

Role of Cys-295 on subunit interactions and allosteric regulation of phosphofructokinase-2 from *Escherichia coli*

Andrés Caniuguir^a, Ricardo Cabrera^a, Mauricio Báez^a, Claudio C. Vásquez^b,
Jorge Babul^a, Victoria Guixé^{a,*}

^a Laboratorio de Bioquímica y Biología Molecular, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

^b Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile

Abstract In a previous work, chemical modification of Cys-238 of *Escherichia coli* Pfk-2 raised concerns on the importance of the dimeric state of Pfk-2 for enzyme activity, whereas modification of Cys-295 impaired the enzymatic activity and the MgATP-induced tetramerization of the enzyme. The results presented here demonstrate that the dimeric state of Pfk-2 is critical for the stability and the activity of the enzyme. The replacement of Cys-238 by either Ala or Phe shows no effect on the kinetic parameters, allosteric inhibition, dimer stability and oligomeric structure of Pfk-2. However, the mutation of Cys-295 by either Ala or Phe provokes a decrease in the k_{cat} value and an increment in the K_m values for both substrates. We suggest that the Cys-295 residue participates in intersubunit interactions in the tetramer since the Cys-295-Phe mutant exhibits higher tetramer stability, which in turn results in an increase in the fructose-6-P concentration required for the reversal of the MgATP inhibition relative to the wild type enzyme.

Keywords: Subunit interaction; SH group; Phosphofructokinase; Allosteric regulation; *Escherichia coli*

1. Introduction

Subunit association in oligomeric proteins and its modulation by ligand binding plays a central role in several biological processes such as regulation of enzymatic activity and signal transduction. For this reason, oligomeric interfaces are important for protein stability, assembly and function. In *Escherichia coli* (*E. coli*) two enzymes catalyze the key commitment step in the glycolytic pathway, phosphofructokinase-1 (Pfk-1) and phosphofructokinase-2 (Pfk-2), which are oligomeric proteins that depend on their subunit interfaces for the regulation of their activities. Pfk-1 is a homotetramer that presents sigmoidal kinetics with respect to fructose-6-P and allosteric regulation by the activators ADP or GDP and by the inhibitor PEP [1–4]. Binding sites for these effectors are located at the

subunit interface and are formed by amino acid residues coming from different polypeptide chains [5]. Thus, the importance of interfacial interactions for the regulation and stability of Pfk-1 activity has been addressed in several studies [6,7].

On the other hand, homodimeric Pfk-2 exhibits hyperbolic kinetics with respect to both substrates, fructose-6-P and MgATP, and does not show sensitivity towards the allosteric effectors that regulates Pfk-1 [4]. However, Pfk-2 activity is regulated by binding of MgATP to an allosteric site that causes inhibition of the enzyme activity at low concentrations of fructose-6-P [8,9]. Also, this inhibition is linked to a dimer–tetramer association process [10,11]. Thus, the structural properties of the dimer–dimer interface should contribute to the molecular mechanism by which the allosteric binding of MgATP is linked to enzyme inhibition. However, in the absence of an experimentally determined three-dimensional structure of the enzyme, this contribution is difficult to assess. For the same reason, little is known about the importance that monomer interactions may play in the activity and stability of native Pfk-2.

Nonetheless, some structural information has been obtained from chemical modification experiments in studies of the role of cysteine residues in the activity and oligomeric structure of Pfk-2 [11,12]. Chemical modification of Cys-238 with pyrene maleimide (PM) generates a monomeric, inactive enzyme, which is converted to a fully active dimeric state in the presence of the substrates. As the native protein, the modified enzyme shows the ability of forming tetramers in the presence of MgATP. On the other hand, specific labelling of Cys-295 with eosine maleimide (EM) results in the impairment of MgATP-induced tetramerization, absence of allosteric binding of MgATP and inactivation of the enzyme [12]. These results suggest that while Cys-238 is probably participating in subunit interactions of dimeric Pfk-2, Cys-295 could be involved in MgATP binding, tetramer formation, and enzymatic activity.

In this work, we present evidence about the importance of intersubunit interactions for the activity and stability of dimeric Pfk-2, and about the contribution of the Cys-238 residue to this interaction. In addition, the involvement of Cys-295 in dimer–dimer interactions and in the allosteric regulation by MgATP was evaluated. The results reported here show that the activity of Pfk-2 is associated to its dimeric state and that Cys-238 is not critical for the maintenance of the oligomeric state or to the stability of the Pfk-2 dimer. However, the introduction of phenylalanine in the 295 position increases the stability of the tetrameric state of the enzyme and the fructose-6-P

*Corresponding author. Fax: +56 2 2712983.
E-mail address: vguixe@uchile.cl (V. Guixé).

