP inhibition [20].

In order to assess if there is a correlation between the binding of MgATP to the allosteric site, determined by changes in the intrinsic fluorescence of the protein, the dynamic light-scattering measurements, and the protection effect observed by this ligand against trypsin digestion, we determined the effect of different nucleotide concentrations upon the proteolytic cleavage. Fig. 3 shows that the concentration needed to achieve half of the maximal protection to trypsin digestion is about 90 μM, which is very similar to the K_d value of 80 μM obtained by measuring the changes in the intrinsic fluorescence properties of Pfk-2 upon binding of MgATP to the allosteric site [4], and to the half-saturation value of 70 μM obtained for the effect of MgATP on the aggregation state of Pfk-2 from dynamic light-scattering measurements. These results suggest that both protection and tetramerization are the result of binding of MgATP to a unique allosteric site and that conformational changes induced in the tetramer are responsible for the protection effect observed in the presence of this ligand using different proteases.

The use of subtilisin, an unspecific protease with a different preferred amino acid contributing the carbonyl group than proteinase K, mainly generates a proteolytic fragment of 28 kDa, which was also observed when trypsin or proteinase K were used. In the presence of 1 mM Fru-6-P there is a major stabilization of this fragment, being almost the only species present after 20 min of incubation with this proteolytic enzyme (Figs. 4A and B). Approximately 25% of the initial 35-kDa protein appears as a 28-kDa fragment, while the rest is further digested. Digestion by subtilisin is probably the result of cleavage at several sites, not all leading to the 28-kDa fragment. In the presence of 1 mM MgATP, almost no digestion of the polypeptide chain is observed until 30 min of incubation. In the absence of ligands the loss of enzymatic activity (not shown) correlates well with the first-order process of disappearance of the intact polypeptide chain. On the other hand, the effect of ligands upon the loss of catalytic activity shows that

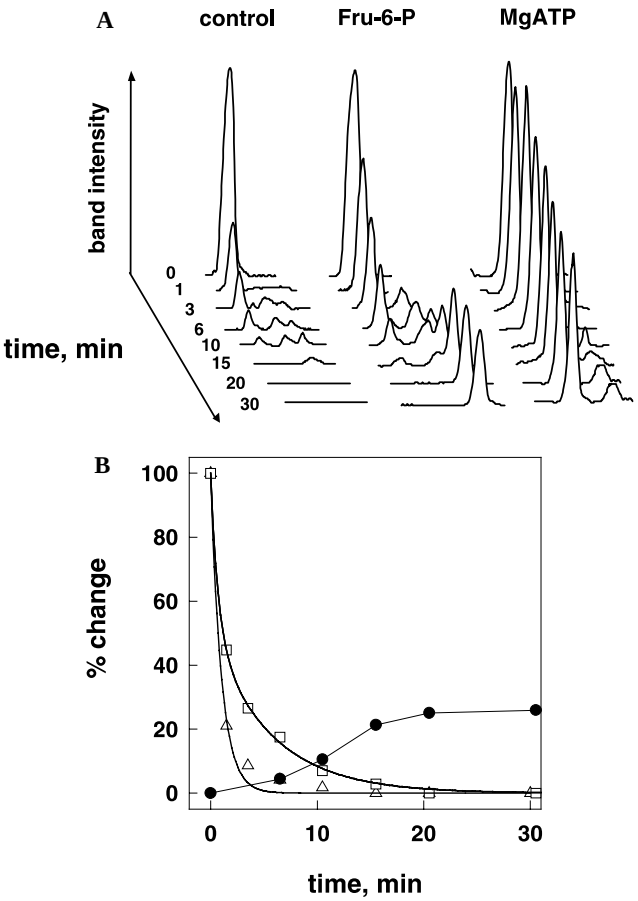

Fig. 4. Limited proteolysis of Pfk-2 with subtilisin. Densitometric analyses of gel patterns obtained after PAGE under denaturing conditions. (A) Densitometric analysis of Pfk-2 digested with subtilisin in the absence and in the presence of ligands (1 mM). Proteolytic digestion was carried out with a molar ratio protease/Pfk-2 of 1/300 under conditions described under Experimental procedures. (B) Densitometric analysis of the 35-kDa polypeptide after different incubation times in the absence of ligands (△) and in the presence of 1 mM Fru-6-P (□). The curves in the absence of ligands and in the presence of Fru-6-P were fitted to a single and double exponential decay, respectively. Densitometric analysis of the appearance of the 28-kDa fragment upon digestion of Pfk-2 in the presence of 1 mM Fru-6-P (●). Values are expressed as percentage of the controls at zero time.

