Conformational State Distributions and Catalytically Relevant Dynamics of a Hinge-Bending Enzyme Studied by Single-Molecule FRET and a Coarse-Grained Simulation

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ABSTRACT Over the last few decades, a view has emerged showing that multidomain enzymes are biological machines evolved to harness stochastic kicks of solvent particles into highly directional functional motions. These intrinsic motions are structurally encoded, and Nature makes use of them to catalyze chemical reactions by means of ligand-induced conformational changes and states redistribution. Such mechanisms align reactive groups for efficient chemistry and stabilize conformers most proficient for catalysis. By combining single-molecule Förster resonance energy transfer measurements with normal mode analysis and coarse-grained mesoscopic simulations, we obtained results for a hinge-bending enzyme, namely phosphoglycerate kinase (PGK), which support and extend these ideas. From single-molecule Förster resonance energy transfer, we obtained insight into the distribution of conformational states and the dynamical properties of the domains. The simulations allowed for the characterization of interdomain motions of a compact state of PGK. The data show that PGK is intrinsically a highly dynamic system sampling a wealth of conformations on timescales ranging from nanoseconds to milliseconds and above. Functional motions encoded in the fold are performed by the PGK domains already in its ligand-free form, and substrate binding is not required to enable them. Compared to other multidomain proteins, these motions are rather fast and presumably not rate-limiting in the enzymatic reaction. Ligand binding slightly readjusts the orientation of the domains and feasibly locks the protein motions along a preferential direction. In addition, the functionally relevant compact state is stabilized by the substrates, and acts as a prestate to reach active conformations by means of Brownian motions.

INTRODUCTION

Proteins are by nature rather flexible macromolecules with a crucial balance between flexibility and rigidity, which is important for a proper functioning. The flexibility of proteins such as enzymes is related to internal motions in time regimes ranging from picoseconds to seconds (1). Global large-amplitude motions as well as local motions are driven by stochastic collisions between the solvent molecules and the protein surface. Although the driving force of these motions is random, enzymes often respond to the thermal kicks with highly specific conformational changes that are related to equally specific catalytic reactions. The character of these large-amplitude functional motions is encoded in the topology of the protein structure, as shown by normal mode analysis (NMA) of protein elastic models (2). In particular, in multidomain proteins, like in various kinases, collective domain motions modulate the accessibility of substrate binding sites and the transient distance between reacting substrates. Often, these enzymes have substrate binding sites at the interface between domains, and large-amplitude interdomain movements are required for the catalytic activity (3). In this respect, it is not only of interest how the domain motions enable the catalytic activity, but also how interacting substrates change the dynamical properties of the enzyme. Related to that, two models have been proposed to describe the ligand-binding mechanism: the traditional induced-fit model, and the more recently described conformational-selection or population-shift model (4,5). A keystone in the development of the aforementioned concepts is composed of the studies on adenylate kinase (6–8), a multidomain enzyme bearing three domains of unequal size with a core domain and two lids. To support and extend the aforesaid ideas, we studied another well-characterized model system, phosphoglycerate kinase (PGK), a hinge-bending protein (9) with two globular structural domains of equal size connected by a highly flexible hinge region (see Fig. 1, which uses the VMD software (10)). In fact, a different dynamic behavior is expected for PGK with respect to adenylate kinase due to the different structural topology, although the two kinases catalyze similar reactions.
Static as well as dynamic properties of the PGK domains have been investigated with different techniques. Early studies on PGK brought out the idea of a hinge-bending enzyme performing a ligand-induced transition between an open and a closed conformation of the domains (11–14). The conformational flexibility of PGK in solution was detected with small-angle scattering experiments (15–17), time-resolved fluorescence energy transfer studies (18), and simulations (19–21). Upon substrate-binding, a decrease of the protein conformational flexibility, a reorientation of the domains, and a slight compaction of the protein were observed. In 2010, the character of the rather fast and large-amplitude interdomain movements in PGK was confirmed by a combined study employing small angle neutron scattering and neutron spin-echo spectroscopy (16).

