Nucleotide Binding to Pig Muscle 3-Phosphoglycerate Kinase in the Crystal and in Solution: Relationship between Substrate Antagonism and Interdomain Communication†

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Received July 23, 2001; Revised Manuscript Received October 12, 2001

ABSTRACT: Binding constants for the nucleotide substrates were determined in two different crystalline forms of pig muscle 3-phosphoglycerate kinase (PGK): the binary complex with 3-phosphoglycerate (3-PG) in which the two domains are in an open conformation (Harlos, Vas, and Blake (1992) *Proteins, 12,* 133−144) and the ternary complex with 3-PG and the Mg salt of the ATP analogue, β,γ-methyleneadenosine-5′-triphosphate (AMP-PCP), the structure of which is under resolution. Competitive titrations have been performed in the presence of the chromophoric analogue of ATP, 2′3′-*O*-(2,4,6-trinitrophenyl)ATP (TNP-ATP), similar to those previously carried out in solution, where a weakening of the binding of the nucleotide substrates in the presence of the other substrate, 3-PG, has been observed (Vas, Merli, and Rossi (1994) *Biochem. J. 301*, 885−891). Here the K_d values for MgADP were found to be 0.096 ± 0.021 and 0.045 ± 0.016 mM, respectively, for the crystals of the binary and ternary complexes. Both K_d values are significantly smaller than the one obtained in solution in the presence of 3-PG (0.38 ± 0.05 mM) and are close to the values determined in solution in the absence of 3-PG (0.06 ± 0.01 mM). Thus, the "substrate antagonism" observed in solution is not present in either of the investigated crystal forms. Further nucleotide binding studies with the solubilized enzyme have shown that 3-PG has no effect on ADP (Mg^{2+}-free) binding ($K_d = 0.34 \pm 0.05$ mM), while it weakens MgADP binding. Thus, 3-PG abolishes the strengthening effect of the Mg^{2+} ion on the binding of ADP. This phenomenon is apparently due to the interaction between the carboxyl group of 3-PG and the protein, since the carboxyl-lacking analogue glycerol-3-phosphate has no detectable effect on MgADP binding. Comparison of the crystallographic data of different PGK binary (with either 3-PG or MgADP) and ternary (with both 3-PG and MgADP) complexes, having open and closed conformations, respectively, provides a possible structural explanation of the substrate antagonism. We suggest that the specific interaction between the 3-PG carboxylic group and a conserved arginine side chain is changed during domain closure, and, through interdomain communication, this change may be transmitted to the site in which Mg^{2+} binds the ADP phosphates. This effect is abolished in the crystals of pig muscle PGK, in which lattice forces stabilize the open domain conformation.

3-Phosphoglycerate kinase (PGK) is a typical two-domain kinase (*1, 2*) catalyzing the transfer of 1-phosphate from 1,3-bisphosphoglycerate to MgADP to produce MgATP. Each domain binds one of the two substrates (*3, 4*), and both solution X-ray scattering (*5*−*7*) and crystallographic data (*8, 9*) are entirely consistent with the occurrence of a large-scale relative domain movement during catalysis that brings the two substrates in the close proximity required for reaction. Furthermore, kinetic evidence for domain closure in the productive ternary complex has been reported (*10*). Using enzymes from different sources, structures of various forms of both "open" (*3, 4, 11*) and "closed" (*9, 12*) conformations have been described at high resolution that contribute to the elucidation of the molecular mechanisms of relative domain movements (*13, 24*).

It has been reported that, in solution, binding of the nucleotide substrate is weakened by the bound substrate, 3-phosphoglycerate (3-PG). This "antagonistic binding" of the substrates could only be interpreted by assuming a conformational transition upon formation of the ternary complexes (*15*). The question arises whether this conformational transition can be identified in the domain closure that, in fact, has only been observed in the crystal structure of

† The work has been supported by grant OTKA (T 029208) of the Hungarian National Research Fund and by a bilateral grant (I-5/98) provided by the Italian Foreign Ministry and the Hungarian National Committee for Science and Technological Development.

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[1] Abbreviations: PGK, 3-phospho-D-glycerate kinase; ATP, 3-phospho-D-glycerate 1-phosphotransferase (EC 2.7.2.3); GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PEG, poly(ethylene glycol); 3-PG, 3-phospho-D-glycerate; G-3-P, DL-glycerol-3-phosphate; AMP-PCP, β,γ-methylene-adenosine-5′-triphosphate; AMP-PNP, β,γ-imido-adenosine-5′-triphosphate; TNP-ATP, 2′3′−*O*−(2,4,6-trinitrophenyl) ATP.

10.1021/bi0115380 CCC: $22.00
Published on Web 12/05/2001

the ternary enzyme−substrate complexes (*9*, *12*) and not (or only to a very small extent) in the respective binary complexes (*3*, *4*). It is also conceivable that the conformational change, occurring during the transition from the binary to the ternary complex, may affect the binding characteristics of the simultaneously bound ligands relative to their individual binding. The binding properties of the substrates in the catalytically competent ternary complex are important for the catalyzed phospho-transfer.

In the present work, we aimed (i) at investigating whether there is a relationship between substrate antagonism and domain closure and (ii) at elucidating the structural basis of the antagonism by testing the importance of individual parts of the substrate molecules in the antagonism and by analyzing the structural details of the enzyme−substrate interaction.

For the first purpose, using the technique of single-crystal microspectrophotometry, we determined the binding constants of MgADP and MgATP to the crystalline state of the binary complex between pig muscle enzyme and 3-PG, the open conformation of which has been determined at high resolution (*3*). It has been shown that the large-scale conformational transition of domain closure is prevented by crystal lattice forces in this binary complex with 3-PG; namely, it maintains the open conformation even after diffusing the nucleotide analogue 5′-adenylylimidodiphosphate (AMP-PNP) through the crystal channels (*11*). Therefore, we should not observe substrate antagonism in the crystals of this binary complex, if this feature depends on domain closure.

We have also cocrystallized pig muscle PGK with both 3-PG and the Mg salt of the ATP analogue, β,γ-methyleneadenosine-5′-triphosphate (MgAMP-PCP), with the aim of obtaining a structure with different domain positions. In the present work, we determined the binding constants of the nucleotide substrate, MgADP, to this new crystalline form of the enzyme, the structure of which is under resolution (Kovári, Flachner, Náray-Szabó, and Vas, unpublished).

