Structural and functional roles of Cys-238 and Cys-295 in *Escherichia coli* phosphofructokinase-2

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Modification of *Escherichia coli* phosphofructokinase-2 (Pfk-2) with pyrene maleimide (PM) results in a rapid inactivation of the enzyme. The loss of enzyme activity correlates with the incorporation of 2 mol of PM/mol of subunit and the concomitant dissociation of the dimeric enzyme. The two modified residues were identified as Cys-238 and Cys-295. In the presence of the negative allosteric effector, MgATP, Cys-238 was the only modified cysteine residue. Kinetic characterization of the Cys-238-labelled Pfk-2 indicates that the enzyme is fully active, with the kinetic constants (K_m , k_{cat}) being almost identical to the ones obtained for the native enzyme. The modified enzyme is a monomer in the absence of ligands and, like the native enzyme, behaves as a tetramer in the presence of the nucleotide. However, in the presence of fructose-6-phosphate (fru-6-P) and ATP⁻⁴,

the enzyme behaves as a dimer, suggesting that the monomers undergo re-association in the presence of the substrates and that the active species is a dimer. Modification of Pfk-2 with eosin-5-maleimide (EM) results in the labelling of Cys-295. This modified enzyme is inactive and is not able to bind to the allosteric effector, remaining as a dimer in its presence. Nonetheless, Cys-295-labelled Pfk-2 is able to bind to the substrate fru-6-P in an hyperbolic fashion with a K_d value that is 6-fold higher than the one determined for the native enzyme. These are the first residues to be implicated in the activity and/or structure of the Pfk-2.

Key words: allosteric regulation, eosin-5-maleimide, phosphofructokinase-2, pyrene N-(1-pyrenyl) maleimide, SH group.

INTRODUCTION

Phosphofructokinase (PFK, EC. 2.7.1.11) is a central enzyme of glycolysis and is found in almost all organisms, from bacteria to higher eukaryotes. Owing to its important role in the control of glycolytic flux and its allosteric properties, PFK has long been a subject of study in terms of both its enzymology and protein chemistry. The enzyme catalyses the conversion of fructose 6-phosphate (fru-6-P) into fructose 1,6-bisphosphate (fru-1,6-bisP), with associated consumption of ATP. In *Escherichia coli*, two different enzymes catalyse this reaction: a Pfk-1, which represents 90 % of the total activity and Pfk-2, which accounts for the rest. Pfk-1 has been studied extensively: it exhibits sigmoidal kinetics with fru-6-P and is subject to allosteric regulation of its activity by different effectors [1–3]. This laboratory has been engaged in the structural and kinetic characterization of the Pfk-2 isozyme, which exhibits hyperbolic kinetics with both substrates and inhibition by MgATP [4,5]. The nucleotide inhibition is a consequence of its binding to an allosteric site, whose presence has been demonstrated through intrinsic fluorescence studies [6]. Another difference between the two isoenzymes is their quaternary structure. Pfk-1, like most of the phosphofructokinases studied, is a tetramer, while Pfk-2 is a dimer which can be converted into a tetramer through the binding of MgATP to the allosteric site [7,8]. Some PP_i-dependent PFKs from bacteria and Archea have been reported recently to exist as dimers [9–12].

Also, Pfk-1 and Pfk-2 differ as to the order in which the substrates are bound to and the products are released from the enzyme. Pfk-2 has an ordered Bi Bi mechanism, with fru-6-P being the first substrate to bind and fru-1,6-bisP the last product

to be released [4]. Nonetheless, a random, non-rapid equilibrium mechanism has been proposed for the Pfk-1 enzyme [13].