In accordance with the early fluorescence measurements (18), this study revealed large-amplitude Brownian fluctuations of the domains on a timescale of as few as 10 ns for the ligand-free and ligand-bound cases (the latter with reduced amplitudes). However, it is still unclear how the interdomain distance, and thereby the intermolecular distances of the involved substrates, evolve during catalysis. In this respect, knowledge about the amplitudes, the geometry, and the related timescales of the rigid-body interdomain movements, and how they are influenced by substrate binding, is very important. Finally, a core issue is the question of whether, and/or how, these structural and dynamical properties are related to substrate binding, product release, and catalytic turnover rates.

A powerful approach to obtain quantitative information about these issues is given by single-molecule Förster resonance energy transfer (smFRET) studies. Besides the possibility to monitor distributions of conformational states, smFRET provides detailed information about conformational transitions on a broad spectrum of timescales (nanoseconds to milliseconds), if fluorescence intensities as well as fluorescence lifetimes are measured (7,22–26), and correlation curves of the FRET filtered photons are calculated (25,27,28). We have therefore used smFRET to investigate interdomain movements in PGK in the absence and in the presence of substrates. To achieve a more meaningful interpretation of our experimental data, we applied NMA and performed a mesoscale hydrodynamics simulation of a coarse-grained model of PGK, which yielded valuable information about possible geometries and fast timescales of the observed interdomain movements (29–31). Based on our results on PGK, we think that this combined approach has the potential to elucidate functionally relevant motions of multidomain proteins in general, with the long-term goal to classify and link these motions to protein function.

**MATERIALS AND METHODS**

**Single-molecule FRET measurements**

To be sensitive to interdomain distance changes induced by substrate binding, we choose two labeling positions, each at the distal end of the N- and the C-domain, respectively (18,32). For this purpose, we employed a cysteine double mutant of PGK from baker’s yeast, which was site-specifically labeled with a donor (Alexa Fluor 488) and an acceptor (Alexa Fluor 647) fluorophore (see Fig. 1 and see Sample Preparation in the Supporting Material). With dyes attached at positions 135 (N-domain) and 290 (C-domain), the distance between the labeling sites is sufficiently close to the Förster radius of the employed dye pair \( R_0 \approx 51 \text{ Å} \); for details, see Characterization of Labeled PGK in the Supporting Material) to measure interdomain distance changes. Diffusing double-labeled proteins were measured at a concentration \( <50 \text{ pM} \) (with an average number \( N \approx 0.03 \) of molecules in the detection volume) in our standard buffer 10 mM MOPS, 50 mM NaCl, 2 mM EDTA, pH 7.4 with 0.003% Tween 20. To obtain PGK species with a high degree of bound substrates, we added 13 mM Mg\(^2\)-ATP (K-ADP) and/or 42 mM 3-PG to the buffer, which is, in both cases, a substrate concentration \( \approx 10 \) times larger than the respective \( K_d \) values (11,14).

For all buffer conditions (with and without substrate), we measured respective quantum yields and time-resolved fluorescence anisotropy decays of protein-bound fluorophores. Photon bursts of single diffusing proteins were measured with a confocal fluorescence microscope by employing pulsed excitation and time-correlated single photon counting for fluorescence lifetime determination (see Measurements in the Supporting Material). Our measurements were performed with pulsed interleaved excitation (33), which reports on the photophysical properties of both fluorophores and allows filtering-out events with nonfluorescent acceptor dyes. Bursts of photons were considered as acceptable single-molecule events if the sum of detected photons for both channels \( (F_A + F_D) \) was larger than (25) counts. For accepted bursts, the energy transfer efficiency between the dyes was determined by

\[
E = \frac{F_A}{F_A + \gamma \cdot F_D},
\]

