Domain Motions and Quaternary Packing of Phosphofructokinase-2 from *Escherichia coli* Studied by Small Angle X-ray Scattering and Homology Modeling*

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The binding of MgATP and fructose-6-phosphate to phosphofructokinase-2 from *Escherichia coli* induces conformational changes that result in significant differences in the x-ray-scattering profiles compared with the unligated form of the enzyme. When fructose-6-phosphate binds to the active site of the enzyme, the pair distribution function exhibits lower values at higher distances, indicating a more compact structure. Upon binding of MgATP to the allosteric site of the enzyme, the intensity at lower angles increases as a consequence of tetramer formation, but differences along higher angles also suggest changes at the tertiary structure level. We have used homology modeling to build the native dimeric form of phosphofructokinase-2 and fitted the experimental scattering curves by using rigid body movements of the domains in the model, similar to those observed in known homologous structures. The best fit with the experimental data of the unbound protein was achieved with open conformations of the domains in the model, whereas domain closure improves the agreement with the scattering of the enzyme-fructose-6-phosphate complex. Using the same approach, we utilized the scattering curve of the phosphofructokinase-2-MgATP complex to model the arrangement and conformation of dimers in the tetramer. We observed that, along with tetramerization, binding of MgATP to the allosteric site induces domain closure. Additionally, we used the scattering data to restore the low resolution structure of phosphofructokinase-2 (free and bound forms) by an *ab initio* procedure. Based on these findings, a proposal is made to account for the inhibitory effect of MgATP on the enzymatic activity.

Oligomeric enzymes frequently require conformational changes within or between subunits for their activity and regulation. Furthermore, allosteric ligands may affect enzymatic activity by means of conformational changes to the tertiary and/or quaternary structure, which may result in metabolic pathway regulation.

Phosphofructokinase activity, the ATP-dependent phosphorylation of fructose-6-P, is an important step in the glycolytic pathway that is subject to strict regulation in a wide variety of organisms. In *Escherichia coli* this activity is accomplished by two isozymes that differ in their kinetic and structural properties. Phosphofructokinase-1 (Pfk-1) has been extensively characterized and belongs to the PfKα protein family that includes higher eukaryotic ATP-dependent phosphofructokinases ATP- and pyrophosphate-dependent bacterial and plant phosphofructokinases (1). The atomic structure of PfK-1 has been solved by x-ray crystallography (2); the enzyme is a homotetramer whose state of aggregation does not change upon ligand binding. The other isozyme, phosphofructokinase-2 (Pfk-2), presents inhibition by the substrate MgATP when the assay is performed at low fructose-6-P concentrations (3). Fluorescence studies demonstrated that MgATP inhibition occurs upon binding of MgATP to an allosteric site in Pfk-2 (4). Also, binding of MgATP promotes oligomerization of Pfk-2, changing from a dimer to a tetramer (5–7). Such features make Pfk-2 an excellent model to study allosteric regulation linked to protein aggregation and enzymatic inhibition.

Pfk-2 has no conserved patterns of sequence associated with the PfKα protein family. However, Pfk-2 is related to the PfKB superfamily of sugar kinases which includes ribokinases, adenosine kinases, fructokinases, and possibly, ADP-dependent glucokinases and phosphofructokinases (8, 9). Several crystal structures have been solved for members of this family (9–12) showing that the overall fold is strongly conserved. For example, the root mean square deviation for superimposed residues between *E. coli* ribokinase and human adenosine kinase is 2.4 Å even though the sequence identity among them is only 22% (11). The protein fold in this superfamily consists of two domains, a large α/β domain and a small domain. In ribokinase this small domain is a β-sheet that acts as a lid over the active site and also as the dimerization interface. Adenosine kinases have α-helical insertions in the small domain so that dimerization is precluded.