Fru-6-P presents a minor protection and that the MgATP protection after 15 min of incubation is not as pronounced as in the case of the other proteases (not shown).

For all the proteases tested there is a good correlation between the loss of the catalytic activity and the disappearance of the protein band corresponding to the intact subunit polypeptide chain, suggesting that ligand binding lowers the rate of proteolysis of active species into inactive ones.

Since the presence of MgATP promotes the tetramerization of the enzyme, this could be the major factor involved in the protection effect afforded by this ligand. On the other hand, the protection effect exhibited by Fru-6-P cannot be explained by changes in the aggregation state of the enzyme. For this case, the most plausible explanation is a higher degree of subunit compactness in the enzyme–ligand complex compared to the enzyme in the absence of ligand, which results in lower exposure of the proteolytic cleavage site to the solvent.

Sequence analysis. The major proteolytic product (28-kDa fragment) obtained upon digestion of Pfk-2 with subtilisin in the presence of Fru-6-P was isolated by SDS–PAGE electrophoresis, transferred to PVDF membranes, and subjected to N-terminal sequencing. The analysis reveals that the fragment removed was located in the C-terminus of the protein (Scheme 1).

Oligomeric structure of the 28-kDa fragment. Size-exclusion chromatography of the fragment in the absence of ligands shows an elution volume corresponding to a dimer, as with the native enzyme. However, in the presence of 0.4 mM MgATP the fragment remains as a dimer (Fig. 5), while Pfk-2 behaves as a tetramer. These results suggest that the C-terminus peptide may contribute to the binding of MgATP to the allosteric site or that it is involved in the ligand-induced conformational change leading to the tetrameric structure. Also, these results emphasize the importance of the integrity of the C-terminal region of the protein for catalysis and indicate that the absence of catalytic activity of the fragment is not due to dimer dissociation.

Ligand-binding properties of the 28-kDa fragment. Since the 28-kDa fragment is inactive and does not form a tetramer in the presence of MgATP, it was of interest to examine whether this fragment presents a loss of sensitivity to Fru-6-P and to the allosteric effector. The 28-kDa fragment produced upon digestion of Pfk-2 with subtilisin in the presence of 1 mM Fru-6-P was purified by ion-exchange chromatography in a Mono Q column. The eluted peak was homogeneous as judged by SDS electrophoresis (not shown). Since this fragment contains the only tryptophan residue of the enzyme (Trp 88), one can measure ligand binding by monitoring changes in the intrinsic fluorescence properties. Fig. 6A shows the fluorescence spectrum of Pfk-2 that has been digested by subtilisin under several different conditions. In the absence of ligands the fragment has an emission maximum at 352 nm, like native Pfk-2 [4], suggesting no

28-kDa fragment	NH_2-MVRIYTLTLAP...
Pfk-2	NH_2-MVRIYTLTLAPSLDSATITPQIYPEG...

Scheme 1. N-terminal sequence of the 28-kDa fragment obtained by digestion of Pfk-2 with subtilisin in the presence of 1 mM Fru-6-P, and of the intact Pfk-2 polypeptide chain.

