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Chemical Modification of SH Groups of *E. coli* Phosphofructokinase-2 Induces Subunit Dissociation: Monomers Are Inactive but Preserve Ligand Binding Properties

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Modification of *Escherichia coli* phosphofructokinase-2 (Pfk-2) with *N*-(1-pyrenil)maleimide results in an enzyme form that is inactive. However, the rate of modification is drastically reduced in the presence of the allosteric effector MgATP. The stoichiometry of the label incorporation was found to be 2.03 ± 0.035 mol of the reagent/mol of subunit, in agreement with the number of titratable SH groups by 5,5′-dithiobis(2-nitrobenzoic acid) in the labeled protein. HPLC gel filtration experiments demonstrate that native Pfk-2 is a dimer in the absence of ligands, while in the presence of MgATP a dimer–tetramer transition is promoted. In contrast, the modified enzyme eluted as a monomer and the presence of MgATP was not able to induce aggregation. Although the modified monomers are inactive, the dissociation constants for the substrates and the allosteric effector MgATP, measured by following the fluorescence of the binding probe, are the same as for the native enzyme. Quenching of pyrene fluorescence emission of labeled phosphofructokinase-2 monomers by acrylamide gave downward curved Stern–Volmer plots, with very similar quenching efficiencies for the control and for the fructose-6-P and MgATP–enzyme complexes. These results show the presence of SH groups in the interface of Pfk-2 subunits, critical for subunit interactions, and that conformational changes occurring through the dimers are essential for catalytic activity. © 2000 Academic Press

Key Words: **phosphofructokinase; SH groups; *N*-(1-pyrenil)maleimide; subunit dissociation.**

Phosphofructokinase (Pfk, EC 2.7.11)[1] catalyzes the transfer of phosphate from ATP to the 1-OH group of fructose-6-P to yield fructose-1,6-bisP and ADP. The enzymatic activity of this enzyme is tightly regulated in a wide variety of organisms. Two forms of this enzyme have been found in *Escherichia coli.* Pfk-1 exhibits sigmoidal kinetics with respect to fructose-6-P, allosteric activation by ADP or GDP, and allosteric inhibition by phosphoenolpyruvate (1, 2). Pfk-2, the minor isoenzyme in the wild type strain, presents hyperbolic kinetics with fructose-6-P and inhibition by MgATP when the assay is performed at low fructose-6-P concentrations (3). Also, Pfk-1 and Pfk-2 differ as to the order in which the substrates are bound to and the products are release from the enzyme. Since Pfk-2 has an ordered Bi–Bi mechanism with fructose-6-P being the first substrate to bind and fructose-1,6-bisP the last product to be released, one would not predict binding of MgATP in the absence of fructose-6-P (4). Recently we have demonstrated, through intrinsic fluorescence studies, the presence of an allosteric site for MgATP in Pfk-2, responsible for the inhibition of the enzyme activity by this ligand (5). Also, the binding of MgATP to Pfk-2 has been linked to a dimer–tetramer association process (6).

The primary structure of Pfk-2, as predicted from the nucleotide sequence, shows no significant relationship to the Pfk-1 family, but appears to be related to another group of kinases, which includes fructose-1-phosphate kinase (7). Since no structural information is available for Pfk-2 and with a view to developing a more detailed understanding of its structure/function relationship, we investigated the involvement of some amino acid residues (SH groups) in the kinetic and structural properties of the enzyme.

Several amino acid residues have been reported to be important for either *E. coli* Pfk-1 catalytic activity (8–12) or allosteric interactions (13–15). For many other phosphofructokinases the presence of thiol groups in the active and allosteric sites has been shown (16–19).

[1] *Abbreviations used:* Pfk, phosphofructokinase; PM, pyrene N-(1-pyrenyl)maleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); fructose-6-P, fructose 6-phosphate; fructose-1,6-bisP, fructose 1,6-bisphosphate; DTT, dithiotreitol.

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The amino-acid sequence of Pfk-2, deduced from the DNA sequence (20, 21), reveals the presence of four cysteine residues and since modification of the enzyme with thiol modifying agents led to a rapid loss of enzymatic activity, we decided to study the involvement of this kind of residues in the catalytic and allosteric properties of the enzyme.

Here we report the specific modification, inactivation and subunit dissociation of Pfk-2 by a fluorescent sulfhydryl reagent, pyrene *N*-(1-pyrenyl)maleimide. The results presented demonstrate the presence of SH residues in the interface of Pfk-2 subunits critical for interactions between them and that conformational changes occurring through dimers are essential for catalytic activity.