The primary structure of Pfk-2, as predicted from the nucleotide sequence, shows no significant relationship to the family to which Pfk-1 belongs, the PfkA family. Instead, Pfk-2 belongs to the PfkB family, which includes several kinases, such as fru-6-P kinase, fructose-1-phosphate kinase, adenosine kinase, tagatose kinase, ribose kinase and inosine kinase [14,15]. The protein fold in this family consists of two domains: a major α/β domain and a small domain. Based on the known structure of ribokinase and using homology modelling, our group built the tertiary structure of the dimeric form of Pfk-2. Furthermore, the relative orientation of the domains in the Pfk-2 model was refined using experimental data from small angle X-ray scattering. The results show that binding of fru-6-P generates a more compact structure and that the binding of MgATP produces both a tetramer formation and changes at the tertiary structure level. These results allowed us to propose a mechanism for the inhibitory effect of MgATP [16].

However, since the detailed three-dimensional structure of Pfk-2 is not known at present, no roles for any particular amino acid in enzyme function or structure have been identified. Recently, we have reported the specific modification of Pfk-2 with a fluorescent sulfhydryl reagent, pyrene N-(1-pyrenyl) maleimide (PM), with a stoichiometry of 2.03 ± 0.04 mol of the reagent/mol of subunit. This modification causes the inactivation of the enzyme and its dissociation from dimer to monomer [17]. In this report, we identify the modified cysteine residues and study the effects of the modification of each amino-acid residue on the catalytic activity and the oligomeric structure of the enzyme. The results clarify the roles of these residues in both catalysis and subunit interactions:

Abbreviations used: DTT, dithiothreitol; EM, eosin 5-maleimide; fru-1,6-bisP, fructose 1,6-bisphosphate; fru-6-P, fructose 6-phosphate; Pfk, *Escherichia coli* phosphofructokinase; PFK, generic phosphofructokinase; PM, pyrene N-(1-pyrenyl) maleimide.

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Cys-238 is a main structural determinant for oligomerization of Pfk-2 and the modification of Cys-295 completely abolishes the enzymic activity and binding of the allosteric effector, without altering the dimeric structure of the Pfk-2 enzyme.

EXPERIMENTAL

Enzyme purification

The enzyme was purified from the *E. coli* strain DF 903 essentially as described by Babul [2].

Reagent concentrations

ATP and fru-6-P concentrations were estimated spectrophotometrically by measuring the amount of fru-1,6-bisP formed in the presence of NADH, using aldolase, triosephosphate isomerase and α -glycerophosphate dehydrogenase as auxiliary enzymes. The concentration of NADH was calculated using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Protein concentration was determined as described by Bradford [18] using bovine serum albumin as a standard.

Enzyme activity

PFK activity was determined spectrophotometrically by coupling the fru-1,6-bisP formation to the oxidation of NADH as described previously [5].

Chemical modification of Pfk-2 and stoichiometry of labelling

Pfk-2 was modified with PM essentially as described by Guixé [17], except for the addition, in some cases, of MgATP in the reaction medium. Labelling of cysteine residues (Cys-238 and Cys-295) was achieved by incubating Pfk-2 solutions with a 4-fold molar excess of PM in the absence of ligands. Specific modification of Cys-238 was achieved by incubating the protein with an equimolar ratio of Pfk-2/PM, but in the presence of 1 mM MgATP. Specific labelling of Cys-295 was difficult to achieve owing to the similar reactivity of both cysteine residues with PM. For this purpose, we tested several probes and found that labelling of this residue was possible with eosin 5-maleimide (EM). Pfk-2, at a concentration of approx. 1 mg/ml, was labelled with EM using variable amounts of the probe at 0 °C in the dark, in the presence of 20 mM Hepes, pH 8.0, 0.1 mM EDTA and 5 mM MgCl₂. At appropriate intervals, aliquots were diluted 20-fold in the presence of 5 mM DTT (dithiothreitol), and then assayed for enzymic activity.

The extent of labelling with PM was determined by measuring the absorption of the pyrene moiety after removing the non-reacted reagent by filtration through a Sephadex G-25 column (NAP-5; Amersham Pharmacia Biotech AB) and determining its concentration, assuming a molar extinction coefficient of $4 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 344 nm. When EM was used, the degree of labelling was determined as described above, except that a molar extinction coefficient of $9.68 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 520 nm was employed.