Abbreviations: PM, pyrene *N*-(1-pyrenyl) maleimide; EM, eosin 5-maleimide; GdnHCl, guanidine hydrochloride; Pfk, phosphofructokinase; Fructose-6-P, fructose-6-phosphate

concentration required to reverse the MgATP inhibition of enzyme activity and of tetramer formation.

2. Materials and methods

2.1. Site-directed mutagenesis

Site directed mutagenesis was carried out using the QuickChange (Stratagene) system using pET21d plasmid (Novagen) containing the *pfk-2* gene as template. Two oligonucleotides were used to construct each mutant, both complementary to opposite strands of the template. Bold letters indicate the substituted bases, underlined bases indicate the triplet codon for the newly introduced amino acid (only 'sense' oligonucleotides are shown): Cys-238-Ala, 5'-GTT GAT AGT GAA AAC **GCT** ATT CAG GTG GTG CCA-3'; Cys-238-Phe, 5'-GGT GTT GAT AGT GAA AAC **TTT** ATT CAG GTG GTG CCA CC-3'; Cys-295-Ala, 5'-CAG GGA ACA CGT CTG **GCC** TCC CAT GAC GAT ACG-3'; Cys-295-Phe, 5'-CAG GGA ACA CGT CTG **TTC** TCC CAT GAC GAT ACG-3'.

2.2. Enzyme expression and purification

Mutant Pfk-2 enzymes were produced in *E. coli* DF1020 since this strain does not express wild type phosphofructokinases [13]. DF1020 strain was previously transformed with plasmid pGP1-2 [14] that allows the expression of the T7 RNA polymerase after heat induction. Cultures were grown at 30 °C in Luria Broth media supplemented with ampicillin and kanamycin to a final concentration of 100 and 75 µg/ml, respectively. Protein expression was induced at a $A_{600} = 0.5$ by heat treatment at 42 °C for 20 min; thereafter the culture was incubated at 37 °C for 4 h before collecting the cells by centrifugation. Wild type and mutant enzymes were purified essentially as described in [4]. The transformed *E. coli* strains produced an average of 10–15 mg of protein (wild type and mutant Pfk-2) per l of culture.

2.3. Enzyme kinetics

Pfk activity was determined spectrophotometrically by coupling the fructose-1,6-bisP production to the oxidation of NADH as described previously [8]. In the guanidine hydrochloride (GdnHCl)-induced unfolding experiments of wild type Pfk-2, Cys-238 and Cys-295 mutants, enzymatic activity was measured by diluting 700 fold, in the assay medium, the enzyme samples that had been previously incubated at different GdnHCl concentrations. Under these conditions, the maximum denaturant agent concentration achieved in the assay was below 4 mM and therefore can be neglected. In addition, enzyme activity was measured during the first 5 min where the renaturation process is negligible (M. Baez, unpublished observations).

2.4. Size exclusion chromatography

Size exclusion chromatography experiments were performed with a Waters 1525 HPLC binary pump system, with a Bio-Sil SEC-250 (7.8 mm × 30 cm) column (BioRad, Hercules, CA, USA) equilibrated in a buffer containing 40 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 200 mM KCl, 2 mM DTT in the absence or in the presence of 0.5 mM MgATP, at a flow rate of 0.8 ml/min. The column was calibrated with the following molecular-mass markers: carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and thyroglobulin (669 kDa). Protein elution was recorded with the use of an online Waters 2487 UV dual detector measuring the absorbance at 280 nm.

2.5. GdnHCl induced unfolding of wild type and mutant enzymes

Denaturation of wild type and mutant forms of Pfk-2 was performed by incubating protein solutions (90 µg/ml) at 20 °C for 48 h in 25 mM HEPES buffer, pH 8.0, 5 mM MgCl₂, 5 mM DTT, containing various concentration of GdnHCl, in the absence or in the presence of 1 mM ATP, so that equilibrium was achieved.

2.6. Dynamic light scattering measurements

Experiments were done at 18 ± 0.1 °C using a DLS DynaPro MSTC014 (Protein Solutions Inc. – Bucks, England). Samples for dynamic light scattering measurements contained 0.5 mg/ml protein in 25 mM Tris-HCl buffer, pH 8.2, 5 mM MgCl₂, and 5 mM DTT and

were incubated for 36 h at the indicated GdnHCl concentrations. Data were collected for 15 µl samples after centrifugation at 10 000 rpm for 10 min in an Eppendorf microfuge to eliminate particulate matter. The intensity scattered by the protein was determined from the regularization histogram used to find the contribution of individual species to the total scattering (by inverse Laplace transformation) using the program DYNAMICS supplied with the instrument.