To characterize nucleotide binding in the crystals of PGK, we have exploited the chromophoric properties of the nucleotide analogue 2′3′-*O*-(2,4,6-trinitophenyl)ATP (TNP-ATP), which has been successfully used with PGK in solution, to substitute for either MgADP or MgATP (*15*) and to use as an affinity label for other ATP-dependent enzymes (*16*−*18*). Using single-crystal microspectrophotometry, we performed competitive titrations of the nucleotide substrates, similar to those previously done in solution (*15*), to determine the dissociation constants of the nucleotide substrates in the various available crystal forms of the pig muscle enzyme.

For the second purpose, that is, to test the importance of specific parts of the substrate molecules, we have performed further experiments on the solubilized enzyme. The effect of 3-PG on the binding of Mg^{2+}-free ADP (a nonsubstrate) was investigated by both competitive titration in the presence of the fluorescent substrate analogue TNP-ATP and equilibrium dialysis. In addition, the effect of glycerol-3-phosphate, the carboxyl-lacking analogue of 3-PG, on the binding of MgADP was also determined. Furthermore, details of the interaction of substrates with specific protein side chains were compared for the open form of the binary complex between pig muscle PGK and 3-PG (*3*), the open form of the binary complex of *Bacillus stearothermophilus* PGK with MgADP (*4*), and the closed form of the MgADP*3-PG ternary complex from *Trypanosoma brucei* PGK (*12*).

From the different lines of investigation, a comprehensive picture has emerged about the control of the Mg^{2+}-mediated electrostatic interactions between the nucleotide phosphates and the protein by the bound 3-PG, an effect that may be an essential feature for both substrate antagonism and catalysis. The results provide evidence for the role of the interdomain communication in the antagonistic binding effect.

MATERIALS AND METHODS

Enzymes and Chemicals. 3-Phosphoglycerate kinase (PGK) was isolated from pig muscle as described in Harlos et al. (*3*). The molar activity of the enzyme used for crystallization varied between 500 and 700 kat/mol using the substrates D-3-phosphoglycerate and MgATP.

Glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase, 3-phosphoglycerate, 3-PG (grade I), glycerol-3-phosphate (G-3-P) ADP, ATP, the ATP analogue β,γ-methyleneadenosine-5′-triphosphate, AMP-PCP, NADH, and phosphoenolpyruvate were Boehringer products. The disodium salt of TNP-ATP was purchased from Molecular Probes. $MgCl_2$ and PEG_{8000} were from Sigma and Hampton Research, respectively. All other chemicals were commercially prepared reagent-grade.

The concentrations of ADP and ATP were measured both spectrophotometrically (using the values of $\epsilon_{260} = 15\,000$ M^{-1} cm^{-1} for ADP and 15 400 M^{-1} cm^{-1} for ATP) and enzymatically using the assay mixtures previously described (*19*). The concentration of TNP-ATP was checked spectrophotometrically using a value of $\epsilon_{408} = 26\,400$ M^{-1} cm^{-1} (*16*).

Crystallization. Crystals of the binary complex with 3-PG were grown from PEG_{8000} at pH 7.0 in the presence of 10 mM 3-PG as described (*3*). A similar hanging-drop procedure was applied in growing crystals of the ternary complex in the presence of 10 mM MgAMP-PCP and 10 mM 3-PG. These crystals were obtained at 23−24% (w/w) PEG and were thin monoclinic plates (space group $P2_1$) with unit cell dimensions $a = 48.0$, $b = 110.3$, $c = 36.6$, and $\beta = 93.9$, and the X-ray structure determination is underway (Kovári, Flachner, Náray-Szabó, and Vas, unpublished).

Properties of 3-Phosphoglycerate Kinase in the Presence of the Crystallization Medium. The activity of 3-phosphoglycerate kinase was assayed spectrophotometrically at 340 nm with 3-phospho-D-glycerate and MgATP as substrates according to (*19*) at pH 7.0 and 20 °C. PEG_{8000} did not affect either the maximal activity of the enzyme or the K_m values of the substrates, at least within the experimental error, up to the concentration of 10% (w/w) compatible with the solubilized state of the enzyme. The inhibitory and binding properties of TNP-ATP toward PGK (*15*) were not affected by the presence of PEG.

Equilibration of Crystals with Nucleotides. Single crystals were washed with 30 mM Tris-HCl buffer, pH 7.0, containing 1 mM EDTA, 10 mM 3-phosphoglycerate, and 27% (w/w) PEG. The higher concentrations of PEG with respect to the crystallization conditions prevent the dissolution of the enzyme, and in this medium the crystals are stable for months. A series of crystals was incubated for 3−5 days at room temperature, in the same solution plus 10 mM $MgCl_2$

and various concentrations of TNP-ATP, to determine the dissociation constant for TNP-ATP. $MgCl_2$ was an essential component as in its absence no binding of TNP-ATP to the crystal could be detected, possibly because the highly negatively charged nucleotides cannot enter into the crystal channels filled with the nonionic medium PEG. Other series of crystals were incubated in a solution containing 10 mM $MgCl_2$, a fixed concentration of TNP-ATP, plus various concentrations of either ADP or ATP, for the determination of the nucleotides binding constant. The equilibration with ligands was checked by subsequent microspectrophotometric spectral measurements (cf. below) repeated at different incubation times.

Spectral Measurements with Microspectrophotometer. Single-crystal polarized light spectra were recorded by using a Zeiss UV-visible MPM 03 microspectrophotometer (*20*). A single crystal (typical dimensions (0.1 mm × 0.1 mm × 0.02 mm) was placed in a quartz cell surrounded by its suspending medium, and absorption of the plane-polarized light was recorded with the beam incident perpendicular to one face of the crystal (usually the large (010) face). The crystal was oriented to have the electric vector parallel to one of the two principal optical directions. Absorbance at 1 nm intervals was calculated from the ratio of intensities of the light transmitted through the suspending medium and through the crystal.