where \( F_A \) and \( F_D \) are the respective detected counts for acceptor and donor photons in each burst, after subtraction of the background and correction for
the donor cross-talk into the acceptor detection channel. The correction factor \( \gamma \) accounts for differences in the donor and acceptor quantum yields and for the differences in detection efficiencies of both channels (see Data Treatment in the Supporting Material). The resulting transfer efficiencies calculated from a large number of bursts (events) are displayed in histograms, which show Gaussian-like distributions peaked at a mean efficiency value, which represents the FRET-averaged interdye distance \( \langle r_{DA} \rangle_E \) (see Fig. 2A). By using pulsed excitation, for each burst, the donor fluorescence lifetime \( \tau_{DA} \) was also determined (see Efficiency Histograms, the subsection Burst Selection, and 2D-Plots in the Supporting Material). As will be discussed in detail below, two-dimensional plots of \[ F' = \frac{g' \cdot F_D}{F_A} \]
versus \( \tau_{DA} \) (see Fig. 2B) yield valuable additional information about populations of different conformational states, transitions between different states, and involved timescales (22,34). Here \( g' \) accounts for the differences in detection efficiencies of both channels. In addition, histograms of the apparent FRET efficiency \[ E' = \frac{S_A}{S_A + S_D} \]
were built for a set of different bin durations as shown in Chung et al. (26) and Gopich and Szabo (35). The total number of donor and acceptor photons \( S_A \) and \( S_D \) detected in one bin without background correction were obtained by trimming the selected bursts in segments of equal duration \( T \). Only bins with a total number of photons \( S_A + S_D \) above a threshold \( N_T \) were used for the analysis. The threshold value was adjusted accordingly to the bin duration to discard bins with significant gaps where only a few photons are detected (35). Finally, the donor autocorrelation \( G_{DD}(\tau) \) and the donor-acceptor cross-correlation \( G_{DA}(\tau) \) functions of the burst-selected photons were calculated with the algorithm described in Wahl et al. (36) (see the subsection Correlation Analysis in the Supporting Material).

Simulations

A C\(_{c}\)-atoms elastic network model of PGK (PDB:1QPG (37)) was used to calculate the normal modes (2.29) and to simulate the protein dynamics with a multiparticle collision-dynamics approach (31). The NMA was performed with the MMTK package (Python Software Foundation, https://pypi.python.org/pypi/MMTK (38)). For the multiparticle collision-dynamics simulation, the elastic constant of the force field was globally scaled to reproduce the interdye distance distribution obtained from our ensemble lifetime FRET experiments. To compare the simulated data with the smFRET and ensemble FRET experiments, the dye linker contribution was considered with a modified version of the accessible volume (AV) algorithm (39).

In our approach, the most realistic dye positions within the AV were weighted by a proper weighting function (weighted AV (wAV) algorithm was used). The parameters required to apply the wAV algorithm were derived from the dyes’ chemical structures, and a calibration measurement performed with double-labeled DNA, an ideal model system. More details about the simulation are given in Coarse-Grained Model in the Supporting Material.

RESULTS

Conformational state distribution and dynamics of ligand-free PGK

A transfer efficiency histogram for ligand-free PGK is given in Fig. 2A. In addition to a dominant peak around a transfer efficiency of \( \sim 0.8 \) (cyan line), a considerable fraction of molecules exhibit much smaller efficiency values. The major population peaked at \( E \sim 0.8 \), represents molecules that exhibit conformations with a mean interdye distance \( \langle r_{DA} \rangle_E = 40.6 \) Å (we will call this population “the compact conformation”). This value is very similar to the distance of 39.3 Å between the respective C\(_{\beta}\)-positions known from the crystal structure (37). Molecules included in the populations that peaked at \( E \sim 0.35 \) (yellow line) exhibit a more open structure, with a mean interdye distance \( \langle r_{DA} \rangle_E = 56 \) Å (we will call this population “the expanded conformation”).

As supported by the results obtained from plots showing a FRET intensity indicator \( F' \) versus the donor lifetime (see Fig. 2B), we gain insights about dynamic properties of the molecules in the respective conformational states as well as about transitions between both conformations (for details, see the sections Data Treatment and 2D-Plots in the Supporting Material). Populations with a static distribution of interdye distances at a timescale of the dwell time of the molecules in the confocal detection volume (approximately milliseconds) would fall on the so-called static line (red dashed line in Fig. 2B). Obviously, the measured population centered at \( \tau_{DA} \sim 1.5 \) ns (the compact conformation) falls below the static line. Furthermore, in the efficiency histogram (Fig. 2A), the compact population does not show any additional broadening (i.e., conformational variance).

**FIGURE 2**  smFRET data from ligand-free PGK. (A) FRET efficiency histograms. Two populations with different mean efficiencies were fitted with Gaussian distributions (black and yellow solid lines). The distribution width caused by shot noise, and variation of the acceptor quantum yield, is given (blue dashed and yellow sawtoothed lines, respectively). (B) The same data is shown in a two-dimensional contour plot of \( F' \) versus the donor lifetime \( \tau_{DA} \). Both populations are falling on the corrected static FRET line (red solid line), which considers fast fluctuations of the interdye distances (linker dynamics and/or rigid body inter-domain movements). (Red dashed line) Uncorrected static FRET line.
of the Gaussian distribution beyond the variance due to shot noise and variations of the acceptor quantum yield (dashed line).