Fluorescence measurements

Fluorescence emission spectra were recorded at room temperature in a PerkinElmer LS 50 fluorimeter. The excitation wavelength was 344 nm, and excitation and emission slits were set at 5 nm. Background readings were subtracted and corrections were made to compensate for protein dilution. Acrylamide quenching experiments were performed in 20 mM Hepes, 0.1 mM EDTA, pH 8.0 and 1 mM MgATP (or in the absence of MgATP). The quenching

data were analysed using the Stern–Volmer plots ($F_0/F = 1 + K_{sv}[Q]$), where F_0 and F are the fluorescence intensities in the absence and presence of the quenching agent respectively, K_{sv} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of the quencher.

Size-exclusion chromatography of native and modified Pfk-2

Size-exclusion chromatography experiments were performed with a Waters 1525 HPLC binary pump system, with a TSK 2000 (7.8 mm $\times$ 30 cm) column (Tosohass, Montgomeryville, PA, U.S.A.) equilibrated in buffer (25 mM Tris–HCl, pH 7.5, 5 mM MgCl₂, 200 mM KCl) and the various MgATP concentrations, at a flow rate of 1 ml/min. The column was calibrated with the following molecular-mass markers: carbonic anhydrase (29 000), alcohol dehydrogenase (150 000), β -amylase (200 000) and thyroglobulin (669 000). Protein elution was recorded with the use of an online Waters 2487 UV dual detector that measured simultaneously the absorbance at 280 and 344 nm for PM-labelled peptides, or at 280 and 520 nm for EM-labelled peptides, unless indicated otherwise.

Proteolysis of the modified Pfk-2 enzymes

A 2 mg/ml solution of the PM- or EM-modified Pfk-2 was denatured for 3 h with 6 M guanidine hydrochloride and 1 mM DTT at 37 °C. After this treatment, the non-reacting cysteine residues were blocked by reacting them with 10 mM iodoacetamide for 3 h at 37 °C in darkness. The reaction was stopped with 1 % (v/v) β -mercaptoethanol, the sample dialysed overnight against water and the pH was adjusted to 8.2 with ammonium bicarbonate. The concentrated protein solution was digested with trypsin, which was added at a final ratio of 1:40 (trypsin/Pfk-2), made in three successive additions, for 24 h at 37 °C.

Separation, purification and sequencing of the labelled peptides

The samples digested with trypsin were resuspended in 0.1 % (v/v) trifluoroacetic acid and the purification of the soluble peptide fragments were carried out by HPLC using a C-18 BioRad RP-318 (4.6 mm $\times$ 250 mm) reverse-phase column at a flow rate of 1 ml/min. Eluent A was 0.1 % (v/v) aqueous trifluoroacetic acid and eluent B was 0.1 % (v/v) trifluoroacetic acid in acetonitrile. The column was equilibrated with eluent A and chromatography was carried out with the following gradient: $T_0 = 100\%$; $T_3 = 85\%$; $T_{13} = 80\%$; $T_{28} = 70\%$; $T_{53} = 60\%$; $T_{83} = 48\%$; $T_{93} = 20\%$ (subscript numbers correspond to time, in min; percentages corresponds to solution A). Peptide elution was monitored by following the absorbance at 214 and 344 nm for the PM-labelled peptides or at 214 and 520 nm for the EM-labelled peptides. The fractions containing the labelled peptides were collected manually and purified further by application to a Beckman C-18 (4.6 mm $\times$ 250 mm) reverse-phase column that was eluted with the above gradient. The amino-acid sequence of each labelled peptide was determined by Edman degradation using an automated protein sequencer (Applied Biosystems International).

[U-¹⁴C]fru-6-P synthesis

[U-¹⁴C]fructose (150 μ Ci) was mixed with 1 mM ATP, 25 mM Tris–HCl, pH 8.2, 10 mM MgCl₂ and 15 m-units of hexokinase. The mixture was incubated for 30 min at 37 °C and the reaction stopped by heating for 4 min at 95 °C. After centrifugation, the supernatant was analysed for purity by HPLC on a CarboPac PA1 column and purified further on a Mono Q column.