Competitive Fluorimetric Titrations. Fluorimetric titrations of 10 μM TNP-ATP with solubilized pig muscle PGK were performed in the absence and in the presence of various constant concentrations of the nucleotide, MgADP, MgATP, or ADP, as described in (*15*). A Jasco FP-777 spectrofluorimeter was used with a thermostatically controlled cell compartment at 20 °C. The excitation wavelength was 408 nm, and the emission was measured at 535 nm. The inherent fluorescence of TNP-ATP in the absence of PGK was subtracted from the emitted intensity. The light scattering of PGK in the absence of TNP-ATP was also taken into account. Evaluation of the titration data was carried out as described earlier (*15*).

Equilibrium Dialysis Binding Studies. Dialysis was carried out in a series of Micro D200 cells of a Dianorm (MSE) equilibrium dialysis apparatus as described in (*21*). On each side of the membrane, 0.15 mL of solution was filled into the cells: on one side, 0.6−1.0 mM PGK was present in all cells, while on the other side, the ligand concentration was varied from cell to cell. The cells were rotated (12 rev/min) for 2 h in a thermostated water bath at 20 °C. At equilibrium, the concentrations of both the enzyme and the ligand were determined in samples withdrawn from each side of the membrane.

RESULTS

Binding of TNP-ATP to Single Crystals of 3-Phosphoglycerate Kinase. Figure 1 shows polarized light absorption spectra of TNP-ATP (complexed with Mg^{2+}) bound to a single crystal of PGK grown in the presence of 3-PG. The spectra are recorded along the extinction direction on two perpendicular faces of the crystal (Figure 1A and B). Of the four spectra, two are identical, since they are recorded along the same direction with respect to the crystal axis. Similar spectra were also obtained for single crystals of a ternary

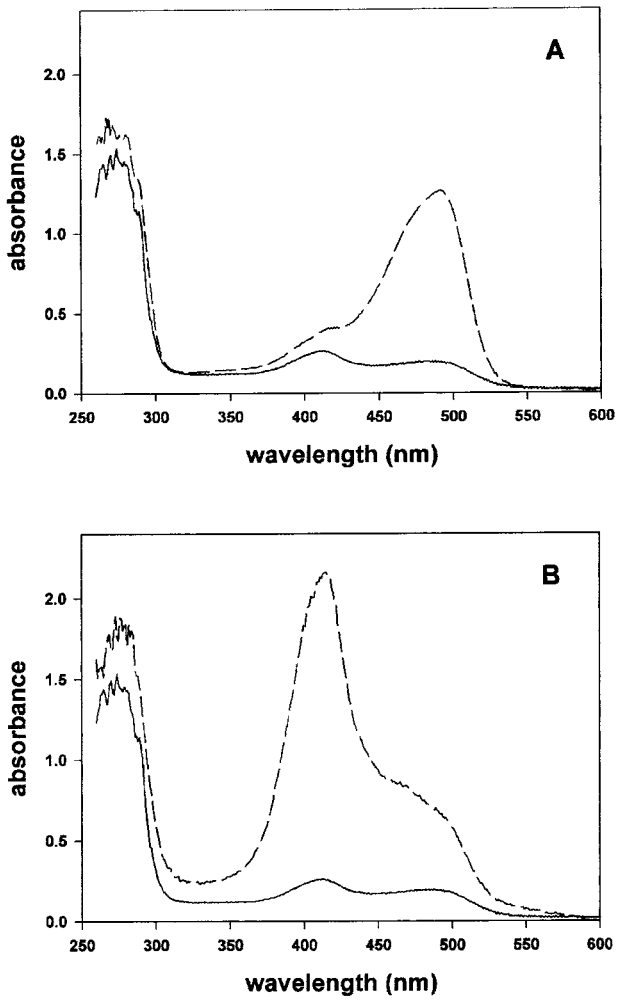

FIGURE 1: Polarized light absorption spectra of TNP-ATP bound to a single crystal of PGK. The spectra were recorded by a microspectrophotometer on two perpendicular faces (A and B) of the crystal grown as a binary complex with 3-PG. Continuous and dashed lines represent spectra, recorded with the electrical vector of the polarized light parallel to the two principal optical directions on each face. The spectra on the two faces are normalized to a thickness of 0.1 mm.

complex of PGK grown in the presence of both MgAMP-PCP and 3-PG (not shown). In both crystal forms, the absorption spectrum of enzyme-bound TNP-ATP exhibits two main peaks, strongly polarized in different directions. This indicates that TNP-ATP binds to the nucleotide binding site in a specifically oriented way.

By measuring the intensity of the absorption spectrum at various TNP-ATP concentrations, its dissociation constant could be determined. In the case of the ternary complex, it is assumed that TNP-ATP completely replaces the originally bound AMP-PCP since both analogues have been found to be competitive inhibitors of MgATP binding ((*15*), Kovári, Flachner, Náray-Szabó, and Vas, unpublished) and TNP-ATP was present in the incubation mixture in a large molar excess with respect to AMP-PCP. The spectra were usually recorded using light polarized along the principal optical direction in which the band at 490 nm exhibits the higher intensity (cf. Figure 1A). The absorbance ratio $OD_{490\ nm}/OD_{280\ nm}$ in these spectra recorded in such direction was used as a measure of the analogue binding level.

Table 1: Nucleotide Binding to PGK in the Crystal and in Solution: Dissociation Constants (K_d, mM) in the Absence and in the Presence of Other Ligands

	crystal		solution		
nucleotide	PGK*3PG	PGK*3PG*MgAMP-PCP	PGK	PGK*3-PG	PGK*G-3-P
MgADP[a]	0.096 ± 0.021	0.045 ± 0.016[b]	0.048 ± 0.009[c,e] 0.060 ± 0.010[d,f]	0.34 ± 0.13[c,e] 0.38 ± 0.05[d]	0.058 ± 0.009[d]
MgATP[a]	0.21 ± 0.02	n.d.	0.27 ± 0.09[c,e] 0.23 ± 0.05[d,f]	0.51 ± 0.22[c,e,g] 0.38 ± 0.06[d,g]	n.d.
ADP	n.d.	n.d.	0.41 ± 0.12[c] 0.34 ± 0.05[d]	0.48 ± 0.14[c] 0.39 ± 0.07[d]	n.d.