MATERIALS AND METHODS

Pfk purification. The strain used for enzyme purification was DF 903, for which construction and growth has been described (22). Essentially, Pfk-2 was purified as reported before (1).

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

Reagent concentration. Fructose-6-P concentration was estimated spectrophotometrically using the aldolase coupling assay. The concentration of NADH was calculated using a molar extinction coefficient of 6.22×10^{-3} M^{-1} cm^{-1}. ATP concentrations were determined spectrophotometrically by the fructose-1,6-bisP formed in the presence of Pfk, fructose-6-P, aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase, and NADH. Protein concentration was determined using the Bio Rad protein assay with the standard curve constructed with bovine serum albumin (23).

Chemical modification of Pfk-2 with PM and stoichiometry of labeling. Stock solutions of PM (Molecular Probes) were freshly prepared in dimethylsulfoxide and the concentration was determined spectrophotometrically using a molar extinction coefficient of 4×10^4 M^{-1} cm^{-1} at 344 nm (24). The added aliquot never exceeded 1% of the final volume. Under the standard conditions of modification, Pfk-2 (0.8–1.0 μM) was incubated with a fourfold molar excess of PM at 0°C in the dark, in the presence of 20 mM Hepes, pH 8.0, 0.1 mM EDTA, and with or without 5 mM $MgCl_2$. At appropriate intervals, aliquots were diluted four- to fivefold in the presence of 5 mM DTT and then assayed for enzymatic activity. For determination of the stoichiometry of labeling, Pfk-2 (4–8 μM) was incubated with a fourfold molar excess of PM at 0°C during 15 or 30 min. The reaction was stopped by adding 5 mM DTT and the nonreacted reagent was eliminated by filtration through a Sephadex-G-25 column (NAP-5 Amersham Pharmacia Biotech AB) equilibrated in the buffer mentioned above. The extent of labeling was determined by measuring the absorption of pyrene moiety and determining its concentration assuming the molar extinction coefficient reported before. Protein concentration was determined as described before.

Total thiol titration of the native and modified enzyme was determined using Ellman's reagent (DTNB) (25). The absorbance at 412 nm of each sample was monitored with time, from which a blank that contained all components of the reaction mixture, except the enzyme, was subtracted. Calculations were done by using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. The number of sulfhydryl groups reacted with PM was given by the difference between the total number of sulfhydryl groups of unlabeled Pfk-2 and the number of DTNB-titratable remaining sulfhydryl groups of PM-Pfk-2.

Fluorescence measurements. The reaction of Pfk-2 with PM was also monitored directly by following the increase in fluorescence intensity of the pireno moiety at 395 nm with an excitation wavelength of 343 nm, which arises upon reaction with the maleimide group. Fluorescence measurements were performed with a Perkin Elmer LS 50 fluorimeter. In order to evaluate the ligand properties of the momomer, samples were filtrated through a Sephadex G-25 column after modification, to eliminate the unreacted reagent. The excitation wavelength was set to 343 nm and fluorescence spectra were collected from 350 to 550 nm and analyzed using a Spectra Calc program (Galactica Corporation, USA). Fluorescent determinations were made at room temperature and excitation and emission slits were set to 5 nm. Backgrounds readings were subtracted, and the wavelength and fluorescence maxima values were recorded. Experiments were performed by adding small aliquots of stock solutions of substrate or effector to the enzyme solution. Corrections were made to compensate for volume changes and enzyme dilution.

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

Acrylamide quenching experiments were performed, with an acrylamide stock solution of 5 M in 20 mM Hepes, 0.1 mM EDTA, pH 8.0, and 5 mM $MgCl_2$ when indicated. The quenching data were analyzed using the Stern–Volmer equation, $F°/F = 1 + K_{sv}[Q]$, where $F°$ and F are the fluorescence intensities in the absence and in the 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. Molecular weight determinations were performed in a TSK 2000 column equilibrated in buffer 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, 200 KCl mM, and the indicated MgATP concentration, at a flow rate of 1 ml/min. Protein elution (A_{280} nm or A_{340} nm) was recorded in an on line Beckman UV detector. The column was calibrated with the following molecular weight markers: carbonic anhydrase (29,000), alcohol dehydrogenase (150,000), β-amilase (200,000), and thyroglobuline (669,000).

RESULTS

Inactivation and labeling of Pfk-2 with PM. Incubation of *E. coli* Pfk-2 with PM at 0°C resulted in a very rapid and almost complete loss of catalytic activity in the presence and in the absence of Mg^{2+} ions (control curve of Figs. 1A and 1B). Under identical conditions, the control without the reagent shows less than 5% inactivation. The second-order rate constant for the reaction was not determined because the rapidness of the reaction precluded the generation of linear second-order plots of the data and because the ratio of inhibitor to enzyme was necessarily low. Other thiol modifying agents were also able to inactivate Pfk-2 (not shown).

