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Elevated μ s-ms timescale backbone dynamics in the transition state analog form of arginine kinase

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Abstract

Arginine kinase catalyzes reversible phosphoryl transfer between arginine and ATP. Crystal structures of arginine kinase in an open, substrate-free form and closed, transition state analog (TSA) complex indicate that the enzyme undergoes substantial domain and loop rearrangements required for substrate binding, catalysis, and product release. Nuclear magnetic resonance (NMR) has shown that substrate-free arginine kinase is rigid on the ps-ns timescale (average $S^2=0.84 \pm 0.08$) yet quite dynamic on the μ s-ms timescale (35 residues with R_{ex} , 12%), and that movements of the N-terminal domain and the loop comprising residues I182-G209 are rate-limiting on catalysis. Here, NMR of the TSA-bound enzyme shows similar rigidity on the ps-ns timescale (average $S^2=0.91 \pm 0.05$) and substantially increased μ s-ms timescale dynamics (77 residues; 22%). Many of the residues displaying μ s-ms dynamics in NMR Carr-Purcell-Meiboom-Gill (CPMG) ^{15}N backbone relaxation dispersion experiments of the TSA complex are also dynamic in substrate-free enzyme. However, the presence of additional dynamic residues in the TSA-bound form suggests that dynamics extend through much of the C-terminal domain, which indicates that in the closed form, a larger fraction of the protein takes part in conformational transitions to the excited state(s). Conformational exchange rate constants (k_{ex}) of the TSA complex are all approximately 2500 s^{-1} , higher than any observed in the substrate-free enzyme ($800 - 1900 \text{ s}^{-1}$). Elevated μ s-ms timescale protein dynamics in the TSA-bound enzyme is more consistent with recently postulated catalytic networks involving multiple interconnected states at each step of the reaction, rather than a classical *single* stabilized transition state.

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Keywords

Arginine kinase; dynamics; NMR; relaxation dispersion; transition state analog

Arginine kinase (AK; EC 2.7.3.3) is a member of the phosphagen kinase family of enzymes and catalyzes the reversible transfer of a phosphoryl group between ATP and arginine, allowing for buffering of ATP levels in cells with high and/or variable energy demands. As well as playing an important physiological role, members of the phosphagen kinase family of enzymes have long been paradigms for understanding bimolecular enzyme catalysis (Ellington, 2001). Due to its amenability to both high resolution X-ray crystallography and nuclear magnetic resonance spectroscopy, arginine kinase from the Atlantic horseshoe crab *Limulus polyphemus*, a 42 kDa monomeric enzyme, is an ideal model system for understanding the link between dynamics and function through the enzymatic cycle (Niu et al., 2011; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998).

Three decades of research on arginine kinase point to the enzyme as being dynamic, existing in multiple well-defined structural states that interconvert during turnover which is on the ms timescale. Solution scattering initially highlighted a decrease in the enzyme's radius of gyration upon substrate binding (Dumas and Janin, 1983). Subsequent crystal structures of arginine kinase in both a substrate-free state, which is open, and a transition state analog (TSA) bound state, which is closed, revealed the locations and magnitudes of these conformational changes (Niu et al., 2011; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998). The TSA is composed of arginine, MgADP, and nitrate bound to arginine kinase, with the planar nitrate anion mimicking the phosphoryl group being transferred between arginine and ADP (Figure 1). Previous NMR, EPR, and enzyme kinetic studies have shown that the nitrate ion, when added to the abortive complex of arginine kinase bound to arginine and MgADP, indeed forms a transition state analog (Buttlaire and Cohn, 1974a; Buttlaire and Cohn, 1974b; Rao et al., 1976). In the 1.2 Å resolution crystal structure of the arginine kinase TSA complex, neither the substrate arginine, ADP, nor the nitrate ion are constrained covalently, yet the nitrate ion is observed in almost the exact position the phosphoryl group would be expected to be in the actual transition state with partial covalent bonding to both nucleotide β -phosphate and arginine guanidinium (Yousef et al., 2002; Zhou et al., 1998). Analogous complexes have been used to solve TSA crystal structures of other phosphagen kinases: glycoamine kinase, taurocyamine kinase, and creatine kinase (Lahiri et al., 2002; Lim et al., 2010; Merceron et al., 2015).

The open and closed crystal structures of arginine kinase show the enzyme to be a two domain enzyme comprising an α -helical N-terminal domain (NTD) and a β -sheet C-terminal domain (CTD) which are connected by a 29 residue flexible linker. Substrate associated motions highlighted by these structures include folding of the NTD and CTD, in addition to a number of loops, over the active site, similar to a hand performing a grasping motion. These conformational changes have also been described in terms of five “dynamic domains,” which are groups of residues whose motion can be described by a single rotation and/or translation and need not be contiguous in primary sequence (Niu et al., 2011) (Figure 1A).