This is indicative for the fact that molecules belonging to this population exhibit interdye distance fluctuations much faster than the dwell time ($\tau_c \ll \text{ms}$) (22,34). Potential sources for fast interdye distance fluctuations are the dye-linker dynamics (39) and fast interdomain rigid body movements, which were already reported earlier in the literature (16,18,19,21). These fast fluctuations are considered by the so-called corrected static FRET line (solid red line) on which the major population is falling. By modeling dye-linker dynamics and rigid body movements of the domains with a coarse-grained model (see Coarse-Grained Model in the Supporting Material), we were able to detect rather fast interdomain motions with amplitudes of $2\sigma_{CC} \sim 10.4$ Å (referred to the labeled $C_a$) on the nanosecond timescale. Like the compact state, the expanded conformation (peaked at $E \sim 0.35$ in Fig. 2A) also falls below the static line, but has a rather large conformational variance and is broadened along the corrected static FRET line (with the center at $\tau_{DA} \sim 2.6$ ns in Fig. 2B). This is an indication of fast linker dynamics superimposed to an underlying distribution of substates, which are static on the milliseconds timescale ($\tau_c \gg \text{ms}$).

To check for the presence of dynamics on the microsecond to milliseconds timescale within the expanded state, and of interconversion between the expanded and the compact populations on the same time regime, we then performed analyses that were more sophisticated:

In a first approach, histograms of the apparent FRET efficiency were built for different bin durations (see Fig. 3A). At long bin times, if the rate of conformational transitions and the bin duration are comparable, a collapse of the interconverting states into a single peak is expected (35). Clearly, the histograms do not change their shapes, indicating a static behavior in the sampled time regime of (0.2–7.5) ms.

In a second approach, we plotted the normalized ratio

$$\frac{G_{DD} (\tau)}{G_{DA} (\tau)}$$

between the correlation functions of burst-selected photons. Here, the diffusion term cancels out and dynamics contributions should appear as additional correlation time-decays (27,28,40). The results for ligand-free PGK (see Fig. 3B, red) are identical to the two intrinsically rigid, double-stranded DNA controls, indicating no dynamics contributions in the time regime of (0.001–10) ms. Altogether, these observations prove that no interconversion takes place between the compact and the expanded state on the time interval of (0.001–10) ms. In addition, the data show that the expanded state consists of a heterogeneous static distribution of conformers. The conformational space sampled by molecules of this population is rather large, with a standard

![FIGURE 3](A) Histograms of apparent FRET efficiencies of ligand-free PGK calculated for different bin durations $T$. The largest bin time was set to 7.5 ms, a value slightly higher than the mean burst duration of ~7.0 ms (see Fig. S11 in the Supporting Material). (B and C) Normalized ratio $G_{DD} (\tau)/G_{DA} (\tau)$ between the donor autocorrelation function $G_{DD} (\tau)$ and the donor-acceptor cross-correlation function $G_{DA} (\tau)$. The data are normalized to the plateau for a better comparison. No difference between ligand-bound/-free PGK and the DNA rigid control is observed. The original curves are shown in Fig. S11 and Fig. S12. Color code for both panels B and C: double-labeled DNA with dyes separated by 10 basepairs (black) and 17 basepairs (gray). Panel B: ligand-free PGK (red). Panel C: ATP-bound (cyan), ADP-bound (orange), 3-PG-bound (green), and 3-PG*ADP-bound (yellow) PGK.
deviation of the distribution of distances of ~9.7 Å (without correcting for the effects of linker dynamics; for details, see Efficiency Histograms in the Supporting Material). From the efficiency histogram, we also obtain the occupation probability of the individual states, which is larger for the compact state ($p = 0.57$) as compared to the expanded state ($p = 0.43$). To summarize, these observations about the ligand-free protein highlight that PGK is intrinsically a highly dynamic and flexible system, which samples a wealth of conformations on a wide range of timescales (from nanoseconds to greater than milliseconds). These features unveil the complexity of the underlying energy landscape characterized by multiple minima.