To investigate further the interaction of PM with Pfk-2, the effect of various ligands on the inactivation reaction was studied. Addition of fructose-6-P, the first substrate to bind to Pfk-2, provided only a minor protection against PM-induced inactivation, while the presence of 1 mM MgATP in the incubation medium completely protects the enzyme activity (Fig. 1A). Since binding of MgATP to Pfk-2 in the absence of fructose-6-P has been demonstrated to occur at an allosteric site of the enzyme (5), the complete protection afforded by this ligand might suggest the presence of a

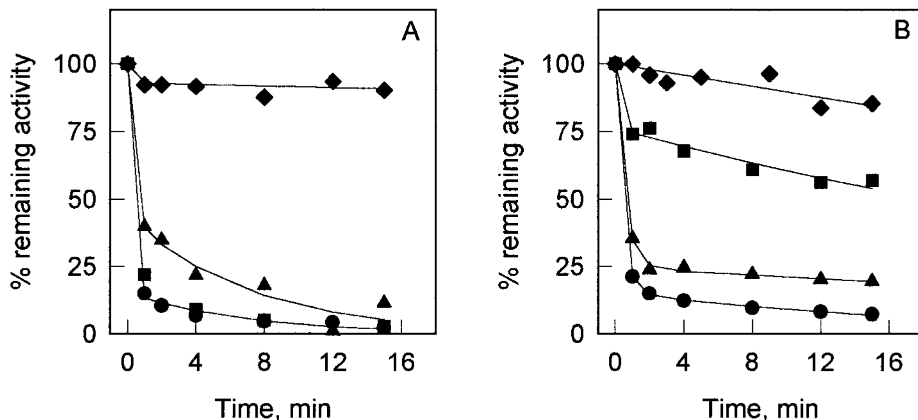

FIG. 1. Effect of ligands on the inactivation of Pfk-2 by PM. (A) The enzyme (0.8 μM) was incubated with a fourfold molar excess of PM at 0°C in the dark, in the absence of ligands and Mg^{2+} ions (●) and in the presence of 1 mM fructose-6-P (▲), 1 mM ATP^{4-} (■), or 1 mM MgATP (◆). Aliquots were removed at the indicated times (0–15 min) and assayed for residual activity as described under Materials and Methods. (B) The experimental conditions were those described in A, except for the addition of 5 mM $MgCl_2$ and the following ATP concentrations: none (●), 25 μM (▲), 50 μM (■), 650 μM (◆).

reactive(s) SH group(s) at this site or that conformational changes, such as the tetramerization process promoted by this ligand, modifies drastically the reactivity of the SH groups. On the other hand, the little protection effect provided by fructose-6-P shows that conformational changes taking place at the active site of the enzyme have a marginal effect on the reactivity of these groups. Also, ATP^{4-} in the inactivation medium does not protect Pfk-2 from PM inactivation (Fig. 1A), in agreement with the kinetic and binding data which show that this ligand does not bind to the enzyme in the absence of fructose-6-P (4, 5).

In order to assess if the MgATP protection effect was achieved by binding of this ligand to the allosteric site of Pfk-2, we determined the effect of different MgATP concentrations on the inactivation rate of Pfk-2. Figure 1B shows that indeed the inactivation rate depends on the MgATP concentration and that 0.65 mM MgATP in the incubation medium almost completely protects the enzyme from inactivation. The MgATP concentration curve obtained for the protection of Pfk-2 against PM inactivation is well correlated with the one obtained for binding this ligand to the allosteric site of the enzyme (5). The initial rapid inactivation of Pfk-2 by PM, however, is not merely due to tight noncovalent binding of the probe, because incubation of the enzyme with a PM–mercaptoethanol conjugate had no effect on catalytic activity. Also, even after denaturation the label still remains in the protein (data not shown).