The comparison of the free and sugar-bound forms of riboki- nase and adenosine kinase structures reveals an important conformational change that can be described as a hinge bending motion, with one domain rotating toward the other by an angle of 17–30° (12, 13). The hinge-like movement helps bind-

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* This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico, Chile Grant 1010648 and Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil Grants 99/09471-7, 01/07798-0, and 98/14138-2. We also thank Programa de Apoio a Núcleos de Excelência (Ministerio de Ciencia e Tecnología, Brazil), Brazil and National Synchrotron Light Laboratory (Campinas, Brazil) for financial support. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: fructose-6-P, fructose-6-phosphate; Pfk, phosphofructokinase; SANS, small-angle x-ray scattering; COM, center of mass.
ing of the second substrate (MgATP) and further catalysis. In Pfk-2, the binding of fructose-6-P promotes a conformational change, as indicated by a 30% increment in the fluorescence emission of the single tryptophan (Trp-88) (4) and enables the subsequent binding of MgATP to the active site, as indicated by the ordered bi-reaction mechanism of Pfk-2 in which MgATP can bind the active site only after fructose-6-P binding (14). On the other hand, when MgATP is bound to the allosteric site, fluorescence quenching with a blue shift in the emission maximum is observed. It is not known whether this last effect can be accounted for by the observed quaternary structural transition only or if it also involves tertiary structural changes.

To determine the effects of fructose-6-P binding to the active site and MgATP binding to the allosteric site on the tertiary and quaternary structure of Pfk-2, we used homology modeling combined with small angle x-ray scattering (SAXS), a technique sensitive to shape and oligomeric state changes. In this work, SAXS data have been used to detect shape changes upon fructose-6-P binding that have been interpreted in terms of quasi-rigid domain movements within the dimeric model of Pfk-2. Also, SAXS data have been used to detect changes in the aggregation state of the enzyme upon MgATP binding, and a model for the subunit arrangement in the tetramer is suggested. Based on our results, a mechanism for the inhibitory effect of MgATP is proposed.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—pET 21-d plasmid (Novagen) containing the cloned original gene (15) was transformed into E. coli strain JM109, and 2 liters of culture were grown in Luria Broth medium supplemented with ampicillin to a final concentration of 100 mg/ml. Protein expression was induced at 1 hr of induction, cells were collected by centrifugation, and the radii of gyration, defined by

$$R_g = \frac{\sum Z_i r_i^2}{\sum Z_i}^{1/2}$$

(2)

where $Z_i$ is the atomic number of the atom located at a distance $r_i$ from the electronic center of mass. The radii of gyration of the Pfk-2 molecules in their different states were calculated from the slopes of the Guinier plots (In $I(q)$ versus $q^2$).

**Ab Initio Shape Determination**—The resolution of the resulting solution x-ray-scattering curve extended to 11.4 Å. The low resolution protein shape was restored using the ab initio procedure described by Svergun (25) as implemented in the program GASBOR. In this method, a dummy residue model is generated by a random-walk Cc chain and is folded in a way to minimize the discrepancy between the calculated scattering curve from the model and the experimental data. The program simulates the protein internal structure, which makes it unnecessary to subtract a constant from the experimental data to ensure Porod’s law. Several runs of ab initio shape determination with different starting conditions $k$ were performed. Purification was used to detect shape changes upon MgATP binding that have been interpreted in terms of quasi-rigid domain movements within the dimeric model of Pfk-2. The structural similarity of the output models, yielding nearly identical scattering patterns and fitting statistics in a stable and self-consistent process. The final shape restoration for the Pfk-2 free protein and with fructose-6-P was performed using 618 dummy residues and 611 waters assuming 2-fold molecular symmetry. In the case of Pfk-2 with an excess of MgATP we used 1236 dummy residues and 988 waters assuming a 222 molecular symmetry.

**Domain Movement Modeling**—The open and closed crystallographic structures of ribokinase (PDB codes 1RRA and 1RKD, respectively) were chosen as templates to model the domain movements in Pfk-2. Because these PDB entries contain coordinates for just one monomer of ribokinase, the second monomeric subunit of the dimeric ribokinase was obtained by application of the appropriate 2-fold crystallographic symmetry operations. The program DynDom (26) was then used to define the quasi-rigid domains, the flexible inter-domain connecting residues, and the screw axes that virtually describe the conformation transition in ribokinase. Using a sliding window of 5 residues, the program DynDom found three dynamic domains, corresponding to the two αβ domains of the dimer (residues 4–13, 42–96, and 118–309 from each monomer, following the 1RKD numbering scheme) and the central β barrel to which both subunits contribute (residues 18–38, 99–113). Residences 14–17, 39–41, 97–98, and 114–117 in each monomer were defined as forming a flexible hinge that rotates one αβ domain relative to the other. The central β barrel has been found as well as the associated rotation (−17°) and translation (−1 Å) parameters. Finally, the Pfk-2 model was superimposed on the ribokinase structure (using just the homologous residues in the central β barrel domain as reference), and the residues corresponding to the two αβ domains were rigidly rotated along the screw axes found by DynDom ribokinase. Several models were generated (program MOLMEN2; x-ray.lmc.uu.se/ usf/molmen2_man.html) rotating the αβ domains from −20° (most closed model) to +40° (most open model) with an angular step of 2°. The theoretical SAXS curves of each rotated model were subsequently fitted to the experimental data as described under “Calculation of SAXS Intensity from Atomic Models.”