[a] 10 mM $MgCl_2$ was present. [b] The analogue MgAMP-PCP is competitively displaced by the nucleotide. [c] Values obtained from fluorimetric titration using TNP-ATP. [d] Values obtained from equilibrium dialysis. [e] Data from (*15*). [f] Data from (*21*). [g] The data of MgATP binding in the presence of 3-PG represents the average K_d of MgATP and MgADP in the equilibrium reaction mixture of the functioning PGK, in which, however, the concentration of MgATP is usually dominant over the concentration of MgADP (*23*).

Titration of crystals of both binary and ternary complexes of PGK with TNP-ATP could be fitted to a single dissociation curve (not shown) with $K_d = 0.029 \pm 0.007$ mM, assuming a 1:1 stoichiometry of binding, according to the equilibrium dialysis binding data obtained with the pig muscle enzyme in solution (*21*).

Binding of MgADP and MgATP to Single Crystals of PGK. TNP-ATP bound to the crystal of the 3-PG binary complex of PGK can be replaced upon incubation with the nucleotide substrates. In a competitive titration, at constant TNP-ATP concentration, a progressive decrease of the amount of the crystal-bound TNP-ATP is observed as the concentration of either MgADP (Figure 2A) or MgATP (not shown) is increased in the incubation medium. Thus, the formation of the ternary complex with the two physiological substrates is compatible with the maintenance of the crystal lattice of the binary complex of PGK with 3-PG. It is remarkable that the stability of the MgATP*3-PG ternary complex implies that in these crystals large conformational changes do not occur, and, therefore, the crystalline PGK is catalytically inactive. The restricted conformational motion of PGK in the crystalline state has been pointed out also by Joao and Williams (*22*).

From these competitive titrations and the knowledge of the previously determined K_d for TNP-ATP, the K_d values for both MgADP and MgATP were determined (Table 1). In the calculations (cf. Appendix), the complete replacement of the analogue at infinite concentration of the nucleotide substrates is assumed.

MgADP binding was also investigated in the ternary complex obtained by crystallizing pig muscle PGK in the presence of both 3-PG and the ATP analogue MgAMP-PCP. For this purpose, in advance, the bound AMP-PCP has been replaced by TNP-ATP. Figure 2B illustrates the gradual replacement of bound TNP-ATP within this crystal in a competitive titration with MgADP.

Table 1 summarizes the various K_d values in the crystal and in solution (cf. below). In comparison with solution, stronger binding of nucleotides could be detected in the presence of 3-PG in both types of crystals. This result indicates that the antagonistic substrate binding, characteristic of the enzyme in solution, is not retained in the investigated crystal forms.

Effects of 3-PG and Its Analogue on the Binding of MgADP and ADP to PGK in Solution. In our previous binding studies by fluorimetric titrations of PGK in solution, a more pronounced weakening effect by bound 3-PG has been observed for MgADP than for ADP (*15*). However, there were some discrepancies between the data obtained by fluorimetric titration and those obtained by equilibrium dialysis, especially in the absence of Mg^{2+} ions. In particular,

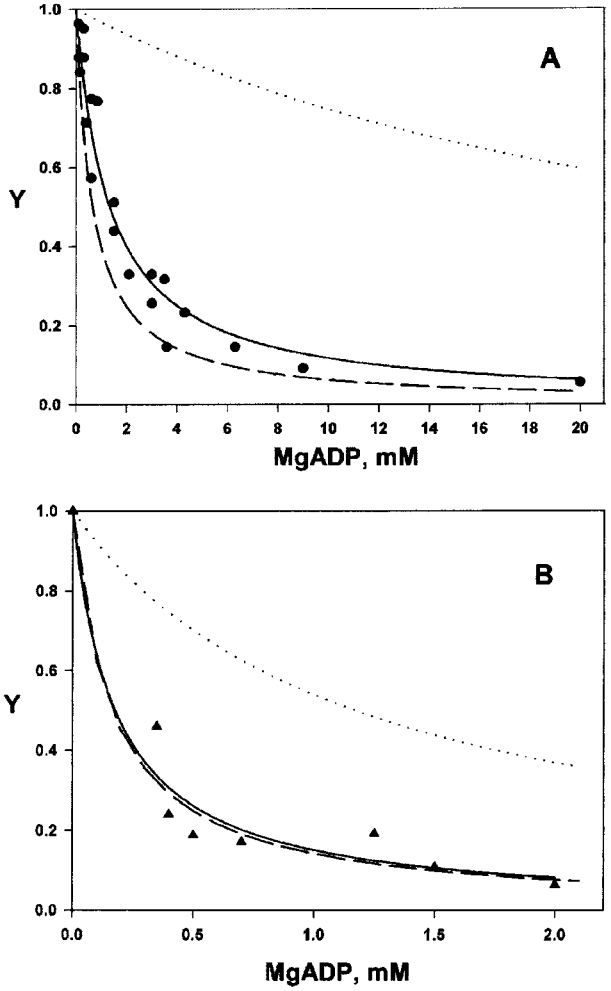

FIGURE 2: Competitive microspectrophotometric titrations of the crystals with the nucleotide substrates in the presence of TNP-ATP. Binding of the chromophoric analogue TNP-ATP in single crystals of the binary complex of PGK with 3-PG (A) and of the ternary complex with MgAMP-PCP and 3-PG (B) at variable concentrations of MgADP. The constant concentration of TNP-ATP was 0.40 mM in (A) and 0.10 mM in (B). The continuous lines represent the best fit to the experimental points, calculated using eq 7. The theoretical curves calculated by using the K_2 values obtained in solution (*15*) for binding of MgADP in the presence of 3-PG (dotted lines) and in the absence of 3-PG (dashed lines) are reported for comparison.