To determine the degree of labeling, samples were incubated with PM for 30 min at 0°C as described under Materials and Methods. Under these conditions labeling stoichiometry was found to be 2.03 ± 0.035 mol of SH/mol of subunit. This was confirmed by titra-

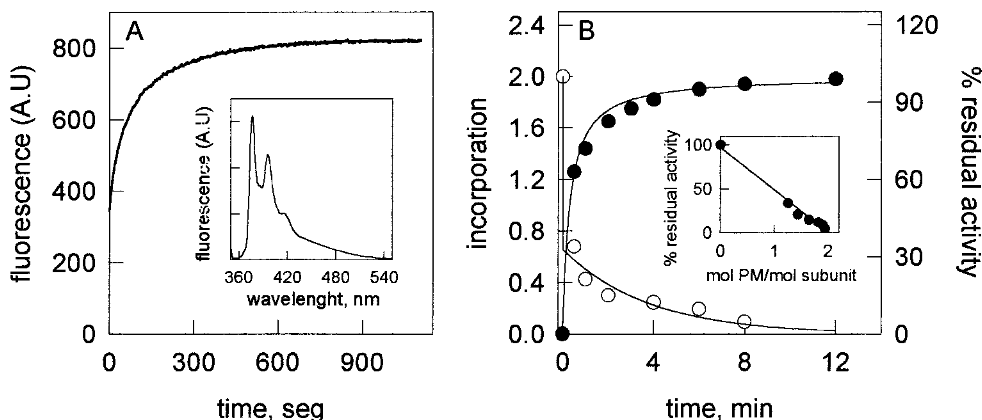

FIG. 2. Relationship between incorporation of PM and Pfk-2 inactivation. (A) Increase in fluorescence intensity (λ_{ex} = 343 nm, λ_{em} = 395 nm) when 0.12 μM Pfk-2 was reacted with 0.48 μM pyrene maleimide. (Inset) The fluorescence emission spectra of Pfk-2 after modification by PM. (B) The stoichiometry of labeling was calculated as described in the text and residual activity was determined as in Fig. 1; (inset) the relationship between these two parameters.

TABLE I

DTNB Titration of Thiol Residues in Native and Modified Pfk-2

Condition	Moles SH/subunit
Native Pfk-2 (control)	3.91 ± 0.08
Pkf-2 labeled with PM	2.02 ± 0.16
Predicted[a]	4.0

[a] Based on the DNA sequence of *E. coli* Pfk-2 (20, 21). Results are given as the means ± SE of three to four individual observations.

tion of the SH groups in the native and modified enzyme (Table I).

The native enzyme reacted with 4 mol of DTNB, indicating the existence of four free SH groups per subunit, in agreement with the number of cysteine residues deduced from the DNA sequence. In contrast, in the modified enzyme there are only two DTNB-titratable SH groups, confirming the modification of these types of residues. This was further confirmed, taking advantage of the fact that PM is essentially nonfluorescent in aqueous solutions but forms intense fluorescent adducts with a long lifetime, upon reaction with protein thiol groups (25). This property allows us to monitor the time-dependent labeling of Pfk-2 with PM by following the increase in fluorescence after the addition of PM to an aqueous solution of Pfk-2 (Fig. 2A). The relationship between the extent of labeling and the extent of inactivation was obtained from the fluorescence response of Pfk-2 upon reaction with PM, assuming a limiting value of 2 mol of reagent/mol of subunit, and from the enzymatic activity remaining, measured under similar conditions (Fig. 2B). A linear correlation was obtained between the amount of PM bound to Pfk-2 and its residual enzymatic activity (Fig. 2B, inset).

Size-exclusion chromatography of native and PM-modified Pfk-2. HPLC gel filtration studies on a TSK 2000 column of native Pfk-2, along with molecular weight standards, show an elution volume corresponding to a dimeric form of the enzyme (Fig. 3A), while in the presence of 1 mM MgATP the formation of a tetramer was observed (Fig. 3B). These results suggest that the binding of MgATP to the allosteric site of Pfk-2, responsible for the inhibition of the enzyme activity, also promotes a dimer–tetramer transition. On the other hand, molecular filtration studies of PM-modified Pfk-2 show that the enzyme elutes as a monomer (either following the absorbance at 280 nm or 340 nm) and that the presence of MgATP was not able to induce any change in its aggregation state (Figs. 3C and 3D).

Characterization of the Pfk-2 PM-modified monomer. Since the data presented so far suggested that inactivation of Pfk-2 by PM results in a loss of enzymatic activity with a concomitant dissociation of enzyme subunits, it was of interest to examine whether these monomers also present a loss of sensitivity to the substrates and to the allosteric effector MgATP. First, the integrity of the active site of the Pfk-2 modified monomer was evaluated by measuring the fluorescence changes induced by the substrate fructose-6-P. Figure 4A shows that the fluorescence response of PM labeled Pfk-2 increases with a hyperbolic dependence on the concentration of fructose-6-P with a K_d value of 13 μM, which is very similar to the one obtained through intrinsic fluorescence measurements (5). This result suggests that the modified monomer binds fructose-6-P with properties similar to those of the native enzyme and that from this point of view, the active site has not been altered upon modification.