**Effects of substrate binding on the conformational states and dynamics of PGK**

PGK is a key enzyme in glycolysis that catalyzes the reversible transfer of phosphate between bPG (1,3-bisphosphoglycerate) and ADP (bPG + ADP 3-PG + ATP). As shown in Fig. 1, the C-domain binds the nucleotides ATP (ADP), whereas the N-domain binds 3-PG (3-phosphoglycerate) or bPG. Therefore, we studied the impact of substrate binding on the dynamic and structural properties of PGK. For this purpose, we added to PGK solutions: in three cases, only one substrate (Mg2-ATP, K-ADP, 3-PG) forming binary complexes; and in one case, two substrates (K-ADP and 3-PG) forming a dead-end ternary complex. Qualitatively, the impact of all substrates (forming either binary or ternary complexes) on the nature of PGK conformations is rather similar, as shown in Fig. 4.

The most striking effect of substrate binding is a reduction of ~30% (for ATP-/ADP-bound PGK) and of ~47% (for 3-PG/3-PG*ADP-bound PGK) of the expanded state occupation probability to $p \sim 0.30$ and $p \sim 0.23$ (see left panel of Fig. 4 and Table S5 in the Supporting Material). A second feature, a shift of the major population (i.e., the compact state) to slightly smaller efficiency values, is related to increased mean interdye distances to $\langle r_{DM} \rangle_E = \langle r_{DM} \rangle_C$. The effect of substrate binding on this state is qualitatively the same as already observed in a previous time-resolved ensemble FRET study by Haran et al. (18). However, the absolute interdye distances were significantly smaller in this study, with values (32–38) Å. On the other hand, in a similar ensemble study (41), an interdye distance of ~39.4 Å was measured for ligand-free PGK, which is in reasonable agreement with our findings (see Fig. S6 in the Supporting Material). Because in both ensemble studies the same pair of dyes (IADENANS and IAF) and labeling positions were used, effects related to dye properties cannot explain the observed discrepancy. To our knowledge, the only difference, which may explain the shorter distances observed in Haran et al. (18), is the measuring temperature of 4°C instead of room temperature.

The often observed decrease of $R_g$ values upon substrate binding (15–17) is also supported by our data, as shown by slightly smaller diffusion times of PGK obtained from fluorescence correlation spectroscopy (see Fig. S2). From the single-molecule perspective, this observation is easily explained by the depopulation of the expanded state, which displays larger $R_g$ values as indicated by the longer mean burst duration $\langle T \rangle$ (see Fig. S5). Concerning the dynamics of the domains in the ligand-bound state of PGK, the only visible effect in our data (Fig. 2 B and Fig. 4) is the reduced shift of the major population with respect to the static FRET line, which indicates a smaller amplitude of motion within the compact conformation, as also observed by molecular dynamics (MD) (19), neutron spin echo (16), and ensemble FRET (18) studies.

Moreover, static heterogeneity, visible on the time regime of (0.001–10) ms, is confirmed for ligand-bound PGK by the correlation analysis (see Fig. 3 C) and by the variation of the bin duration of the apparent FRET efficiency histograms (see Fig. S12). Also, the extension of the conformational space visited by the domains in the expanded state is almost unchanged, with only a small decrease of the standard deviation of the interdye distance distribution to ~8.1 Å for 3-PG/3-PG*ADP-bound PGK (see Fig. S4).

Based on the resulting occupancies $p$ for the observed states, we derived a transition scheme for PGK, as shown in Fig. 5. Possible biases of the occupation probability values due to difference in the brightness or in diffusion coefficients between the low and high efficiency peaks can be excluded, as proved in the subsection Multi-Gaussian Fit in the Supporting Material. Therefore, for ligand-free PGK, a Gibbs free energy difference

$$
\Delta G = -0.2 \text{ kcal/mol}
$$

between the compact and the expanded state conformation was calculated. For ligand-bound PGK, values of

$$
\sim -0.5 \text{ kcal/mol}
$$

for ATP/ADP and

$$
\sim -0.7 \text{ kcal/mol}
$$

for 3-PG/3-PG*ADP were determined showing that 3-PG is more effective in stabilizing the compact state with respect to ATP and ADP. The relative equilibrium constants $K_{eq}$ were also derived and are given in Fig. 5.