Figure 1 Incorporation of PM into Pfk-2 and inactivation of the enzyme

The enzyme subunit (22 μ M) was incubated with different amounts of PM (0.11–88 μ M) for 20 min at 4 °C. At the end of the incubation period, aliquots were diluted 5-fold in the presence of 5 mM DTT and then assayed to determine enzymic activity and the level of incorporation of PM, as described in the Experimental section.

Binding of [U- 14 C]fru-6-P to native and Cys-295-modified Pfk-2

Binding analyses were performed in a micropartition kit (Amicon, Beverly, MA, U.S.A) as follows: 500 μ l of 11.4 μ M protein, either native or Cys-295-modified Pfk-2, was incubated at 25 °C with different concentrations of [U- 14 C]fru-6-P (3.2–200 μ M) for 30 min in 40 mM Hepes buffer, pH 8.0, 5 mM DTT and 10 mM MgCl₂. Bound and free labelled fru-6-P were separated by centrifugation for 2 min at 1000 *g* and aliquots removed from the supernatant and filtrate, and counted in a Packard 1600 TR liquid-scintillation analyzer. Binding data were analysed using the following equation:

$$[\text{fru-6-P}]_{\text{bound}} = \text{maximal binding } [\text{fru-6-P}]_{\text{free}} / (K + [\text{fru-6-P}]_{\text{free}})$$

where *K* represents the dissociation constant. After curve fitting, *n*, the number of ligand binding sites per monomer of enzyme was calculated from:

$$n = \text{maximal binding} / \text{Et}$$

where Et represents the total subunit concentration.

Binding of [U- 14 C]ATP to native and Cys-295-modified Pfk-2

[U- 14 C]-labelled ATP was purchased from Du Pont NEN Products, Boston, MA, U.S.A. For the native enzyme, binding experiments were performed using the Hummel-Dryer method in a TSK column (Tosohass), which was equilibrated with different MgATP concentrations. However, since the Cys-295-modified enzyme presented some problems in the gel-filtration experiments and considering that this enzyme is unable to form tetramers in the presence of MgATP, we employed the micropartition method described above to estimate MgATP binding. For the native enzyme, binding data were analysed using the Scatchard equation.

Figure 2 Reverse-phase HPLC resolution of labelled tryptic peptides

(A) Pfk-2 modified with PM in the absence of ligands. (B) Pfk-2 modified with PM in the presence of 1 mM MgATP. (C) Pfk-2 modified with EM in the absence of ligands. Modification conditions are described in the Experimental section. All samples were digested with trypsin and the resulting peptides were separated on a C-18 reverse-phase column using a gradient of acetonitrile in 0.1% trifluoroacetic acid. The absorbances of 344 or 520 nm were used to monitor the PM- and EM-containing peptides respectively.

RESULTS AND DISCUSSION

Identification of the PM-modified cysteine residues

As reported previously [17], reaction of Pfk-2 with PM in the absence of ligands resulted in the inactivation of the enzyme, the modification of 2 mol of SH/mol of subunit, and the dissociation of the dimeric enzyme. As shown in Figure 1, complete inactivation is achieved when 2 mol of PM are incorporated per mol of subunit. The modification of both residues and the accompanying loss of activity are characterized by a single rate-constant. Therefore, interpretation of what causes the inactivation of the enzyme is complicated by both the formation of two PM adducts per subunit and the destabilization of the dimeric enzyme. Since there is no evidence as to whether the unmodified monomeric enzyme is catalytically active, it is not possible to attribute enzyme inactivation to the modification of a catalytically important group or to the dissociation process induced by incorporation of

Table 1 Sequence of Pfk-2 labeled peptides

Sequence of labelled peptides generated by modification of Pfk-2 with PM in the absence and in the presence of 1 mM MgATP, and with EM in the absence of MgATP, followed by trypsin digestion. Peptides were separated and purified as described in the Experimental section. The percentage of acetonitrile required to elute each peptide is indicated. The numbers at the beginning and at the end of the sequence indicate the position of the respective amino-acid residues in the primary structure of the enzyme and X represents the modified residue. The sequences presented correspond to the number of sequenced residues and are partial sequences of longer peptides.