2.7. Fluorescence measurements

Fluorescence emission spectra were recorded at room temperature in a Perkin Elmer LS 50 fluorimeter. The excitation wavelength was 295 nm and the emission was recorded from 305 to 500 nm. Excitation and emission slits were set at 5 nm. The experiments were performed with 200 µg/ml of protein in 25 mM Tris-HCl buffer, pH 8.2, 5 mM MgCl₂, and 2 mM DTT. Titration experiments were done by adding small aliquots of stock solutions of ligand to the enzyme solution. Background readings were subtracted and corrections were made to compensate for protein dilution. Data analysis was carried out with the Grams/386 (Galactic Industries Corp, Main Street Salem, NH, USA). The fractional saturation binding was determined from the intensity change with ligand concentration by using the formula: $(F^0 - F)/(F^0 - F^\infty)$, where F^0 and F^∞ represent the emission intensity in the absence and in the presence of saturating concentrations of ligand, respectively, and F is the intensity after addition of a given concentration of ligand.

3. Results and discussion

3.1. Structural and kinetic characterization of Cys-238 and Cys-295 mutant enzymes

As previously reported, chemical modification of Pfk-2 with PM and EM suggests that Cys-238 is important for the maintenance of the dimeric state of Pfk-2, and that Cys-295 is involved in the catalytic activity of the enzyme [12]. In order to have a more detailed information about the role of these residues in the oligomeric structure and catalytic activity of Pfk-2, site-directed mutagenesis was used to replace them for either Ala or Phe. Cys-Ala substitution was intended to evaluate the importance of the presence of a thiol group in the side chain while the Cys-Phe substitution was carried out to mimic the size and hydrophobic characteristics of the PM and EM chemical probes. In agreement with chemical modification experiments the kinetic parameters of the Cys-238 mutant enzymes are similar to those exhibited by the wild type Pfk-2 (Table 1). However, the k_{cat} values of the Cys-295-Ala and Cys-295-Phe mutants are 2 and 7-fold lower than that of the wild type enzyme, respectively, while the K_m values for MgATP and fructose-6-P are approximately between 3 and 6-fold higher than those found for the wild type enzyme. These results show that, although Cys 295 is not required for enzymatic activity, as suggested previously, its substitution affects the active site properties. The location of Cys-295 near to the active site in the major domain of the molecular model of

Table 1
Kinetic parameters of wild type Pfk-2 and Cys-238 and Cys-295 mutants

	Wild type	Cys-238 substituted by		Cys-295 substituted by	
		Ala	Phe	Ala	Phe
k_{cat} , s ⁻¹	90	70	70	39	13
K_m fructose-6-P, µM	31	31	47	88	81
K_m MgATP, µM	17	17	21	61	107

dimeric Pfk-2 [15] supports this observation. Size exclusion chromatography experiments demonstrate that both Cys-295 and Cys-238 mutant enzymes elute as dimers in the absence of ligands, whereas in the presence of MgATP the tetramer is the dominant species for both mutants, showing the same behaviour of the wild type enzyme under the same conditions (Fig. 1). Also, circular dichroism spectra of the mutants were identical to that of the wild type enzyme, indicating no significant alterations in the secondary structure of the enzyme due to residue replacement (not shown).

3.2. The dimeric state of Pfk-2 is critical for stability and enzymatic activity

We have previously shown that modification of Cys-238 with PM produces an active enzyme with an elution volume similar to that expected for the globular monomeric form of Pfk-2, indicating that the chemical probe is somehow affecting monomer-monomer interactions. However, the PM-modified enzyme elutes as the native dimer in the presence of fructose-6-P and ATP⁻⁴, indicating that disrupting monomer-monomer interactions does not prevent dimerization and cannot be used for the assessment of monomer's catalytic activity. The observed monomerization upon chemical modification suggests that the PM probe can induce a conformational change that affects the dimer interface. An analogous situation has been described for the luciferase enzyme from *Vibrio harveyi*, where mutations at the subunit interface of the dimer alter the stability of a region in one of the subunits that is distant from the interface [16]. Insights about the relationship between the dimeric form and catalytic activity can be drawn from the homology model of dimeric Pfk-2. In each monomer, the small β -sheet domain is involved in the formation of both, the subunit interface and the cleft that contains the active site [15]. Therefore, disrupting the monomer-monomer interface should affect the active site structure, and hence, the catalytic activity. In order to explore the relevance of subunit interactions for the stability and catalytic activity of Pfk-2, and to assess the contribution of Cys-238 and Cys-295 to these interactions, we used GdnHCl and protein dilution experiments to induce protein unfolding and subunit dissociation. For each condition used, the resulting changes in the catalytic activity and oligomeric structure were evaluated.