**Tetramer Modeling**—Two copies of each dimeric model (the above) were aligned putting their centers of mass (COMs) at the origin of an orthogonal reference system, with their 2-fold symmetry axes aligned along x and their major inertia axis aligned along y (program MOL- MEN2). After rotation of one of the dimers by 180° along y, the dimers
were translated in opposite directions along x, obtaining the two major crystallographic orientations of the tetramer (tetramer-I and tetramer-II) depending on the direction of translation. Several tetrameric models were obtained applying different separations (along x) between the COMs of the dimers (31–39 Å for tetramer I, 26–34 Å for tetramer II, 0.333-Å translation step). Furthermore, several shear angles (rotations in opposite directions along x) were applied to each dimer (0–44° with 4° step). For each configuration, the fit against SAXS data from Pfk-2 in complex with MgATP was evaluated as described below.

Calculation of SAXS Intensity from Atomic Models and Estimation of the Uncertainty of the Fitted Parameters—The theoretical SAXS curves were determined from the atomic models using the program CRYSS (27). The program calculates the SAXS intensity through the equation,

\[ I(q) = (A_q(q) - \rho_A + \delta q \rho_b A_q(q))^2 \] (Eq. 3)

where \( A_q(q) \) is the amplitude scattered by the protein (calculated from the atomic structure factors), \( \rho_A(q) \) is the amplitude produced by the excluded volume (determined using dummy Gaussian spheres placed at the atomic positions), and \( \rho_b \) is the electron density of the solvent. The first solvation shell is modeled by a hydration shell that yields an amplitude \( \delta q \rho_b \). The solvent density is approximated by a 3-Å-thick uniform layer placed 2 Å away from the protein envelope. The symbol \( < > \) indicates spherical averaging.

Two parameters, the excluded volume of the particle \( V \) and the electron density in the hydration layer \( \rho_h \), are optimized by the program CRYSS to minimize the discrepancy \( \chi \) defined as,

\[ \chi = \frac{1}{N-1} \sum_{j=1}^{N} \left( \frac{I(q_j) - \langle I(q_j) \rangle}{\sigma(q_j)} \right)^2 \] (Eq. 4)

where \( \langle I(q_j) \rangle \) and \( \sigma(q_j) \) denote the experimental SAXS intensity of the \( j \)th point and its standard deviation, respectively, and \( N \) is the number of experimental points. The excluded volume is varied around the value predicted from the molecular mass by changing the average displaced volume per dummy atom to account for the uncertainty in its partial specific volume.

To estimate the errors in the parameters fitted during dimer and tetramer modeling (i.e., the α/β domain rotation angle, the dimer shear angle, and the dimer COM distance), the F-statistics method was used (28). The F-statistics parameter, as applied to the present work, can be written as,

\[ F_r = \frac{(N - n_m - n_a)^2}{n_a(1-n_a)} \chi^2(n_a) - \chi^2(n_a+n_m) \chi^2(n_m+n_a) \] (Eq. 5)

where \( N \) is the number of experimental points used by the fitting procedure (\( N = 89 \) in this work), \( n_m = 2 \) is the number of the CRYSS fitted parameters (i.e. \( V \) and \( \rho_h \)), and \( n_a \) is the number of parameters varied during modeling \( n_a = 1 \) for dimer modeling and \( n_a = 3 \) for tetramer modeling). \( F_r \), as defined in Equation 5, is a measure of the relative improvement of \( \chi \) when the simple CRYSS fitting procedure with a single “fixed” molecular structure is substituted by the evaluation of several “variable” structures. A given increase in \( F_r \) (rewritten in \( \chi \)) due to the introduction of \( n_m \) additional parameters is statistically more than 95% significant if \( F_r > 9.55 \) (for \( n_m = 1 \)) or \( F_r > 5.79 \) (for \( n_m = 3 \)) (28). The parameter errors reported in this work are relative to the 95% confidence limit of the F-statistics.