Modification conditions	Peak	Acetonitrile for elution (%)	Peptide sequence
PM, no ligands	I	28	L ₂₉₄ XSHDDTQ ₃₀₁
	II	36	V ₂₂₁ VVSLGPQ ₂₂₈
PM + 1 mM MgATP	II	36	V ₂₂₁ VVSLGPQGALGVDS _{ENX} I ₂₃₉
EM, no ligands	I	30	L ₂₉₄ XSHDDTQ ₃₀₁

the probe. To identify the amino-acid residues involved, the PM-modified enzyme (2 mol of SH/mol of subunit) was subjected to proteolytic degradation by trypsin, as described in the Experimental section, and the resulting peptide mixture was separated by reverse-phase HPLC. The proteolytically cleaved protein separated into two major labelled peaks (I and II in Figure 2A) each of which was composed of two closely migrating components. Peaks I and II were collected and repurified by reverse-phase chromatography in a different column, thereby making it possible to collect the individual components of both peaks. Amino-acid sequence determinations of the two components of peak I showed identical sequences and the modified amino-acid residue was identified as Cys-295. The two components of peak II also showed identical sequences and in this case Cys-238 was the labelled residue (Table 1). The origin of the two components of the PM-labelled peaks may be explained on the basis of partial succinimido-ring cleavage during the digestion of the labelled enzyme samples by trypsin, which leads to peptides with different

elution times, as observed with PM-modified peptides from phosphoenolpyruvate carboxykinase and inositol monophosphatase [19,20].

Differential labelling of Cys-238 and Cys-295

In order to determine the differential role of each cysteine residue in the inactivation and dissociation of Pfk-2, we searched for conditions under which only one of them would be labelled. This goal was achieved by including 1 mM MgATP in the PM modification reaction (1.1 ± 0.05 mol PM/mol subunit) or by using a different probe, EM, in the absence of ligands (1.1 ± 0.15 mol of EM/mol of subunit). The enzyme modified with PM in the presence of MgATP retained full activity, but the EM-modified enzyme was inactivated rapidly (Figure 3A). Correlation between the loss of enzymic activity and the number of cysteine residues modified with EM showed that complete inactivation was observed when only one residue was modified (Figure 3B).

Figures 2(B) and 2(C) show the reverse-phase chromatograms of the proteolysed protein that was modified under the conditions described above. After labelling Pfk-2 with PM in the presence of 1 mM MgATP (Figure 2B) the HPLC profile shows that the extent of labelling of peak I was greatly decreased, with labelling occurring almost exclusively in peak II. Comparison of the sequence obtained from the labelled peptide with the predicted sequence of Pfk-2 showed that it corresponds to amino-acid residues 221–239 and that the PM moiety was attached covalently to Cys-238 (Table 1). On the other hand, modification of Pfk-2 with EM in the absence of ligands resulted mainly in one labelled peak (Figure 2C) which contained Cys-295 (Table 1).

Effect of Cys-238 modification on the kinetic properties and quaternary structure of Pfk-2

As shown above, reaction of Pfk-2 with PM in the presence of the nucleotide ligand results in the modification of only one residue, Cys-238. In order to study the effect of this modification on the

Figure 3 Modification of Pfk-2 with PM in the presence of MgATP and the stoichiometry of inactivation of Pfk-2 by EM in the absence of ligands

(A) Pfk-2 (38 μ M subunit) was incubated with 76 μ M EM at 4 °C in darkness in the absence of ligands (●) and with 152 μ M PM in the presence of 1 mM MgATP (○). At various time intervals, samples were withdrawn for determining residual activity. (B) The enzyme subunit (20 μ M) was incubated with different amounts of EM (19–152 μ M) for 20 min at 4 °C. Aliquots were assayed for residual activity and stoichiometry of labelling as described in the Experimental section.