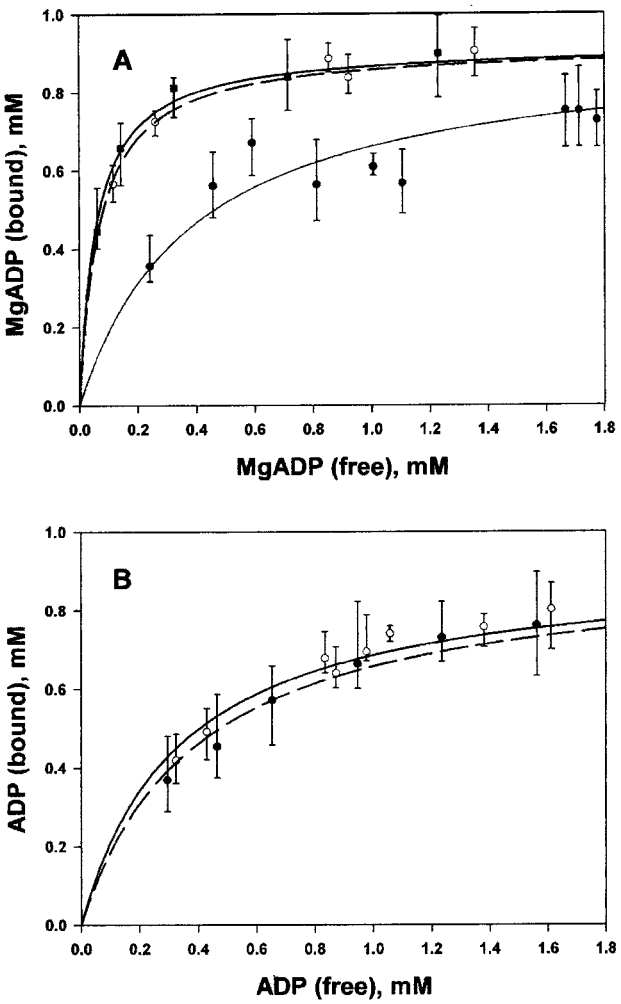

FIGURE 3: Effect of 3-PG on binding of MgADP (A) and of ADP (B) to the solubilized PGK as determined by equilibrium dialysis. One-half of each dialysis cell contained 0.916 mM PGK, and the other half contained different initial concentrations (from 0.6 to 4.0 mM) of ADP with 20 mM $MgCl_2$ (A) and without it (B). Experiments were carried out in the absence (○) and presence (●) of 14 mM 3-PG, as well as in the presence of 20 mM G-3P (■). The concentration of free ADP was measured in the enzyme-free compartment of the dialysis cells by both UV absorption and enzymatic testing (cf. Materials and Methods). The curves were obtained by fitting the experimental data to a binding equation that assumes a single binding site. The respective K_d values that gave the best fit are 0.058, 0.068, and 0.384 mM (A) as well as 0.346 and 0.394 mM (B). The bars represent the whole range of the experimental values, and the points are the means of the data.

equilibrium dialysis measurements were not performed in the presence of 3-PG. For these reasons, there were some uncertainties in the K_d values, mainly for Mg^{2+}-free ADP, and the effect of 3-PG on nucleotide binding needed clarification.

In this work, we have extended binding studies on PGK in solution by using both independent methods: fluorimetric titration and the more direct equilibrium dialysis. The results, shown in Figure 3A and B and summarized in Table 1, confirm the weakening effect of 3-PG on MgADP binding and, in addition, clearly indicate that, within the experimental error, 3-PG has no effect on the binding of Mg^{2+}-free ADP. It is also notable that the fluorimetric titration and the equilibrium dialysis methods lead to essentially the same results.

It is remarkable that in the presence of 3-PG the affinities of MgADP and ADP for the enzyme become similar. Therefore, 3-PG abolishes the previously observed strengthening effect (*21*) of the Mg^{2+} ion on ADP binding. Another possibly relevant finding is that MgAMP and adenosine are respectively mixed-type and noncompetitive inhibitors (Flachner, Szilágyi, and Vas, unpublished), while MgADP (as a product) is apparently a competitive inhibitor toward the substrate 3-PG (*21*).

These experiments have shown that either without the complete phosphate chain of ADP or without the Mg^{2+} ion that forms a complex with it, no antagonistic effect of 3-PG on nucleotide binding could be observed. At the same time, the present results with Mg^{2+}-free ADP argue against the electrostatic repulsion between the negatively charged phosphate and carboxyl groups of the bound nucleotide and 3-PG, respectively, as a possible reason for the observed antagonism with MgADP.

The effect of the carboxyl-lacking structural analogue of 3-PG, glycerol-3-phosphate, on MgADP binding has also been investigated by equilibrium dialysis. Glycerol-3-phosphate has been found to be a good competitive inhibitor of the PGK-catalyzed reaction with respect to 3-PG (*19*). As seen in Table 1, glycerol-3-phosphate, in contrast to 3-PG, does not weaken the binding of MgADP.

Table 1 also shows an appreciably weaker binding of MgATP with respect to MgADP in solution in the presence of 3-PG. In this case, an equilibrium mixture of substrates and products is present, although the equilibrium is far shifted toward MgATP and 3-PG (*23*) (in our conditions, only 4−10% of the substrates are converted to products). The observed effect, therefore, can be attributed to the weakening effect of 3-PG on the binding of MgATP, with a minor contribution of the much lower concentration of the product 1,3 bis-phosphoglycerate (*15*).

DISCUSSION

To interpret the above results on nucleotide binding to PGK, that is, the antagonistic effect of 3-PG in solution and the absence of this effect in the investigated crystal forms, we have inspected the existing X-ray crystallographic structural data. Thereby, we expected to identify the structural basis of substrate antagonism and to understand its possible relevance in the catalytic process.

The absence of substrate antagonism in the crystal of the binary complex with 3-PG, having an open domain conformation (*3*), is consistent with the assumption that substrate antagonism is related to domain closure. Furthermore, the absence of antagonism in solution, when 3-PG is replaced by glycerol-3-phosphate, is in line with the possible importance of the 3-PG carboxylate group in the interdomain communication leading to domain closure. These assumptions are supported by both the absence of strong interactions of 3-PG carboxylate with the protein and the absence of the H bond between Gly 372(N) of helix 13 and Ser 392(O) of the β-strand L in this structure (Figure 4A), which have been shown to be part of the network of H bonds essential for operation of the main hinge point at this region (*24*).