RESULTS

Homology Modeling

The three-dimensional structure of dimeric Pfk-2 was constructed by homology modeling (program MODELLER-6) (17) using the crystal structure of E. coli ribokinase as template (37% similarity), as described under “Experimental Procedures.” Ten models of Pfk-2 were built starting from different random initial atomic positions at the beginning of the optimization and evaluated by pseudo-energy parameters. The Ca-chain trace of the 10 models of Pfk-2 are superposed in Fig. 1a, showing the positional variability between the models (only the monomer is shown for simplicity). The probability of good global folding, \( p(G) \) (20), was around 0.999 for each model, and the average three-dimensional-one-dimensional profile score (pro-

![Fig. 1. Homology modeling of Pfk-2. a, superposition of the Ca-traces of 10 models of monomeric Pfk-2 generated by MODELLER. b, average Verify3D score profile for the models shown in a. The dotted line indicates a score of 0.1. The molecular models were drawn using the program PyMOL (Delano Scientific, San Carlos, CA, www.pymol.org).](image)

![Fig. 2. Solution scattering curves of free and ligand-bound Pfk-2. Total SAXS curves obtained by merging the experimental results corresponding to two sample-to-detector distances (see “Experimental Procedures”). The continuous lines are the best fitting of ab initio calculations obtained using GASBOR. The \( q \) values of the fits are 1.22, 0.86, and 1.05 for free Pfk-2, Pfk-2 bound to fructose-6-P, and Pfk-2 bound to ATP, respectively. The inset displays the Guinier plots from which the radii of gyration were obtained. a.u., arbitrary units.](image)

![Fig. 3. Solution small angle X-ray scattering curves of Pfk-2.](image)
FIG. 3. Distance distribution function for free and ligand-bound Pfk-2. The curves were obtained by indirect Fourier transformation of the experimental SAXS curves (Fig. 2) using the program GNOM.

FIG. 4. Modeling of fructose-6-P induced conformational changes. a, top, Co-traces of the Pfk-2 homology model showing rigid rotations around the calculated rotation axes (solid arrows). Bottom, Co-traces of the most open (40°, left) and most closed (∼−20°, right) structures. b, discrepancy (x) between the experimental data and the simulated scattering curves as a function the α/β-domain rotation angle. α was made using PyMOL.
Fig. 5. The two types of tetrameric models. Within a tetramer, the two central β-barrels (made up by the β-domains of adjacent monomers in the dimers) are darkened. The 222 symmetry of the tetrameric models is represented by the x, y, and z axes, with the x axis 2-fold symmetry axis of the dimers. The three degrees of freedom (domain opening, shear angle between the dimers, and COM distance) used for the refinement of the domain orientations are illustrated. This figure was prepared using PyMOL.

This is not totally unrealistic; a comparison of the substrate-bound forms of adenosine kinase and ribokinase reveals that the former is more open by a rotation angle of −6 degrees, indicating that the closed conformations of this family of proteins may have different orientations of the αβ domains. Alternatively, the enzyme may present movements that are different from those proposed in our model.

MgATP-bound Form; Tetrameric Packing of Pfk-2—It is known that MgATP binding induces tetramerization in Pfk-2, but the quaternary structure of the tetrameric enzyme in solution remains unknown. Using our SAXS data, we have modeled the tetrameric arrangement of the enzyme as being a “dimer of dimers.” This implies a 222 point symmetry for the tetramer (D2, according to Schoenflies notation), where one of the 2-fold axes corresponds to the symmetry axis of both dimers (the x axis in Fig. 5). This axis passes through the two central β-barrels.

Two opposite orientations of the dimers are possible (Fig. 5); one (which we called tetramer-I) with the active sites looking outward from the tetramer and the other (tetramer-II) with the active sites looking inward. Fig. 6a shows the χ-fit to the experimental SAXS data for the tetrameric models of type I and II. The tetrameric models have been obtained using the dimeric models previously described in this article, i.e., using different opening angles of the monomers (rotation of the αβ domains, see Figs. 4a and 5). The χ-fit in Fig. 6a refers to optimized tetrameric models. The distances between the COMs of the dimers and the shear angle (Fig. 5) of the dimers along their symmetry axis have been refined to get the best fit to the SAXS data (Fig. 6a). These are the only rigid movements of the dimers that are allowed in order to maintain the 222 symmetry of the tetramer.