The activity of Pfk-2 samples incubated at different GdnHCl concentrations is shown in Fig. 2A. The inactivation profile shows a single transition in a GdnHCl concentration range of 0.2–0.5 M, above which no enzyme activity was detected. Since loss of activity could be due to subunit dissociation along with unfolding, we measured the light scattering intensity as a function of GdnHCl concentration to observe the dissociation of the Pfk-2 dimer (Fig. 2A). Since scattering intensity (I) is proportional to both, molecular weight (m) and particle concentration (C), a decrease in the scattered intensity ($I \propto mC$) could be associated to dimer dissociation provided that the

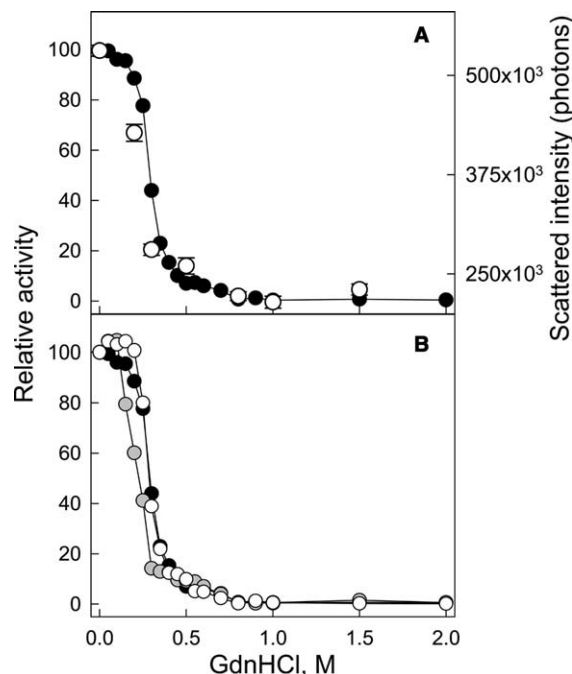


Fig. 2. GdnHCl-induced unfolding of wild type Pfk-2, Cys-238-Phe and Cys-295-Phe mutant enzymes. (A) Relative changes of the enzymatic activity of Pfk-2 (●) and associated changes in light scattering (○) as a function of GdnHCl concentration. (B) Effect of mutations on Cys-238-Phe (○) and Cys-295-Phe (●) on the loss of activity induced by GdnHCl. Wild type (●). The samples (90 μ g/ml) were incubated for 48 h at 20 °C at different GdnHCl concentrations.

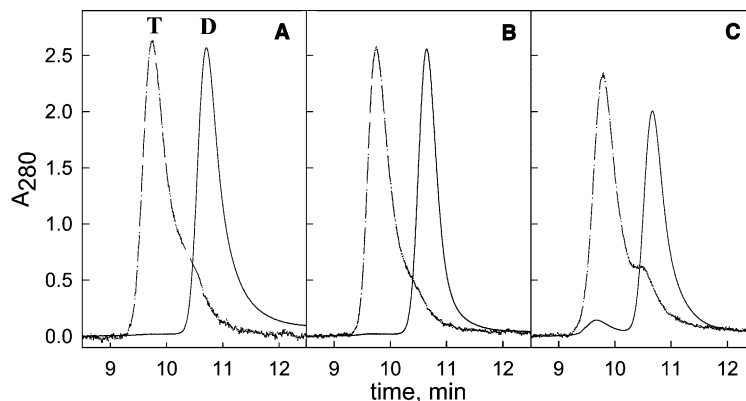


Fig. 1. Effect of MgATP on the aggregation state of wild type Pfk-2, Cys-238-Phe and Cys-295-Phe mutant enzymes. Size exclusion chromatography was performed in a Bio Rad Bio Sil SEC-250 column in the absence of ligands (continuous line) and in the presence of 0.5 mM MgATP (dashed line). Protein samples were preincubated under the same conditions. A, B and C panels represents wild type, Cys-238-Phe and Cys-295-Phe mutants, respectively. T and D denote tetramer and dimer, respectively.

protein concentration has been held constant. The scattered intensity correlated well with the loss of enzymatic activity, dropping to less than half of the initial value at the same GdnHCl concentration range where loss of activity was observed. Since unfolding proceeds from the native dimeric state, a less stable interface induced by mutation of Cys-238 or Cys-295 residues should require lower GdnHCl concentrations to provoke the unfolding of the mutant enzymes. Equilibrium unfolding experiments show that there are no significant differences between the wild type and the Cys-238-Phe mutant (or Cys-238-Ala, data not shown) (Fig. 2B). However, in the case of the Cys-295-Phe mutant the loss of activity occurs at slightly lower GdnHCl concentrations compared with wild type Pfk-2 (Fig. 2B).