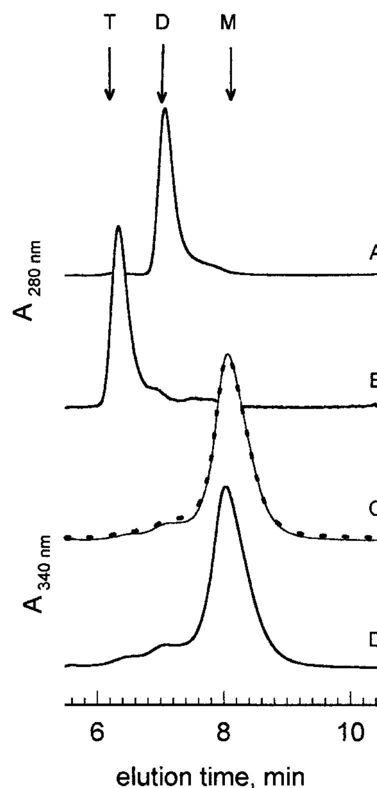

FIG. 3. Effect of MgATP on the aggregation state of native and PM-modified Pfk-2. (A) HPLC elution in a TSK 2000 column of native Pfk-2 in the absence of ligands. (B) HPLC elution of native Pfk-2 in the presence of 1 mM MgATP. The column was equilibrated at the indicated MgATP concentration and the enzyme was preincubated under the same conditions. (C) Pfk-2 modified with PM in the absence of Mg^{2+} ions. Absorbance at 280 nm (. . .) and at 340 nm (—), corresponding to the attached probe, was followed. (D) The column was equilibrated in 1 mM MgATP and the PM-modified Pfk-2 was incubated with the same MgATP concentration. The arrows indicate the elution times of the tetramer (6.1 min), dimer (7.0 min), and monomer (8.1 min) of Pfk-2 derived from a calibration of the column using the following molecular weight standards: carbonic anhydrase (29,000), alcohol dehydrogenase (150,000), β-amilase (200,000), and thyroglobuline (669,000).

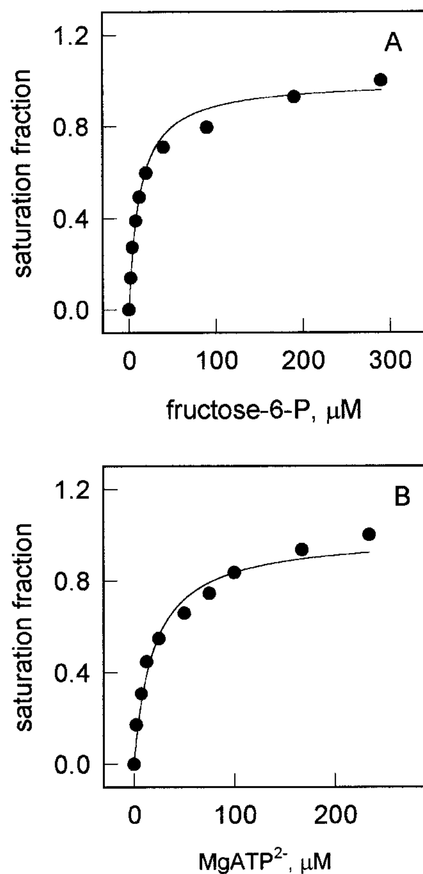

FIG. 4. Effect of ligands on the fluorescence properties of the Pfk-2 PM-modified monomer. (A) Binding of fructose-6-P to PM-modified Pfk-2 as followed by the decrease in fluorescence intensity. (B) Binding of MgATP to PM-modified Pfk-2 as followed by the concomitant change in fluorescence intensity. Saturation fraction in A and B were calculated as described under Materials and Methods. Lines represent the fit of the experimental points to a hyperbola equation.

On the other hand, if the MgATP allosteric site of Pfk-2 has been altered by the modification, one would expect no binding of this ligand to the modified monomer. This is not the case and, as shown in Fig. 4B, the fluorescence response of PM-labeled Pfk-2 to different MgATP concentrations is hyperbolic with a K_d of 20 μM. This value is somewhat lower than the value of 70 μM obtained using intrinsic fluorescence measurements. Also, the saturation curve is hyperbolic, in contrast with the cooperative behavior observed in the intrinsic fluorescence experiments (5).