Table 2 Kinetic constants for native and Cys-238-modified Pfk-2

Kinetic constant	Pfk-2	Cys-238 Pfk-2
K_m ATP (μ M)	42	44
K_m fru-6-P (μ M)	58	63
k_{cat} (s^{-1})	9.24×10^3	9.32×10^3

kinetic properties of the enzyme, the stability of the modified enzyme was assayed under two conditions: the enzyme maintained in the presence of 1 mM MgATP after modification and the enzyme with MgATP removed after modification. Under both conditions, the enzyme maintained approx. 90 % of the original enzymic activity after 24 h. The modified enzyme also displays the same MgATP inhibition as the native enzyme (results not shown). The kinetic constants, k_{cat} and K_m for the substrates MgATP and fru-6-P, show almost identical values for native Pfk-2 and the Cys-238-modified enzyme, demonstrating that this residue is not involved in substrate-binding and/or catalysis (Table 2).

Since Cys-238-modified Pfk-2 is a fully active enzyme with its allosteric site unaltered, we asked if this modification affects the quaternary structure of the enzyme. Size exclusion chromatography of the modified enzyme in the absence of the nucleotide shows the presence of a monomer (Figure 4B), conditions under which the native enzyme is a dimer (Figure 4A). On the other hand, the modified enzyme in the presence of MgATP behaves as a tetramer, i.e. like the native enzyme (Figures 4A and 4C). The presence of 1 mM fru-6-P in the equilibrating buffer does not promote the dimerization of the enzyme, although the elution peak sharpens (results not shown). Considering that the modified enzyme is a monomer, but that it retains enzymic activity, it is possible that the active species is monomeric or that the activity is due to re-association of the monomers in the assay medium. In order to avoid the problems that arise from the simultaneous presence of substrates and products in the incubation medium, we produced an enzyme ternary complex by using fru-6-P and ATP⁻⁴. ATP⁻⁴ acts as a strong competitive inhibitor of Pfk-2, with respect to MgATP at the catalytic site [5]. Under these conditions (ternary-complex formation), the Cys-238 modified enzyme elutes as a dimer in size-exclusion chromatography experiments (Figure 4D), a result that supports the idea that the enzyme activity observed with the modified enzyme probably corresponds to the dimeric species formed by re-association of monomers induced by the presence of substrates.

Solvent accessibility of PM-Cys-238 in monomeric and tetrameric Pfk-2

Solute quenching of protein fluorescence is a technique that can yield information regarding the solvent exposure of the intrinsic or extrinsic fluorophore. In order to test the solvent accessibility of the PM probe that covalently attaches to tetrameric and monomeric Pfk-2, we studied the quenching of the pyrene moiety attached to the cysteine-modified enzyme by neutral acrylamide in the absence and in the presence of MgATP (Figure 5). A linear relationship between fluorescence intensity and quenching reagent concentration was observed, which indicates a single class of fluorophores that are all equally accessible to the quencher. The calculated Stern–Volmer constants are $3.1 M^{-1}$ and $0.47 M^{-1}$ for the enzyme in the absence and in the presence of MgATP respectively, indicating that the probe is less easily quenched in the tetramer and is therefore considerably shielded from the solvent.

Figure 4 Size-exclusion chromatography of PM-Cys-238-modified Pfk-2 in the absence and in the presence of MgATP

(A) Unmodified Pfk-2 in the absence of ligands (peak D) and in the presence of 0.8 mM MgATP (peak T). (B) Cys-238-modified Pfk-2 in the absence of ligands (peak M). (C) Cys-238-modified Pfk-2 in the presence of 0.8 mM MgATP (peak T). (D) Cys-238-modified Pfk-2 in the presence of 0.8 mM fru-6-P and 0.8 mM ATP⁻⁴ (peak D). The column was equilibrated and eluted at the indicated concentrations of ligands and the enzyme was pre-incubated under the same conditions. M, D and T denote monomer, dimer and tetramer respectively.