The correlation between the aggregation state of Pfk-2 and enzymatic activity suggests that the dimer is the only active specie (Fig. 2A). If this is correct, subunit dissociation induced by protein dilution should cause a decrease of enzyme activity. To evaluate this hypothesis, wild type and mutant enzymes were incubated at different protein concentrations (from 30 μ M to 30 nM) and the specific activity was determined using a constant amount of 3 pmol of protein in the reaction mixture. Fig. 3 shows that wild type Pfk-2 specific activity tends toward zero as the protein concentration decreases. This means that the fraction of active enzyme diminishes with protein dilution probably because the dissociated protein is not active. The same behaviour was observed for the mutant enzymes, whether Cys-238 was replaced either by Phe (Fig. 3) or Ala (data not shown) indicating that this residue is not involved in monomer–monomer interactions. Since the activity of the Cys-295-Phe mutant is more sensitive to dilution and less stable to GdnHCl induced-unfolding than the wild type enzyme (Fig. 2), we suggest that this residue may contribute to monomer interactions in the Pfk-2 dimer. When considering the location of Cys-295 in the molecular model of dimeric Pfk-

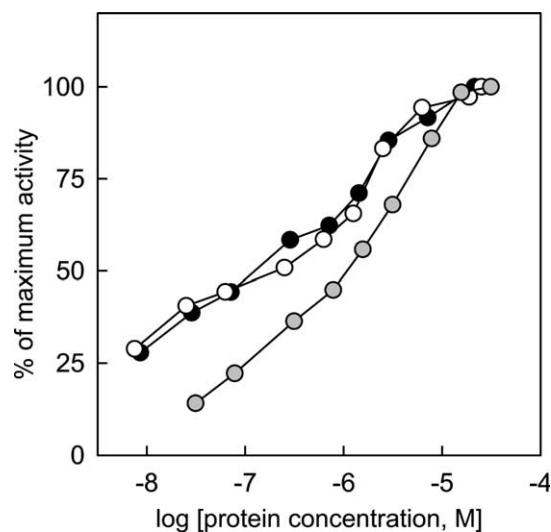


Fig. 3. Relative changes in specific activity as a function of protein concentration. Samples of wild type Pfk-2 (●), Cys-238-Phe (○) and Cys-295-Phe (○) mutants were incubated at different protein concentrations, between 0.0003 and 1.0 μ g/ μ l, corresponding to 30 nM and 30 μ M respectively, for 3 h at 20 °C in 25 mM Tris–HCl buffer pH 8.2, 5 mM MgCl₂ and 10 mM DTT. Then, enzyme activity was measured using 3 pmol of each sample.

2 [15] these results are not easy to explain, unless long range structural effects caused by the mutation should be claimed. On the other hand, results from kinetic and structural studies obtained with the Cys-238 mutants are compatible with the molecular model of Pfk-2, which predicts a location for this residue far from the active site and from the monomer–monomer interface.

3.3. MgATP-induced inhibition of the enzyme activity is modulated by the stability of dimer–dimer interactions

The inhibition of Pfk-2 activity by binding of MgATP to an allosteric site has been demonstrated to be an important regulatory feature for the in vivo carbohydrate metabolism of *E. coli* under gluconeogenic conditions [17]. Chemical modification of Cys-295 suggests a role of this residue in the binding of MgATP to the allosteric site and also in the MgATP-induced tetramer formation. However, its importance for the MgATP-induced inhibition of the enzyme activity could not be evaluated because the chemically modified enzyme was inactive.

In order to determine the effect of mutations of this residue by Ala or Phe on the regulation of Pfk-2 activity, we studied the MgATP-induced inhibition of enzymatic activity at low (0.1 mM) and high (1 mM) fructose-6-P concentrations. Fig. 4B shows that the Cys-295-Ala mutant enzyme displayed the same behaviour as the wild type enzyme (Fig. 4A) and the Cys-238 mutants (not shown), that is, inhibition of enzyme activity at low fructose-6-P and the reversal of this inhibition at higher fructose-6-P concentrations. However, although the Cys-295-Phe mutant is inhibited at 0.1 mM fructose-6-P, the reversal of the inhibition requires higher fructose-6-P concentrations (>5 mM) as compared to the wild type enzyme. Since the allosteric inhibition of Pfk-2 by MgATP has been related to the association of dimers to form tetramers [10,11] the reversal of this inhibition by high fructose-6-P concentrations could be explained by the dissociation of tetramers into active dimers due to binding of the sugar-phosphate to the active site. The observed result with Cys-295-Phe could arise from differences in the affinity of this enzyme for fructose-6-P as compared to