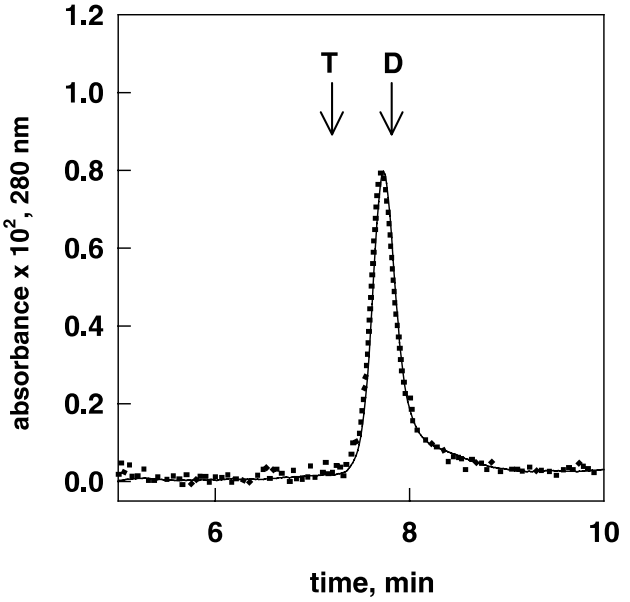

Fig. 5. Size-exclusion chromatography of the 28-kDa fragment in the presence of MgATP. Pfk-2 was treated with subtilisin under conditions described under Experimental procedures, for 60 min at 37 °C in the presence of 1 mM Fru-6-P, and then subjected to size-exclusion chromatography in the absence of ligands (—), and in the presence of 0.4 mM MgATP (···). T and D represent the elution time of tetrameric and dimeric Pfk-2, respectively.

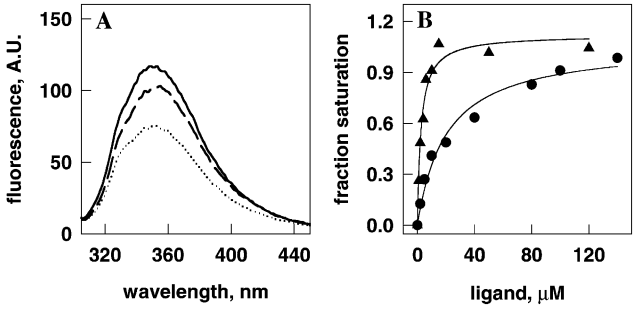

Fig. 6. Effect of ligands on the intrinsic fluorescence properties of the 28-kDa fragment. (A) Fluorescence emission spectra of the fragment in the absence and in the presence of ligands: in the absence of ligands (—), in the presence of 120 μM Fru-6-P (- - -), in the presence of 120 μM MgATP (···). (B) Binding of Fru-6-P (▲) and MgATP (●). Saturation fraction was calculated as described under Experimental procedures. Excitation wavelength was set to 295 nm.

major changes in the degree of exposure of this residue to the solvent after digestion. Upon addition of saturating concentrations of Fru-6-P (120 μM), the fluorescence intensity decreases 14% with respect to that of the fragment in the absence of ligands, in contrast with the response observed with the native enzyme where binding of Fru-6-P produces an increase of fluorescence up to 30% [4]. MgATP also modifies the intrinsic fluorescence of the fragment. Saturating concentrations of this ligand (120 μM) cause a quenching of fluorescence of about 39% of its initial value, with no shift in the emission maximum. For the native enzyme, binding of MgATP to the allosteric site results in a quenching of the fluorescence of about 30% with a blue shift of 7 nm in the emission maximum [4], indicating a lower exposure to the solvent of the tryptophan residue in this enzyme–ligand complex. In contrast, the Trp residue in the fragment shows no changes in solvent exposure as a consequence of MgATP binding.

Titration of the 28-kDa fragment with Fru-6-P shows a hyperbolic dependence on ligand concentration with a K_d of 3 μM (Fig. 6B), similar to the value obtained with the native enzyme, 6 μM [4]. The fluorescence response of the fragment to different MgATP concentrations is also hyperbolic with a K_d of 20 μM. However, the value obtained for the binding of the allosteric effector to the native enzyme is 80 μM, and the response is cooperative instead of hyperbolic [4]. These results indicate that the cooperative response and the blue shift in the emission maximum observed for the native enzyme upon MgATP binding reflect tetramer formation, which is not observed in the fragment, in agreement with the HPLC molecular filtration studies.

The results presented demonstrate major conformational changes in *E. coli* Pfk-2 upon ligand binding and establish for the first time segments of the polypeptide chain involved in binding of Fru-6-P and allosteric effector, important for catalytic activity and MgATP-induced tetramerization.

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