With respect to the question of how substrate binding is coupled to functional relevant interdomain movements, three key results can be identified:

1. Substrate binding increases the compact state population and decreases the expanded state population. This
behavior can be explained by a conformational selection or population shift model. In fact, a prerequisite for the population shift model is a preexisting equilibrium of at least two states and with at least one state able to bind the substrate. This seems to be the case for PGK, where the substrates shift the equilibrium toward the compact state (which behaves as a prestate to reach catalytically competent conformations).

2. Substrate binding induces a conformational change of the compact state with an increase of the interdye distances. This feature is more consistent with properties described by the induced-fit model (4), although we cannot completely rule out a conformational selection mechanism. The potential relevance of this conformational change will be addressed in the Discussion.

3. Fast interdomain rigid body motions with fairly large amplitudes (see next subsection) are present in compact states of both the liganded and the ligand-free forms of PGK. Finally, although the structure of the compact state is slightly altered upon substrate binding, the conformational space sampled on the fast (\(>\text{\(\text{ns}\)}\)) timescale and on the very slow (\(>\text{\(10 \text{ ms}\)}\)) timescale is not altered substantially upon substrate binding.

This latter result supports the notion that the possibility to reach all catalytically relevant conformations is an intrinsic property of the three-dimensional protein structure (and its dynamical properties), and substrate binding is not required to reach these conformations (2,6,7). Nevertheless, small changes of the protein conformations are induced by the
substrates, which stabilize the conformation most suited for catalysis.

**NMA and mesoscale hydrodynamics simulation**

To achieve a more detailed picture of the interdomain motions within the compact state of ligand-free PGK, we performed NMA (3,20,38). The NMA was performed on the basis of the yeast PGK crystal structure (PDB:1QPG (37), Fig. 1) with substrate molecules omitted (see Coarse-Grained Model in the Supporting Material). Three major rigid-body movements correspond to the modes with the lowest frequencies, as reported in Inoue et al. (16) and Guilbert et al. (20). The geometries of these large-amplitude interdomain movements depend on the protein topology and are characterized by a hinge bending motion (mode 7), a propeller twist (mode 8), and a rocking motion (mode 9) (see Fig. 6A).

Because NMA inherently cannot give any information about absolute values of amplitudes and correlation times, we performed coarse-grained simulations (31,42). For this purpose, PGK is modeled by an elastic network embedded in a particle-based mesoscale solvent, to identify and characterize conformations sampled by PGK within the catalytically relevant compact state. Here, the multiparticle collision dynamics approach (see Coarse-Grained Model in the Supporting Material) is employed for the solvent. The preexponential factor of the restitution constant determining the force between pairs of particles was globally adjusted such that the calculated amplitude of the distance fluctuations between the dyes of $\sigma_{DA} = (9.0 \pm 0.1)$ Å matches the value of $\sigma_{DA} = (8.8 \pm 0.4)$ Å, which we measured with an ensemble lifetime FRET experiment (see Coarse-Grained Model in the Supporting Material). By deconvoluting the dye linker contribution from the rigid body motion of the domains with a wAV algorithm, which was independently calibrated with DNA standards (see the subsection Weighted Accessible Volume in the Supporting Material), we find that the simulation results agree with MD simulation studies (21). In this MD study, the authors obtained $\sigma_{CC} \sim 5.5$ Å for the amplitude of fluctuations between the C $\alpha$-atoms at position Q135 and S290, while we obtain a value of $(5.2 \pm 0.2)$ Å.

In addition, the mean interdye distances obtained from the simulation and from the smFRET measurement are in good agreement for the compact state conformation ($\langle r_{DA}\rangle^{\text{exp}} = 40.6$ Å and $\langle r_{DA}\rangle^{\text{sim}}_{E} = 39.4$ Å). Therefore, we used the results of the simulation performed on ligand-free PGK to analyze the structural fluctuations of the compact state in more detail. In this respect, we focused on structural fluctuations that can reduce the distance ($r_{PP}$) between the 3-PG phosphate group and the $\gamma$- or $\beta$-phosphate of ATP (or ADP) to a limiting value of ~4 Å, which is required for the phosphate-transfer reaction (13). To do so, atomistic representations of the rigid domains including bound substrates and the trajectory resulting from the protein dynamics simulation were superimposed. In this way, we were able to calculate the $r_{PP}$ trajectory. This trajectory in Fig. 6B (black) shows a mean $r_{PP}$ value (~12 Å) that is much larger than the limiting value. However, Brownian fluctuations of the domains results quite frequently in $r_{PP}$-distances reaching or breaking the limit of ~4 Å. In addition, this analysis shows that mainly the hinge bending motion (blue line) is responsible for the functional relevant fluctuation of the compact state. Therefore, functionally relevant large-amplitude Brownian motions are performed by PGK domains already in the ligand-free form.