The discrepancy from the experimental SAXS data obtained for the best fitted model is small (χ = 1) both for tetramer-I and for tetramer-II. It is evident that, no matter which orientation (tetramer-I or -II) is used, the best fits with SAXS data are achieved with closed conformations of the monomers, similar to what is observed for the complex with fructose-6-P. Although the χ curve for tetramer-II shows lower values than that for tetramer-I, inspection of the three-dimensional models indicates that these lower χ values are reached at the expense of unrealistic clashes between the two dimers in tetramer-II (not shown). Furthermore, tetrameric models of type II, built with a reasonable distance between the dimers to prevent steric hindrance, poorly fit the SAXS data (χ = 3). On the other hand, the minimum χ for tetramer-I reflects a configuration that could reasonably represent (within the limits of our rigid-body modeling of the domains configuration) the real structure.

Fig. 6b shows the χ-fit as a function of the COM distance and the shear angle between the dimers in tetramer-I (using a domain opening of 7°). Considering the three variables of our rigid-body modeling for the tetramer (COM distance between the dimers, shear angle of the dimers, monomer aperture), the region around the minimum χ appears to be relatively flat. As a matter of fact, the 95% confidence limits, according to the F-statistics, correspond to COM distances between 34.7 and 36.3 Å, shear angles between 4 and 16°, and monomer opening angles between 1 and 9° around the minimum (maximum χ variation from 1.19 to 1.23). The parameter that mostly affects χ is the distance between the dimers. The opening and the shear angle, within the ranges mentioned above, do not significantly modify the χ value. However, the discrepancy increases quickly outside these ranges.

Inspection of the proposed tetrameric model indicates that the dimer-dimer interface should be formed by contacts between the αβ domains from opposite monomers, although the fine details of this interaction cannot be deduced from our simple, low resolution model.

Ab Initio Low Resolution Structures

The SAXS data from the free, fructose-6-P, and MgATP-bound forms of Pfk-2 were used to restore the low resolution shape of the protein using the dummy residue model method of Svergun et al. (25), as described under “Experimental Procedures.” The restored shape for each conformation of Pfk-2 yields a good fit to the experimental data (see the legend of Fig. 2), indicating that imposed symmetry restrictions actually reflect prevalent features in the structure. The molecular envelope of each ab initio model is shown superimposed to the
corresponding refined homology model in Fig. 7, showing good agreement between both kinds of structures.

**DISCUSSION**

Domain closure induced by sugar binding has been demonstrated in crystallographic high resolution studies on ribokinase and adenosine kinase (12, 13). These domain motions are characterized by large changes in main chain torsion angles of a small number of residues that comprise the hinge that separates the small and large domains of these enzymes. In adenosine kinase, comparison of dihedral angles of the free and sugar-bound structures reveals that two glycines (Gly-68 and Gly-69) undergo the large torsional changes necessary for hinge bending. When sugar binds to its site, displacement of these glycines occurs to avoid steric hindrance. The nearly absolute conservation of these glycine residues throughout the PkB superfamily indicates that they probably play the same critical role in hinge bending. In addition, sugar binding affects the nearby nucleotide binding site in ribokinase, shifting it toward a conformation that is observed when nucleotide is bound, possibly increasing the affinity for this substrate. The main feature of domain closure is the complete occlusion of the sugar site from bulk solvent.

These observations suggest a general mechanism for the reaction catalyzed by kinases in this superfamily; sugar binds initially in an open active site, favoring domain closure, affecting the nucleotide binding site, and therefore, increasing its affinity for ATP. Three independent lines of evidence corroborate these predictions about structural changes of Pfk-2 upon sugar binding. First, previous kinetic studies show that a compulsory ordered kinetic mechanism occurs in Pfk-2, in which MgATP binds the active site only after fructose-6-P binding (14); therefore, sugar binding increases the affinity for MgATP in the active site. Second, and supporting domain closure, the structure of Pfk-2 in solution appears to be more compact when it is bound to fructose-6-P, as suggested by limited proteolysis experiments (7), where binding of fructose-6-P increases the resistance to cleavage by several proteases. And third, rigid body αβ domain closure of the homology model improves the agreement with the experimental SAXS data of the fructose-6-P-bound form.