Since Pfk-2 PM-modified monomers are inactive but able to bind the substrate fructose-6-P and the allosteric effector MgATP, one can ask if the observed loss of catalytic activity is due to impairment in the binding of the second substrate. To test this idea we analyzed the effect of ATP^{4-}, a compound known to bind only to the active site, on the fluorescence response of the monomer–fructose-6-P complex. Figure 5 shows the effect of increasing ATP^{4-} concentrations upon the fluorescence

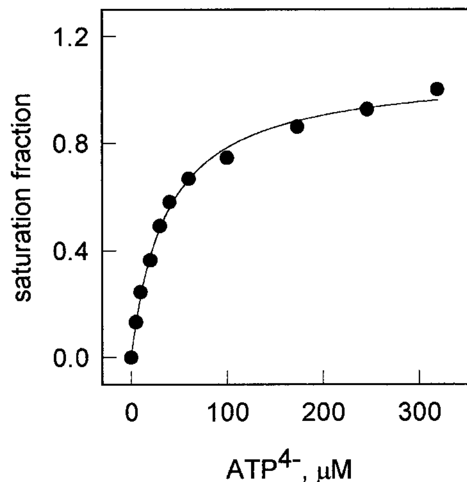

FIG. 5. Effect of ATP^{4-} concentration on the fluorescence of the fructose-6-P PM-labeled monomer complex. Pfk-2 labeled-monomer was incubated with 200 μM fructose-6-P and then binding of ATP^{4-} to the complex was followed by the decrease in fluorescence intensity of the attached probe. The curve is fitted to the hyperbola equation. The fluorescence change was calculated considering as the control, the fluorescence in the presence of 200 μM fructose-6-P and in the absence of ATP^{4-}.

of PM-labeled monomer of Pfk-2 in the presence of 200 μM fructose-6-P. The nucleotide produces a hyperbolic response with a K_d of 37 μM in good agreement with the value of 56 μM obtained for the ATP^{4-} binding to the Pfk-2–fructose-6-P complex through intrinsic fluorescence measurement (5). These results suggest that binding of substrates to the monomeric species of Pfk-2 is possible, but that conformational changes occurring through the dimers are essential for catalytic activity.

Acrylamide fluorescence quenching. Solvent accessibility to the probe covalently attached to the Pfk-2

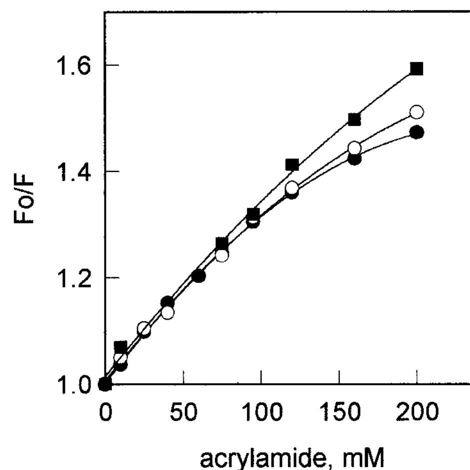

FIG. 6. Stern–Volmer plots for acrylamide for different Pfk-2 PM-monomer–ligand complexes. Free enzyme (●), Pfk-2 PM-monomer in the presence of 0.5 mM MgATP (○), Pfk-2 PM-monomer in the presence of 0.5 mM fructose-6-P (■).

monomer was studied by quenching the pyrene Pfk-2 fluorescence emission in different enzyme–ligand complexes. The emission quenching of the steady state fluorescence gave downward curved Stern–Volmer plots, while the quenching efficiency for the control and for the fructose-6-P and MgATP complexes, were very similar (Fig. 6). The downward curvature of Stern–Volmer plots can be explained in terms of the heterogeneous population of the fluorophores, which differ significantly in their individual susceptibility to the quencher. These results suggest that although binding of ligands to the Pfk-2 labeled monomer can be detected through the emission quenching fluorescence of the probe, these conformational changes do not result in significant changes in the solvent accessibility of the pyrene probe in the different enzyme–ligand complexes.

DISCUSSION

Pyrene maleimide has been used in a number of systems to investigate protein–ligand interactions (26). The reagent is essentially nonfluorescent until it has reacted with thiol groups (27). Upon reaction there is a large increase in quantum yield from this group when excited at 343 nm, as occurs when it reacts with Pfk-2. The emission maxima observed of 377 and 395 nm (λ_{ex} = 343 nm) for the pyrene–maleimide-modified Pfk-2 (Fig. 2A, inset) are similar to those for pyrene maleimide adducts of other proteins (27). The stoichiometry of incorporation of the label into Pfk-2 was found to be 2 mol/mol subunit, suggesting that two thiol groups are being modified, observation that was confirmed by titration of the SH groups present in the native and modified enzyme (Table I). These residues, however, seem not be in close proximity in modified Pfk-2 since no formation of excimers was observed. Pyrene moieties form excimers, characterized by a fluorescence emission maximun at about 470 nm, when adjacent to each other. Excimer formation has been used to test the proximity of SH groups in other proteins (28–30).