**FIGURE 5** Transition scheme of PGK conformers between a compact (PGK$_c$) and an expanded (PGK$_e$) state with (+S) and without substrates. Occupation probabilities $p$ were determined from the FRET efficiency histograms to derive the equilibrium constants $K_{eq}$. Corresponding values of these two parameters are unknown for the transitions between the ligand-free and liganded form of PGK, which are represented here by vertical transitions. The pairs of $p$ and $K_{eq}$ values reported for the ligand-bound protein refer to ADP-/ATP-bound and 3-PG-/3-PG*ADP-bound PGK.

**FIGURE 6** (A) Radius of gyration $R_g$ and the FRET-averaged interdye distance $\langle r_{DA}\rangle_{E}$ calculated along the three slowest normal modes: the hinge bending (blue line), the rocking (red line), and the propeller twist (green line) motions. (B) The $r_{PP}$-distances (black line) and their projections on the normal modes (for color code, see panel A) are shown as a function of time (for details, see Coarse-Grained Model in the Supporting Material). The whole trajectory is shown in Fig. S7.
DISCUSSION

Although numerous studies on substrate-induced conformational changes of the PGK structure were published in the last decades (for an overview, see Vas et al. (14)), our study is, to our knowledge, the first on the single-molecule level. This approach made it possible to uncover, to our belief for the first time, the conformational state distributions of PGK, as well as the effect of substrate binding on this distribution. Furthermore, our study imparts knowledge about structural fluctuations within the respective states and about the interconversion between states. In addition, the combination of smFRET measurements with a coarse-grained model calibrated with our experimental data allows us to obtain a detailed picture of the catalytically relevant motions on a quantitative level, and to generalize our findings to a class of proteins with structural features similar to PGK.

In the past, it was often assumed that large conformational changes in PGK only occur when induced by ligand binding (11,13). Here, we demonstrate that large-amplitude interdomain movements, and thereby functionally relevant conformational changes, are occurring already in ligand-free PGK. These motions are encoded in the architecture of the native three-dimensional protein structure, as observed for some other proteins (1,2). An expanded and a compact conformation were observed. These conformations do not interconvert on the time interval of (0.001–10) ms that is accessible by our experiments. Compared to previous studies on PGK, where open and closed states were observed (11,13,17), the expanded state reported in this study is wider-stretched than these open states, and represents an ensemble of static conformers.

At the moment, the functional role of the expanded state is unclear. We also cannot rule out that the expanded state may be related to a locally unfolded conformation of the hinge region, which would allow the domains to be quite far apart from each other and which could represent a late step of folding. However, CD data indicate that the secondary structure is barely affected with respect to the compact state (32). Furthermore, there is some indication from previous studies that the balanced equilibrium between the expanded and compact states is most probably not only affected by substrate binding but also by other environmental conditions, like crowding (43) and/or buffer composition (32).

By combining our experimental results with the results of the hydrodynamics simulation, the compact state itself also exhibits large-amplitude fluctuations, but on a timescale of a few nanoseconds (see Fig. S10). However, the surprisingly fast timescale for these interdomain motions seems not to represent the rate-limiting step for the rather low catalytic turnover rates (10^2–10^3) s^-1 as observed for PGK (14). As shown in Fig. 6 B, mainly a hinge bending movement is driving PGK from its well-populated compact state to a fully closed conformation, facilitating a catalytically important requirement, namely, a close distance of the involved phosphate groups. In this respect, our compact state is more similar to the open state, as described in Pickover et al. (11), Bernstein et al. (13), and Zerrad et al. (17). Similar to a spring-loaded release mechanism proposed in 2011 (17), our results indicate that PGK adopts a fully closed conformation (i.e., rPP ~ 4 Å) for only short time periods and resides in the open (compact) conformation for much longer time periods (see Fig. 6 B).