Characterization of EM-Cys-295-modified Pfk2

In order to study the effect of Cys-295 modification on the quaternary structure of Pfk-2, we performed size-exclusion chromatography experiments of the EM-modified enzyme in the absence and in the presence of MgATP. Under both conditions the enzyme elutes as a dimer (Figures 6A and 6B). Since the absence of tetramer could be due to loss of the MgATP-binding capacity of the modified enzyme, we assayed MgATP binding to the native and modified enzymes (Figure 6C). The results show that the native enzyme binds co-operatively the metal–nucleotide

Figure 5 Fluorescence quenching of PM-Cys-238-labelled Pfk-2

Stern-Volmer plots of PM fluorescence quenching. Various concentrations of acrylamide were added sequentially to solutions containing PM-labelled Pfk-2, in the absence of ligands (●) or in the presence of 1 mM MgATP (■). The decrease in fluorescence intensity was measured after each quencher addition. The curves were obtained by fitting the data to the Stern-Volmer equation, as described in the Experimental section.

complex, with a dissociation constant of $70 \mu\text{M}$, which is in good agreement with the value observed by intrinsic fluorescence measurements [6] and light-scattering experiments [8]. However, the Cys-295-modified enzyme does not bind the allosteric effector, which explains the absence of tetramer formation observed in the presence of MgATP.

On the other hand, in order to find out if the lack of enzymic activity was due to impairment in substrate binding, we studied

Figure 7 Binding of [^{14}C]fru-6-P to EM-Cys-295-modified Pfk-2

Binding of fru-6-P to native (●) and Cys-295-modified Pfk-2 (○) was performed and analysed as described in the Experimental section.

the binding of fru-6-P in native and in Cys-295-modified Pfk-2 (Figure 7). Both enzymes bind the substrate in an hyperbolic fashion (with n -values of 1.0 and 0.83 for the native and modified enzymes respectively), in accordance with the kinetic data. Nevertheless, the dissociation constant obtained for the modified enzyme ($12.4 \mu\text{M}$) was 6-fold higher than the one obtained for the native enzyme ($2.0 \mu\text{M}$). The value obtained for the native enzyme is in good agreement with the value of $6 \mu\text{M}$ obtained for the binding of fru-6-P to native Pfk-2 through intrinsic fluorescence measurements [6].

Figure 6 Aggregation state and [^{14}C]MgATP binding of EM-Cys-295-modified Pfk-2

(A) HPLC elution of native Pfk-2 in the absence of ligands. (B) Elution of native (---) and modified (—) Pfk-2 in the presence of 1 mM MgATP. Protein elution was followed at 280 nm. (C) Binding of [^{14}C]MgATP to native (●) and Cys-295-modified Pfk-2 (○).

The absence of labelling of Cys-238 when the enzyme is modified with EM may be explained by the fact that this probe has a more polar character than PM, which supports the idea that Cys-238 is located in a hydrophobic environment, probably at the subunit interface. The loss of activity upon Cys-295 modification may be due to the location of this residue in or near to the active site, or to steric hindrance of the bulky eosin moiety. However, ligand binding studies demonstrate that the modified enzyme is able to bind fru-6-P, a result that supports the idea that Cys-295 probably has a catalytic role. Another possibility may be that modification of this residue produces a conformational change that locks the enzyme in an already inactive conformation similar to that obtained by binding of the allosteric effector, since the metal-nucleotide complex is not able to bind to the modified enzyme.

The molecular model of dimeric Pfk-2 shows that Cys-238 and Cys-295 are located in the major domain and that both are accessible to the solvent. Cys-295 is located in the vicinity of the active site and its modification can potentially inactivate the enzyme. Interestingly, this residue also lies near the subunit interface in the tetramer and its chemical modification in the dimeric state of the enzyme should prevent the tetramerization observed upon MgATP binding. On the other hand, Cys-238 is located in a β -strand just before a big loop that surrounds the nucleotide site. However, its location does not allow us to extrapolate a role for this residue in the maintenance of the dimeric form of the enzyme.