To have a closer insight into the structural basis of substrate antagonism, comparison of the nucleotide−protein interaction in the binary and ternary complexes with open

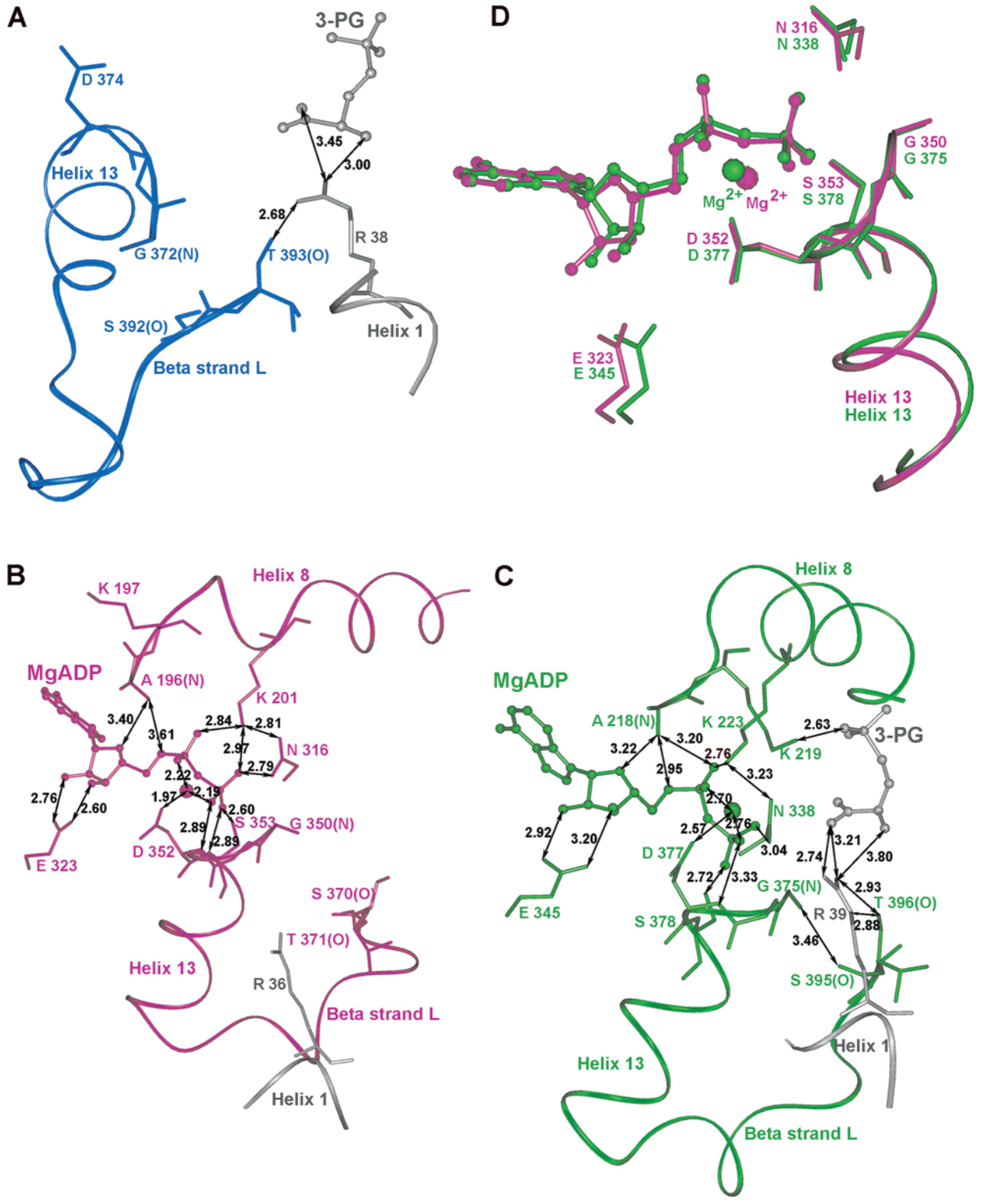

FIGURE 4: Surroundings of the bound substrates in the X-ray crystallographic structures of PGKs of various origin. Parts of the structures, represented by ribbon diagrams with the stick model of a few critical side chains around the bound substrates (ball-and-stick models), are shown: the binary complex of pig muscle PGK with 3-PG (A) (*3*); the binary complex of *B. stearothermophilus* PGK with MgADP (B) (*4*), and the ternary complex of *T. brucei* PGK with MgADP and 3-PG (C) (*12*). A superimposition of the two MgADP-bound structures, optimizing the alignment of all atoms of the conserved N-terminal residues of helix 13, is also shown (D). The bound nucleotide and the part of the C-terminal domain are colored according to the structures of different origins: blue (pig muscle), red (*B. stearothermophilus*), and green (*T. brucei*) PGK. The bound 3-PG and the respective part from the N-terminal domain are shown in gray. The indicated distances are in angstroms (Å). The conserved amino acid residues having corresponding structural positions possess different numbering due to their different positions in sequence of PGKs of various origin. The figures were made on a Silicon Graphics workstation with the software Insight II 95.0, using the coordinates of the respective structures (PDB accession numbers for *B. stearothermophilus* and *T. brucei* PGKs are 1php and 13pk, respectively).

and closed conformations, respectively, would be required. However, crystallographic data could not be determined until now for either of these complexes of the pig muscle enzyme. Instead, high-resolution data are available for the binary complex with MgADP of *B. stearothermophilus* PGK in the open conformation (*4*) and the 3-PG*MgADP ternary complex of *T. brucei* PGK in a closed form (*12*). Since the PGK structure is highly conserved at all levels (*25*, *26*) and only conserved side chains are involved in enzyme−substrate interactions (*24*), it is reasonable to compare in detail the interactions between MgADP and the protein in the binary and the ternary complexes, even if their structures are obtained from different sources (Figure 4B and C, respectively).

All of the following considerations refer to the unproductive ternary complex containing bound 3-PG (substrate) and MgADP (product) but can be extended to the productive ternary complex as also assumed in the crystallographic studies on the closed structure of the 3-PG*MgADP ternary complex of *T. brucei* PGK (*8*, *12*). The fact that 3-PG exerts an antagonistic effect toward the ATP analogue MgAMP-PCP (Kovári, Flachner, Náray-Szabó, and Vas, unpublished) as well as toward ATP (Table 1) supports this assumption.

In the binary complex of *B. stearothermophilus* PGK with MgADP (open conformation), the Mg^{2+} ion is strongly coordinated with both the phosphate oxygen atoms of ADP and the carboxyl group of the conserved Asp352 (Figure 4B), and the distances of the ligands fulfill the requirements of the coordination sphere of Mg^{2+}, that is, 2.0−2.2 Å (*27*). In the closed ternary complex of *T. brucei* PGK, the coordination of Mg^{2+} is weakened (Figure 4C). It is worth noting that Asp352 of *B. stearothermophilus* PGK (corresponding to Asp377 and Asp374 in *T. brucei* and pig muscle PGKs, respectively) is at the N-terminus of helix 13 that, in the closed conformation, is hydrogen-bonded to the β-strand L.