The reaction of Pfk-2 with this thiol-modifying agent produces a rapidly inactivation of the enzyme, while complete protection against inactivation is afforded by MgATP. The concentration curve for the protection effect of MgATP against PM inactivation is well correlated with the one obtained for the binding of this ligand to the allosteric site of Pfk-2. Also, as shown in Fig. 3 (A–D), binding of MgATP to the allosteric site of the enzyme promotes a dimer to tetramer transition. Then, it is possible that MgATP protection of thiol groups against PM modification might be a consequence of the tetramerization process. The binary complex formation between Pfk-2 and fructose-6-P provides only a minor protection against inactivation. Considering that data from the kinetic mechanism of Pfk-2 indicates that fructose-6-P is able to bind to the free enzyme, these results suggest that conformational changes, taking place at the active site have only little effect on the reactivity of the sulfhydryl groups of the enzyme. On the other hand, binding of fructose-6-P to the active site of the modified enzyme can be monitored through fluorescence changes associated with the incorporated label. Since this substrate is able to bind to the active site of the modified enzyme, one must conclude that the modified SH groups of Pfk-2 are not in this site, in agreement with the effect of the sugar substrate in the enzyme activity protection experiments and therefore supporting the localization of these groups at the subunit interface and its relationship to the tetramerization process.

In order to understand what causes enzyme inactivation, we analyzed the ligand binding properties of the monomer. The PM-modified monomer of Pfk-2 binds the substrate fructose-6-P and the allosteric effector MgATP with affinities similar to the ones described for the native dimer through intrinsic fluorescent measurements (5), which establish that the modification has not occur at the active or allosteric site of the enzyme. However, for the case of MgATP, the binding curve of this ligand to the monomer does not present the cooperative behavior observed for the dimer. Since, binding of MgATP to the allosteric site of Pfk-2 promotes the formation of a tetramer, the cooperative response observed probably represents the change in the aggregation state of the enzyme. These results pointed out the importance of the SH groups in the establishment of the interface interactions responsible for the maintenance of the dimeric structure and also in the formation of new subunits interactions, as in the case of the MgATP induced tetramerization process.

The integrity of the catalytic site of the PM-monomer of Pfk-2 was further studied analyzing the binding of ATP^{4-}, an analog of the second substrate, to the enzyme–fructose-6-P complex. In this case, as for the native enzyme (4), the nucleotide binds to the fructose-6-P complex in a hyperbolic fashion with a K_d value of 37 μM, indicating that, although the substrates can bind to the monomer, conformational changes along the dimeric structure are essential for catalytic activity.

The modification of thiol groups at the active and allosteric sites of several phosphofructokinases has been reported, including the enzyme from *Ascaris suum,* rabbit muscle, yeast, and *E. coli* (16–19, 29). In the case of *E. coli* Pfk-1, reaction of the enzyme with several thiol modifying agents led to a loss of activity associated with the modification of a cysteine residue in position 119 in the protein sequence and whose reaction was protected by the substrate fructose-6-P (31). For *A. suum* Pfk, modification of the ATP inhibitory site with 2′,3′-dialdehyde ATP results in an inac-

tive form of the enzyme, with no changes in the conformational integrity of the active site, suggesting that the modification locks the enzyme in an inactive T state that cannot be activated. Gel filtration HPLC studies show that the modified enzyme retains its tetrameric form and that the enzyme is insensitive to the allosteric effector fructose-2,6-bisphosphate, as shown by tryptophan fluorescent measurements and circular dichroic spectra. Also, modification of the enzyme with the thiol reagent 4-4′-dithiodipyridine indicates the presence of one of these residues in the ATP portion of the active site (16).

Recently, participation of SH groups in subunits interactions of several proteins, have been reported. In the case of the F1-ATPase of *E. coli,* the enzyme contains two cysteine residues (positions 64 and 140 of the amino-acid sequence). Labeling of Cys^{64} with or without concomitant labeling of Cys^{140} resulted in a reconstitutively inactive enzyme. However, labeling of Cys^{64} was accompanied by dissociation of the δ subunit from the enzyme, suggesting a role for the microenvironment of this residue in the interactions of the δ subunit with other subunits in the enzyme (28). On the other hand, Bochkareva *et al.* (32) have found that modification of Cys^{458} of GroEL with 4,4′-dithiodipyridine results in disassembly of the protein. This residue belongs to the equatorial domain of GroEL, located at the intersubunit interface within the rings and close to the ring–ring contact area, in a location that a priori seems sterically inaccessible to the external probe. Also, calcium-sensing receptors are present in membranes as dimers that can be reduced to monomers with sulfhydryl reagents (33).