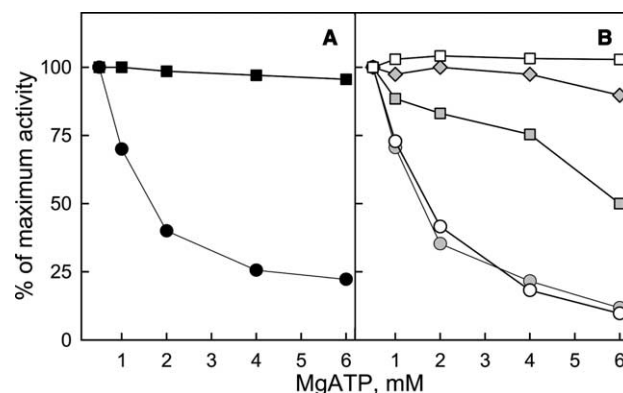


Fig. 4. MgATP inhibition of wild type Pfk-2 and Cys-295-Ala and Cys-295-Phe mutants at different fructose-6-P concentrations. (A) Effect of MgATP concentration on the activity of wild type Pfk-2 at 0.1 mM fructose-6-P (●) and at 1 mM fructose-6-P (■). (B) Effect of MgATP concentration on the activity of Cys-295-Ala at 0.1 mM fructose-6-P (○) and 1 mM fructose-6-P (□) and effect of MgATP concentration on the activity of Cys-295-Phe at 0.1 mM fructose-6-P (○), 1 mM fructose-6-P (□) and 5 mM fructose-6-P (◇).

the wild type enzyme. To test this hypothesis we measured the binding of fructose-6-P to the wild type and to the Cys-295-Phe mutant enzymes using intrinsic fluorescence measurements (Fig. 5). In each case, the fitted curves correspond to a hyperbolic function with an average K_d value of 20 μ M for both, Cys-295-Ala and Cys-295-Phe mutants. These values, although higher than that obtained for the wild type enzyme [9], cannot explain why only the Cys-295-Phe mutant requires higher fructose-6-P concentrations for the reversal of the MgATP inhibition of the enzyme activity.

A second possibility is that replacement of Cys by Phe could affect the strength of dimer–dimer interactions in the tetramer. This alternative was tested by comparing the stability of wild type and Cys-295-Phe tetramers to GdnHCl-induced unfolding in the presence of MgATP. Fig. 6 shows that the Cys-295-Phe mutant requires a higher concentration of the denaturant agent ($C_{m1/2}$ 0.63 M) to unfold the protein, as compared to the wild type and Cys-295-Ala enzymes ($C_{m1/2}$ 0.42 and 0.40 M, respectively). Thus, there is a correlation between the degree of MgATP inhibition of enzyme activity and the stability of the interactions between dimers in the tetramer. Because the Cys-295-Phe dimer is less stable than the wild type dimer, the higher stability of the mutant tetrameric form could be associated to an effect over the dimer–dimer interface.

If the Cys-295 residue is directly involved in dimer–dimer interface, its replacement by a bulky hydrophobic residue such as Phe could increase the stability of the tetramer by decreasing the desolvation energy around the interface, in the absence of other long range structural effects. However, factors determining the role of specific amino acid residues at the subunit interface are not easy to establish. Inlow and Baldwin studied the role of the subunit interface in the conformational stability of a bacterial luciferase collecting data from equilibrium unfolding experiments for different mutants [16]. Although certainly, the extensive hydrophobic surface at the interface would be expected to confer significant stability, neither of

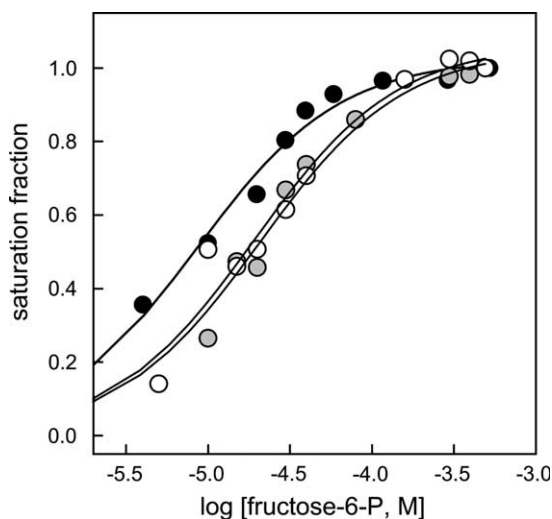


Fig. 5. Binding of fructose-6-P to wild type Pfk-2 and Cys-295 mutants. The binding of fructose-6-P to wild type Pfk-2 (●), Cys-295-Ala (○) and Cys-295-Phe (○) enzymes was followed by the increase in the intrinsic fluorescence as a function of the sugar-phosphate concentration. The excitation wavelength was 295 nm. Lines represent the fit of the experimental points to a hyperbola equation. Fructose-6-P concentrations are shown in a logarithmic scale for a clear presentation of the data.