A further contribution to the stronger nucleotide binding in the open structure is given by the interaction between Lys201(Nϵ) (equivalent to Lys223 in *T. brucei* PGK) and the negatively charged phosphates (Figure 4B). On the other hand, only the H-bond interactions of the nucleotide with Ala196(N) look slightly weaker in the open structure (Figure 4B) than do the equivalent interactions with Ala218(N) in the closed structure (Figure 4C).

There are also remarkable changes in the interactions with 3-PG, when one compares the respective binary (Figure 4A) and ternary (Figure 4C) complexes, possessing open and closed conformations. In the closed structure of *T. brucei* PGK, the 3-PG carboxylate group forms stronger interactions with the conserved Arg39 (corresponding to Arg36 in *B. stearothermophilus* and Arg38 in the muscle PGKs, respectively), a residue making a direct link between the two domains (Figure 4C). These interactions are weaker if not absent in the open structure of the pig muscle PGK binary complex with 3-PG (Figure 4A).

This H-bond network represents a continuous link from the 3-PG carboxyl group through Arg39 to the most important hinge at β strand L and thereby to the N-terminus of helix 13, as can be visualized in Figure 4C. This network largely contributes to the interdomain communication in the ternary complex and may perturb the Mg^{2+}-ion linked interaction of the nucleotide phosphates with the N-terminus of helix 13. In fact, the position of Mg^{2+} ions in the two conformations differs by about 0.8−1.0 Å (and, similarly, the phosphates of the nucleotide occupy different positions) when the respective PGK molecules are superimposed (Figure 4D).

These changes around the metal ion may be brought about not only by the immediate changes in the interactions between 3-PG and the contacting conserved side chains, as described above. A more complex, highly coordinated conformational change may be propagated from the 3-PG site to the nucleotide site, concomitant with domain closure, which may also be responsible for release of the Mg^{2+}-bound phosphate chain of ADP from the N-terminus of helix 13. The present experimental finding of the absence of any effect of 3-PG on the binding of Mg^{2+}-free ADP (Table 1) strongly argues in favor of these suggestions.

The question arises on what would be the significance of the weakening of the metal-mediated coordination of the nucleotide phosphates caused by bound 3-PG.

In general, it is assumed that the coordination pattern of the metal ion to the nucleotide changes during the enzyme-catalyzed phospho-group transfer, concomitant with the ADP-ATP transformation. There are still controversial results concerning this pattern, as well as about the role of the metal ion in catalysis, but, in most cases, Mg^{2+} is found to be coordinated to both the α- and the β-phosphates of ADP and to both the β- and the γ-phosphates of ATP. This implies the movement of the Mg^{2+} ion during catalysis, which is apparently consistent with the accumulating structural observations in various kinases, including PGK (*11*, *12*, *28*). Different roles have been proposed for the Mg^{2+} ion: either to provide a template in orienting the reacting substrates of PGK or simply to shield the negative charges of the nucleotide phosphates and, thereby, to assist departure of the leaving group (*8*, *9*, *29*).

Here we suggest that the substrate antagonism observed by us reflects the initial weakening of the Mg^{2+}-mediated link between the bound nucleotide phosphates and PGK, which may be a prerequisite for the occurrence of phospho-transfer. In light of the above structure-based analysis, we can also interpret the binding data obtained in the new crystal of the ternary complex of pig muscle PGK with MgAMP-PCP and 3-PG. Cocrystallization with both these ligands could have stabilized a different conformation of the protein in a ternary complex. However, the present results indicate the absence of substrate antagonism, and the dissociation constant of the chromophoric nucleotide analogue is similar to that obtained for the binary complex. Thus, in contrast to expectation, it is likely that the enzyme exhibits an open conformation also in this new crystal form of a ternary complex.

It is notable that, so far, the pig muscle enzyme has not been crystallized in the closed conformation. Another ternary complex of pig muscle PGK with MgADP and 3-PG has also been observed in an open conformation (*24*), unlike the similar ternary complex of *T. brucei* PGK (*8*), crystallized in identical conditions. The primary structure of *T. brucei* PGK differs by about 50% from that of the muscle enzyme (*24*). It has been suggested that many conformers of PGK are present in solution, and the rate of conformational change depends on many anions (*22*). It is likely that differences in sequence direct the selection of different conformers in the crystal lattice. The possible selection of a specific conformer

by lattice interactions is a problem of general interest in the comparison of protein conformation in solution and in the crystal (*30*−*32*). We are currently inspecting the nature of protein−protein interactions in the crystal packing of PGK from various sources in the hope of understanding the forces that prevent domain closure in the various crystal forms of pig muscle PGK.

APPENDIX

Equations Used for Evaluating the Microspectrophotometric Titration Data. Analysis of ligand binding data for single crystals is simple, as the concentration of free ligand in the medium is practically the same as the concentration of the total ligand due to the comparatively negligible number of enzyme sites in the crystal. Therefore, the binding equations for the calculation of K_d values of the crystalline enzyme are independent of protein concentration as in solution when [S] ≫ [E] (e.g., *33*).

To take into account the different thicknesses of the crystals, the fractional saturation of the enzyme with TNP-ATP (Y) was calculated by relating the ratio of absorbance at 490 nm and at 280 nm (R) and the same ratio at saturating TNP-ATP concentration (R_{max}), that is, $Y = R/R_{max}$. The polarized spectrum in which the higher intensity at 490 nm is observed was used for calculation.

1. *Dissociation Constants for TNP-ATP.* The dissociation equation for TNP-ATP binding follows:

$$K_1 = ([E_T] - [EA])[A]/[EA] \quad (1)$$

By substituting $[EA] = Y[E_T]$ into eq 1, one can get the following:

$$K_1 = (1 - Y)[A]/Y \quad (2)$$

Thus, K_1 can be calculated, in principle, by determining the saturation level (Y) of the protein crystal with TNP-ATP at a fixed total concentration [A] of the analogue without knowing the enzyme concentration. The measurements were repeated at different concentrations of the total analogue, and the experimental points were fitted to a theoretical dissociation curve given by eq 2.