In summary, the chemical-modification studies presented here show that Cys-238 is a main structural determinant for oligomerization of Pfk-2, and that the modification of Cys-295 completely abolishes the enzymic activity, without altering the dimeric structure of the enzyme and its fru-6-P-binding capacity. These results will be complemented with site-directed mutagenesis studies in order to allow better understanding of the role of these residues in the structure and function of this enzyme.

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REFERENCES

- Hellinga, H. W. and Evans, P. R. (1985) Nucleotide sequence and high level expression of the major *Escherichia coli* phosphofructokinase. *Eur. J. Biochem.* **149**, 363–373
- Babul, J. (1978) Phosphofructokinases from *Escherichia coli*: purification and characterization of the nonallosteric isozyme. *J. Biol. Chem.* **253**, 4350–4355
- Blangy, D., Buc, H. and Monod, J. (1968) Kinetics of allosteric interactions of phosphofructokinase from *Escherichia coli*. *J. Mol. Biol.* **31**, 13–35
- Campos, G., Guixé, V. and Babul, J. (1984) Kinetic mechanism of phosphofructokinase-2 from *Escherichia coli*. *J. Biol. Chem.* **259**, 6147–6152
- 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
- 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. *Biochemistry* **37**, 13269–13275
- 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
- Cabrera, C., Guixé, V., Alfaro, J., Rodríguez, P. H. and Babul, J. (2002) Ligand-dependent structural changes and limited proteolysis of *E. coli* phosphofructokinase-2. *Arch. Biochem. Biophys.* **406**, 289–295
- Ding, Y. R., Ronimus, R. S. and Morgan, H. W. (2000) Sequencing, cloning and high level expression of the *pfk* gene, encoding a PPI-dependent phosphofructokinase from the extremely thermophilic eubacterium *Dictyoglomus thermophilum*. *J. Bacteriol.* **182**, 4661–4666
- Siebers, B., Klenk, H. P. and Hensel, R. (1998) PPI-dependent phosphofructokinase from *Thermoproteus tenax*, an archeal descendant of an ancient line in phosphofructokinase evolution. *J. Bacteriol.* **180**, 2137–2143
- Ding, Y. R., Ronimus, R. S. and Morgan, H. W. (2001) *Thermotoga maritima* phosphofructokinases: expression and characterization of two unique enzymes. *J. Bacteriol.* **183**, 791–794
- Deng, Z., Roberts, D., Wang, X. and Kemp, R. G. (1999) Expression, characterization, and crystallization of the pyrophosphate-dependent phosphofructo-1-kinase of *Borrelia burgdorferi*. *Arch. Biochem. Biophys.* **371**, 326–331
- Wang, X. and Kemp, R. G. (2001) Reaction path of phosphofructo-1-kinase is altered by mutagenesis and alternative substrates. *Biochemistry* **40**, 3938–3942
- Bork, P., Sander, C. and Valencia, A. (1993) Convergent evolution of similar enzymatic function on different protein folds: the hexokinase, ribokinase, and galactokinase families of sugar kinases. *Protein. Sci.* **2**, 31–40
- Spychala, J., Datta, N., Takabayashi, K., Datta, M., Fox, I., Gribbin, T. and Mitchell, B. (1996) Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1232–1237
- 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 modeling. *J. Biol. Chem.* **278**, 12913–12919
- 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
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- Krautwust, H., Encinas, M. V., Marcus, F., Latshaw, S. T., Kemp, R. G., Frey, P. A. and Cardemil, E. (1995) *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase: revised amino acid sequence, site-directed mutagenesis, and microenvironment characteristics of cysteines 365 and 458. *Biochemistry* **34**, 6382–6388
- Greasley, P. J., Hunt, L. G. and Gore, M. G. (1994) Bovine inositol monophosphatase. Ligand binding to pyrene-maleimide-labelled enzyme. *Eur. J. Biochem.* **222**, 453–460

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