The results presented in this paper, together with the examples mentioned above, point out the growing evidence for participation of SH groups in subunit interactions in proteins.

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REFERENCES

1. Babul, J. (1978) *J. Biol. Chem.* **253,** 4350–4355.
2. Blangy, D., Buc, H., and Monod, J. (1968) *J. Mol. Biol.* **31,** 13–35.
3. Guixé, V., and Babul, J. (1985) *J. Biol. Chem.* **260,** 11001–11005.
4. Campos, G., Guixé, V., and Babul, J. (1984) *J. Biol. Chem.* **259,** 6147–6152.
5. Guixé, V., Rodríguez, P. H., and Babul, J. (1998) *Biochemistry* **37,** 13269–13275.
6. Guixé, V., and Babul, J. (1988) *Arch. Biochem. Biophys.* **264,** 519–524.
7. Bork, P., Sander, C., and Valencia, A. (1993) *Protein. Sci.* **2,** 31–40.
8. Wang, X., and Kemp, R. G. (1999) *Biochemistry* **38,** 4313–4318.
9. Berger, S. A., and Evans, P. R. (1992) *Biochemistry* **31,** 9237–9242.
10. Hellinga, H. W., and Evans, P. R. (1987) *Nature* **327,** 437–439.
11. Laine, R., Deville-Bonne, D., Auzat, I., and Garel, J. R. (1992) *Eur. J. Biochem.* **207,** 1109–1114.
12. Berger, S. A., and Evans, P. R. (1990) *Nature* **343,** 575–576.
13. Lau, F. T. K., and Fersht, A. R. (1989) *Biochemistry* **28,** 6841–6847.
14. Lau, F. T. K., and Fersht, A. R. (1987) *Nature* **326,** 811–812.
15. Kundrot, C. E., and Evans, P. R. (1991) *Biochemistry* **30,** 1478–1484.
16. Rao, G. S. J., Cook, P. F., and Harris, B. G. (1991) *Biochemistry* **30,** 9998–10004.
17. Latshaw, S. P., Bazaes, S., Randolph, A., Poorman, R. A., Heinrikson, R. L., and Kemp, R. G. (1987) *J. Biol. Chem.* **262,** 10672–10677.
18. Kemp, R. (1969) *Biochemistry* **8,** 3162–3168.
19. Tijane, M. N., Chaffotte, A. F., Seydoux, F. J., Rouccous, C., and Laurent, M. (1980) *J. Biol. Chem.* **256,** 10188–10193.
20. Daldal, F. (1983) *J. Mol. Biol.* **168,** 285–305.
21. Daldal, F. (1984) *Gene* **28,** 337–342.
22. Daldal, F., and Fraenkel, D. (1981) *J. Bacteriol.* **147,** 935–943.
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
24. Wu, C. W., Yarbrough, L. R., and Wy, Y. H. (1976) *Biochemistry* **15,** 2863–2868.
25. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77.
26. Gupta, S. S., and Lane, L. K. (1983) *J. Biol. Chem.* **258,** 5005–5012.
27. Weltman, J. K., Searo, R. P., Frackelton, A. R., Jr., Dowben, R. M., Bunting, J. R., and Cathow, R. E. (1973) *J. Biol. Chem.* **248,** 3173–3177.
28. Ziegler, M., Xiao, R., and Penefsky, H. S. (1994) *J. Biol. Chem.* **269,** 4233–4239.
29. Betcher-Lange, S. L., and Lehrer, S. S. (1978) *J. Biol. Chem.* **253,** 3757–3760.
30. Sen, A. C., and Chakrabarti, S. (1990) *J. Biol. Chem.* **265,** 14277–14284.
31. Banas, T., Gontero, B., Drews, V. L., Johnson, S. L., Marcus, F., and Kemp, R. G. (1988) *Biochim. Biophys. Acta* **957,** 178–184.
32. Bochkareva, E., Safro, M., and Girshovich, A. (1999) *J. Biol. Chem.* **274,** 20756–20758.
33. Pace, A. J., Gama, L., and Breitwieser, G. E. (1999) *J. Biol. Chem.* **274,** 11629–11634.