2. *Dissociation Constants for the Nucleotide Substrates.* When the nucleotide substrate (MgADP or MgATP) is bound to the crystal in the presence of TNP-ATP, the following equations are valid, assuming that they bind to the same protein site:

$$K_1 = [E][A]/[EA] \quad (3)$$

$$K_2 = [E][N]/[EN] \quad (4)$$

where K_2 is the dissociation constant for the binding of either MgADP or Mg ATP to the crystal, while [EN] and [N] stand for the bound and the total concentrations, respectively, of the nucleotide substrate. By solving both equations for the concentration of free enzyme and equalizing, it follows that,

$$K_1[EA]/[A] = K_2[EN]/[N] \quad (5)$$

and after rearrangements and substituting the saturation function,

$$K_2 = (K_1[N]/[A])([EA]/[EN]) \quad (6)$$

that is,

$$K_2 = (K_1[N]/[A])(Y/(1 - Y)) \quad (7)$$

Thus, knowing K_1, the dissociation constant for the nucleotide substrate K_2 can be calculated from the experimentally determined Y at given concentrations of TNP-ATP (A) and of the substrate (N). The measurements were carried out at a fixed concentration of TNP-ATP and different concentrations of either MgADP or MgATP. The experimental points were fitted by a theoretical curve given by eq 7.

REFERENCES

1. Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Hardy, G. W., Merrett, M., and Phillips, A. W. (1979) *Nature 279*, 773−777.
2. Watson, H. C., Walker, N. P. C., Shaw, P. J., Bryant, T. N., Wendell, P. L., Fothergill, L., Perkin, R. E., Conroy, S. C., Dobson, M. J., Tuite, M. F., Kingsman, A. J., and Kingsman, S. M. (1982) *EMBO J. 1*, 1635−1640.
3. Harlos, K., Vas, M., and Blake, C. C. F. (1992) *Proteins 12*, 133−144.
4. Davies, G. J., Gamblin, S. J., Littlechild, J. A., Dauter, Z., Wilson, K. S., and Watson, H. C. (1994) *Acta Crystallogr. D50*, 202−209.
5. Pickover, C. A., McKay, D. B., Engelman, D. M., and Steitz, T. A. (1979) *J. Biol. Chem. 254*, 11323−11329.
6. Ptitsyn, O. B., Pavlov, M. Y., Sinev, M. A., and Timchenko, A. A. (1986) in *Multidomain Proteins* (Patthy, L., and Friedrich, P., Eds.) pp 9−25, Akadémiai Kiadó, Budapest.
7. Sinev, M. A., Razgulyaev, O. I., Vas, M., Timchenko, A. A., and Ptitsyn, O. B. (1989) *Eur. J. Biochem. 180*, 61−66.
8. Bernstein, B. E., Michels, P. A. M., and Hol, W. G. J. (1997) *Nature 385*, 275−278.
9. Auerbach, G., Huber, R., Grättinger, M., Zaiss, K., Schurig, H., Jaenicke, R., and Jacob, U. (1997) *Structure 5*, 1475−1483.
10. Geerlof, A., Schmidt, P. P., Travers, F., and Barman, T. (1997) *Biochemistry 36*, 5538−5545.
11. May, A., Vas, M., Harlos, K., and Blake, C. C. F. (1996) *Proteins 24*, 292−303.
12. Bernstein, B. E., and Hol, W. G. (1998) *Biochemistry 37*, 4429−4436.
13. Bernstein, B. E., Williams, D. M., Bressi, J. C., Kuhn, P., Gelb, M. H., Blackburn, G. M., and Hol, W. G. J. (1998) *J. Mol. Biol. 279*, 1137−1148.
14. Reference deleted.
15. Vas, M., Merli, A., and Rossi, G. L. (1994) *Biochem. J. 301*, 885−891.
16. Hiratsuka, T., and Uchida, K. (1973) *Biochim. Biophys. Acta 320*, 635−47.
17. Rao, R., Al-Shawi, M. K., and Senior, A. E. (1988) *J. Biol. Chem. 263*, 5569−73.
18. Faller, L. D. (1990) *Biochemistry 29*, 3179−86.
19. Tompa, P., Hong, P. T., and Vas, M. (1986) *Eur. J. Biochem. 154*, 643−649.
20. Rivetti, C., Mozzarelli, A., Rossi, G. L., Henry, E. R., and Eaton, W. A. (1993) *Biochemistry 32*, 2888−906.
21. Molnár, M., and Vas, M. (1993) *Biochem. J. 293*, 595−599.
22. Joao, H. C., and Williams, R. J. P. (1993) *Eur. J. Biochem. 216*, 1−18.
23. Nageswara-Rao, B. D., Cohn, M., and Scopes, R. K. (1978) *J. Biol. Chem. 253*, 8056−8060.
24. Szilágyi, A. N., Ghosh, M., Garman, E., and Vas, M. (2001) *J. Mol. Biol. 306*, 499−511.

25. Mori, N., Singer-Sam, J., and Riggs, A. D. (1986) *FEBS Lett. 204*, 313−317.
26. Watson, H. C., and Littlechild, J. A. (1990) *Biochem. Soc. Trans. 18*, 187−90.
27. Harding, M. M. (1999) *Acta Crystallogr., Sect. D 55*, 1432−43.
28. Raghunathan, V., Chau, M. H., Ray, B. D., and Rao, B. D. (1999) *Biochemistry 38*, 15597−605.
29. Pappu, K. M., and Serpersu, E. H. (1994) *J. Magn. Reson., Ser. B 105*, 157−166.
30. Mozzarelli, A., and Rossi, G. L. (1996) *Annu. Rev. Biophys. Biomol. Struct. 25*, 343−65.
31. Carugo, O., and Argos, P. (1997) *Protein Sci. 6*, 2261−3.
32. Cao, Y., Musah, R. A., Wilcox, S. K., Goodin, D. B., and McRee, D. E. (1998) *Protein Sci. 7*, 72−8.
33. Mozzarelli, A., Ottonello, S., Rossi, G. L., and Fasella, P. (1979) *Eur. J. Biochem. 98*, 173−9.

BI0115380