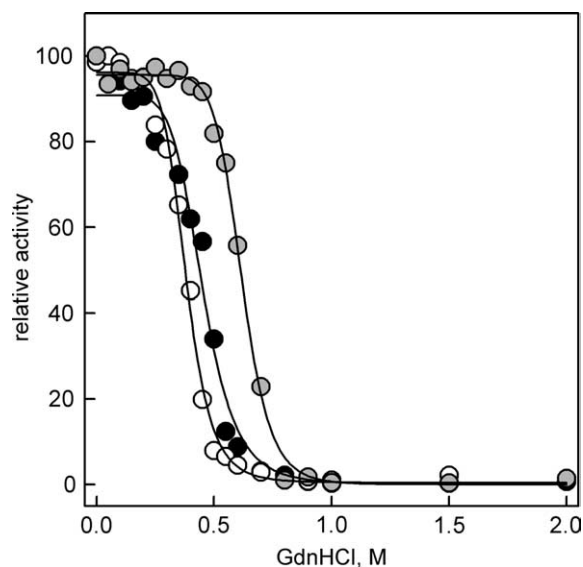


Fig. 6. GdnHCl-induced unfolding of wild type Pfk-2 and Cys-295 mutant enzymes in the presence of MgATP. Protein samples (90 μ g/ml) equilibrated in 1 mM MgATP were diluted into buffer containing the indicated GdnHCl concentrations and the same concentration of MgATP. Samples were incubated for 48 h at 20 °C before enzyme activity measurements. Wild type Pfk-2 (●), Cys-295-Ala (○) and Cys-295-Phe (○).

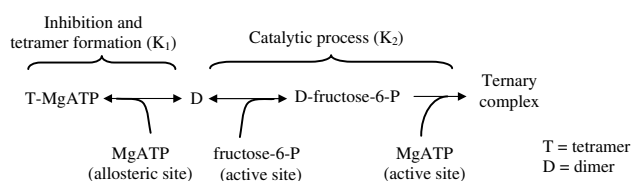
the interface mutants affects the dissociation of the dimeric luciferase under non-denaturing conditions. Moreover, when a loop deletion mutant that does not affect the hydrophobic portions of the interface was studied, it was found that there is a large effect on subunit equilibrium, thus demonstrating that intersubunit affinity is not due only to subunit interactions. Also, when a Phe-Tyr replacement at the subunit interface of glutathione *S*-transferase was made, the weight average molecular mass was found to be characteristic of a dimer. However, when Phe was changed to a small residue such as Ala, the weight average molecular mass was reduced and the equilibrium shifted toward the monomer [18]. The three-dimensional model of the tetramer form of Pfk-2, constructed by using homology modelling combined with solution X-ray scattering in the presence of MgATP [15], shows that although Cys-295 is not located at the dimer–dimer interface, it occupies a nearby location so it could modulate dimer–dimer interactions.

4. Conclusion

The results presented here show that the dimeric state of Pfk-2 is critical for the stability and the activity of the enzyme, as demonstrated by the correlation between enzyme dissociation and enzymatic activity in GdnHCl-induced unfolding and protein dilution experiments. Cys-238 has no apparent role in monomer–monomer interactions, as shown by enzyme activity, allosteric inhibition, dimer stability and oligomeric structure experiments using the Ala and Phe mutants. On the other hand, the mutation of the Cys-295 residue produced a decrease in the k_{cat} value and an increment in the K_m values for both substrates, more markedly in the case of the Phe substitution. This probably occurs through a conformational change that affects the active site instead of a direct

participation of this amino acid in the catalytic mechanism. The involvement of Cys-295 in dimer-dimer interactions was supported by the fact that higher fructose-6-P concentrations are required for the reversal of the MgATP allosteric inhibition of enzyme activity and also by an increase in tetramer stability relative to the wild type tetrameric form of Pfk-2. Studies are in progress in order to gain further insights on the role of subunit interactions in the conformational stability of the Pfk-2 protein.

The following scheme summarizes the role of the oligomeric states on the activity and allosteric behavior of Pfk-2.