From our knowledge of possible geometries of the involved interdomain movements (based on NMA), we have no indication that the experimentally observed increase of \( \langle r_{DA} \rangle_E \) induced by the substrates is directly related to the required reduction of the phosphate distance rPP. Also, the fact that different substrates induce slightly different increases in \( \langle r_{DA} \rangle_E \) (see Fig. 4 and Fig. S6) is not fully understood at the moment. Nevertheless, we assume that the observed feature is related to a structural adjustment making the fast interdomain motions (which appear in the compact state) catalytically more efficient. Such a mechanism was proposed by a study based on MD simulations (19).

In this study, it is proposed that bound substrates restrain the hinge bending to only one dominant hinge point in the vicinity of the substrates. As a result of the reduced overall flexibility of the hinge, the interdomain motions become more directional in bringing the substrates close to each other. A more detailed experimental characterization of the motions, which brings both substrates into a catalytically competent conformation, can be obtained with the following:

1. By employing additional reference positions with distance closer to the Förster radius of our FRET pair, to be more sensitive to interdye distance changes and better resolve the geometry of the domain motions;
2. By measuring the enzyme in the fully closed state stabilized by AlF^4+ (17) and during turnover; and

In fact, this last approach may allow us to experimentally determine correlation times of the fast interdomain movements for PGK.

CONCLUSIONS

The major findings of this article are not limited just to the investigated enzyme PGK, but may have some general application to explaining the interplay between conformational changes and ligand binding, and their relation to catalysis. For another well-studied model system, namely the adenylate kinase, very similar mechanisms were discovered in 2007 (6,7). Although the adenylate kinase has a quite different topology compared to PGK, with three domains of
unequal size (a core domain and two lids), both of these phosphate-transferring kinases share the following general features:

1. Large-amplitude, interdomain movements occur on a broad spectrum of timescales, from the micro- to milli- seconds time-regime, to motions on a nanosecond timescale. These movements include specific motions that occur on trajectories in the direction toward the catalytically competent (often fully closed) state and enable the covering of the entire conformational space required for a complete catalytic reaction cycle. Therefore, the character of those motions is an intrinsic property of the proteins encoded in the three-dimensional structure, which is most probably evolved to accomplish the enzymatic functions with high efficiency.

2. Substrate binding does not generate totally new conformational states, because all possible conformations are already fully sampled in the absence of substrates. In fact, substrate binding induces only small local structural adjustments, possibly accompanied by a restriction of possible motions, to increase the probability to reach the catalytically competent conformation.

Beyond that, adenylate kinase shows catalytically relevant large-amplitude motions on timescales comparatively similar to inverse catalytic rates (microseconds to milli- seconds), indicating that these motions play a rate-limiting role in catalysis (6,7). In contrast, PGK, which features a rather different structural topology with two large do- mains connected by a flexible hinge region, exhibits fast large-amplitude motions (nanoseconds), apparently too fast to be rate-limiting for the enzymatic reaction. Therefore, because these motions are structurally encoded, we would expect a similar behavior from protein with comparable structural features, i.e., compact rigid domains connected by flexible connecting regions (45). Despite that, classes of proteins with different functional roles, but with a similar structural topology as compared to PGK, show functional motions on a wide range of timescales. Examples are the fast large-scale conformational motions with interconversion rates on the nanosecond timescale simulated for periplasmic binding proteins (46,47), and much slower transition rates (in the regime of 10–200 ms) identified experimentally for the binding domain of a glutamate receptor (48) and SlyD, a member of the peptidyl-prolyl cis-trans isomerases class of en- zymes (28).

To our understanding, this apparent contradiction underlines how the geometry of the domain motions is dictated by the protein structural topology, while the extent, the timescales, and the coordination of these motions are adapted to the specific functional role of the protein. Therefore, we think that the presented approach, which allows one to probe scores of different timescales, represents a valuable tool to systematically study the dynamics of multidomain proteins to reveal a common thread between structural and functional properties.

**SUPPORTING MATERIAL**

Thirteen figures, eight tables, and additional supplemental information are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00886-8.

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**REFERENCES**