Based on the present results, we also propose a model for the effect of MgATP on the enzymatic activity of Pfk-2. As can be seen from Fig. 5b, a closed structure is locked when MgATP is bound to the allosteric site no matter what configuration (tetramer-I or -II) is used to fit experimental data. By analogy with the ribokinase and adenosine kinase closed forms, it might be expected that occlusion of the sugar site from solvent hinders either fructose-6-P binding or product release, thus giving the first structural support to a mechanism for MgATP enzymatic inhibition in this enzyme.

It should be noticed that if domain closure exposes surface determinants needed for tetramerization, oligomerization would be expected also as a consequence of fructose-6-P binding, but this is not the case. Unless MgATP itself takes part in the interaction surface, this ligand must induce another conformational change different from the αβ domain closure to induce tetramerization. In this regard, the calculated scattering curves from tetrameric models (not shown) does not fit very well the valley around q = 0.2 Å⁻¹ in the experimental data from the Pfk-2-MgATP complex (Fig. 2). In ribokinase, the dimer interface bears striking similarity to domains of two ligand binding and transport proteins that are built up from orthogonal β sheets (29), but ribokinase lacks an internal space large enough to hold a small molecule ligand. Unfortunately, the existence of such appropriate internal space for MgATP binding in the Pfk-2 dimer interface cannot be established by the present work.

Chemical modification studies suggest that cysteine 295 is involved in the dimer-dimer interface. Mapping this amino acid onto the three-dimensional structure of the proposed tetramer-I model indicates that, although this residue is not located at the interface, it occupies a nearby location.

In previous fluorescence studies of the single tryptophan in Pfk-2 (Trp-88), three states have been reported (4), one with a high quantum yield (Pfk-2 saturated with fructose-6-P), another with an intermediate emission (Pfk-2 without ligands), and a third one with a low quantum yield state (Pfk-2 in presence of MgATP). Acrylamide quenching of protein fluorescence demonstrates that the tryptophan solvent accessibility is reduced when Pfk-2 is bound to MgATP as compared with the free enzyme and the Pfk-2-fructose-6-P complex, whose accessibilities are similar. Our SAXS results fully agree with these previous observations; in the model proposed here, Trp-88 is located in the αβ domain near the hinge; thereby this intrinsic probe could directly detect different openness of domains in monomers. In the tetramer model, Trp-88 is located near the dimer-dimer interface; thus, solvent accessibility might be reduced as a consequence of tetramerization.

Our model refinement of dimeric Pfk-2 in its free and fructose-6-P-bound states using SAXS results indicates that the sugar promotes a domain closure with ~12 degrees of rotation. Results of our modeling indicate that the tetrameric structure of Pfk-2 complexed with MgATP is composed by two parallel or slightly misaligned dimers located at a distance of around 34 Å between each COM, with the active sites looking outward from the tetramer and the monomeric subunits in an almost closed conformation. This tetrameric model provides satisfactory agreement with previous studies on intrinsic fluorescence and chemical modification of the enzyme. It represents the result of a combination of theoretical homology modeling and experimental low-resolution structure determination, which we consider to be valuable in the absence of a crystal structure.

Most hinge-bending proteins appear to display a dynamic equilibrium between their open and closed states, the latter stabilized by ligand binding. Because the present SAXS measurements of E. coli Pfk-2 were taken under equilibrium conditions, our observations could be interpreted as follows. In solution, with no ligands added, equilibrium is displaced toward an open conformation. When fructose-6-P binds the active site, closed structures become populated, helping the subsequent binding of MgATP to the active site, the first step toward catalysis. However, binding of MgATP to the allosteric site promotes, along with tetramerization, a domain closure that occludes the active site, and as a consequence, this impedes the entrance of fructose-6-P to the active site (or the release of products), thus producing the observed enzymatic inhibition.

**Acknowledgments**—We acknowledge Mauricio Baez and Dmitri Svergun for useful discussions.

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Domain Motions and Quaternary Packing of Phosphofructokinase-2 from Escherichia coli Studied by Small Angle X-ray Scattering and Homology Modeling
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J. Biol. Chem. 2003, 278:12913-12919.
doi: 10.1074/jbc.M212137200 originally published online January 14, 2003

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