The left side of the scheme shows tetramer formation (allosteric MgATP binding and dimer association) controlled by K_1 . The right side shows the catalytic process (binding of fructose-6-P and ternary complex formation upon MgATP binding) controlled by K_2 . Therefore, the balance between K_1 and K_2 will determine the modulation of Pfk-2 inhibition. We here postulate that Cys-295-Phe mutation produces structural changes that favour the allosteric inhibition by increasing tetramer stability.

Acknowledgments: The light-scattering measurements were performed at Laboratorio de Cristalografía, Instituto de Física de São Carlos, Universidade de São Paulo, Brazil. M.B. is a recipient of a doctoral fellowship from Conicyt (Comisión Nacional de Investigación Científica y Tecnológica, Chile). This work was supported by a grant from the Fondo Nacional de Desarrollo Científico y Tecnológico (Fondecyt 1010645).

References

- [1] Shirakihara, Y. and Evans, P. (1988) Crystal structure of the complex of phosphofructokinase from *Escherichia coli* with its reaction products. *J. Mol. Biol.* 204, 973–994.
- [2] Blangy, D., Buc, H. and Monod, J. (1968) Kinetics of the allosteric interactions of phosphofructokinase from *Escherichia coli*. *J. Mol. Biol.* 31, 13–35.
- [3] Rypniewski, W.R. and Evans, P.R. (1989) Crystal structure of unliganded phosphofructokinase from *Escherichia coli*. *J. Mol. Biol.* 207, 805–821.
- [4] Babul, J. (1978) Phosphofructokinases from *Escherichia coli*. Purification and characterization of the nonallosteric isozyme. *J. Biol. Chem.* 253, 4350–4355.
- [5] Schirmer, T. and Evans, P.R. (1990) Structural basis of the allosteric behaviour of phosphofructokinase. *Nature* 343, 140–145.
- [6] Le Bras, G., Auzat, I. and Garel, J.R. (1995) Tetramer-dimer equilibrium of phosphofructokinase and formation of hybrid tetramers. *Biochemistry* 34, 13203–13210.
- [7] Auzat, I., Le Bras, G. and Garel, J.R. (1995) Hypercooperativity induced by interface mutations in the phosphofructokinase from *Escherichia coli*. *J. Mol. Biol.* 246, 248–253.
- [8] Guixé, V. and Babul, J. (1985) Effect of ATP on phosphofructokinase-2 from *Escherichia coli*. A mutant enzyme altered in the allosteric site for MgATP. *J. Biol. Chem.* 260, 11001–11005.
- [9] Guixé, V., Rodríguez, P.H. and Babul, J. (1998) Ligand-induced conformational transitions in *Escherichia coli* phosphofructokinase 2: evidence for an allosteric site for MgATP⁻². *Biochemistry* 37, 13269–13275.
- [10] Guixé, V. and Babul, J. (1988) Influence of ligands on the aggregation of the normal and mutant forms of phosphofructokinase 2 of *Escherichia coli*. *Arch. Biochem. Biophys.* 264, 519–524.
- [11] Guixé, V. (2000) Chemical modification of SH groups of *E. coli* phosphofructokinase-2 induces subunit dissociation: monomers are inactive but preserve ligand binding properties. *Arch. Biochem. Biophys.* 376, 313–319.
- [12] Báez, M., Rodríguez, P.H., Babul, J. and Guixé, V. (2003) Structural and functional roles of cysteines 238 and 295 in *Escherichia coli* phosphofructokinase-2. *Biochem. J.* 376, 277–283.
- [13] Daldal, F. (1983) Molecular cloning of the gene for phosphofructokinase-2 of *Escherichia coli* and the nature of a mutation, pfkB1, causing a high level of the enzyme. *J. Mol. Biol.* 168, 285–305.
- [14] Tabor, S. and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Nat. Acad. Sci. USA* 82, 1074–1078.
- [15] Cabrera, R., Fischer, H., Trapani, S., Craievich, A.F., Garrat, R.C., Guixé, V. and Babul, J. (2003) Domain motions and quaternary packing of phosphofructokinase-2 from *Escherichia coli* studied by Small Angle X-ray Scattering and homology modelling. *J. Biol. Chem.* 278, 12913–12919.
- [16] Inlow, J.K. and Baldwin, T.O. (2002) Mutational analysis of the subunit interface of *Vibrio harveyi* bacterial luciferase. *Biochemistry* 41, 3906–3915.
- [17] Torres, J.C., Guixé, V. and Babul, J. (1997) A mutant phosphofructokinase produces a futile cycle during gluconeogenesis in *Escherichia coli*. *Biochem. J.* 327, 675–684.
- [18] Vargo, M.A., Nguyen, L. and Colman, R. (2004) Subunit interface residues of glutathione S-transferase A1-1 that are important in the monomer-dimer equilibrium. *Biochemistry* 43, 3327–3335.