Complementing these crystallographic snapshots, which provide information about the magnitudes of conformational changes and states at either end of an equilibrium, have been recent NMR-based measurements of the rates of enzyme dynamics in the absence of substrates. Lipari-Szabo model-free analysis highlights a single substrate binding loop (residues 308-322) as being intrinsically dynamic on the ps-ns timescale. ^{15}N spin relaxation dispersion measurements indicate that the backbone of substrate-free arginine kinase is significantly involved in μs -ms timescale dynamics, with approximately 12% of the enzyme exhibiting dynamics on this timescale. These studies also established a link between the dynamics and function of arginine kinase. The most pronounced protein dynamics in the substrate-free form were mostly at the hinges and contact surfaces between dynamic domains that were independently seen to be displaced on substrate binding in the crystal structures. Furthermore, the rates of intrinsic conformational dynamics were shown to correspond to the rate of enzyme turnover, providing the first hints that these conformational changes may be rate-limiting for enzyme function (Davulcu et al., 2009). The similarity between subsequently measured activation energies of both the intrinsic conformational dynamics and of enzyme turnover further expounded upon the link between dynamics and turnover and provides supporting evidence for intrinsic dynamics being rate-limiting upon the arginine kinase catalytic cycle (Davulcu et al., 2011; Davulcu et al., 2009; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998).

Recently, the dynamics of selected side-chains have been measured across a number of states representative of different points along the reaction cycle. Side-chain methyl groups of isoleucines, leucines, and valines were used as probes. Only six of a total of 71 ILV residues were observed to exhibit side-chain dynamics in the substrate-free form of arginine kinase. This number increased greatly in bound forms of the enzyme. 15 side-chain methyl groups were shown to undergo conformational exchange in a product complex analog; 34 in a Michaelis complex analog, and 25 in the transition state analog. It was shown that the rates of exchange in the TSA were more than four times greater than that of the Michaelis complex analog. The Michaelis complex analog and the TSA were shown to exhibit a reciprocal relationship in which the conformational ensembles of one complex samples the dominant conformation of the other as a higher-energy excited state, suggesting that the relative free energies of these protein conformations may be modulated as the reaction proceeds (Peng et al., 2017).

In this work, NMR-based ^{15}N spin relaxation measurements are employed to probe now the backbone dynamics of the transition state analog (TSA) bound form for a more comprehensive characterization of conformational exchange throughout arginine kinase. For this purpose, the enzyme was bound to arginine, MgADP, and nitrate, which mimics the phosphoryl group that is transferred between arginine and ATP in the active enzyme, under conditions similar to those of the crystal structure of the enzyme (Zhou et al., 1998). Model-free analysis of NMR laboratory frame ^{15}N T_1 , T_2 , and ^1H - ^{15}N NOE data indicate that the TSA complex is relatively rigid on the ps-ns timescale, which is similar to the substrate-free enzyme (Davulcu et al., 2009). ^{15}N spin Carr-Purcell-Meiboom-Gill relaxation dispersion analysis, however, identifies 77 residues undergoing μs -ms timescale dynamics in the closed form of the enzyme. These residues are located both within and distal to the active site and,

in total, amount to a near doubling of the number of dynamic residues as compared to the substrate-free form of arginine kinase (Davulcu et al., 2009).

Materials and Methods

Sample Preparation

All NMR experiments were performed using arginine kinase isotopically enriched in both ^{15}N (uniform) and ^2H (~75%). Enzyme was expressed and purified as previously described (Davulcu et al., 2005). A TSA complex (AK:MgADP:L-arginine: NO_3^-) was prepared using 0.8 mM enzyme in a buffer containing 10 mM d_4 -imidazole (pH 6.5), 6 mM ADP, 8 mM MgCl_2 , 60 mM L-arginine, 20 mM NaNO_3 , 1 mM DTT, 0.2 mM NaN_3 , 90% H_2O , and 10% D_2O . At these concentrations, greater than >99% of enzyme is bound with TSA components (MgADP:L-arginine: NO_3^-).

NMR Spectroscopy

NMR relaxation data were recorded using Varian INOVA (500 and 600 MHz) and Agilent DirectDrive (800 MHz) NMR spectrometers. The 600 MHz instrument is equipped with a cryogenic HCN triple resonance probe and the 500 and 800 MHz instruments are equipped with RT Nalorac HCN probes. All experiments were recorded at 25 °C; the temperature was calibrated using a methanol standard (Raiford et al., 1979). ^1H chemical shifts were referenced to the DSS methyl protons at 0 ppm and ^{15}N chemical shifts were referenced indirectly (Markley et al., 1998). NMR data were processed with NMRPipe or FELIX2004 (Felix NMR, Inc) and analyzed with SPARKY (Goddard and Kneller). Backbone amide resonance assignments for arginine kinase bound to the transition state analog have been previously reported (Davulcu et al., 2014).

^{15}N T_1 , T_2 , and heteronuclear ^1H - ^{15}N NOE values were measured with relaxation experiments modified for TROSY detection (Farrow et al., 1994). At 500 MHz, ^{15}N T_1 was measured using relaxation delays of 0.01, 0.05*, 0.1, 0.2, 0.5*, 1.0, and 2.5 second delays (asterisks denote points that were measured in duplicate); ^{15}N T_2 was measured using relaxation delays of 10, 30, 50, 70, and 90 ms; the steady-state ^1H - ^{15}N NOE was measured using a ^1H saturation time of 12 seconds. At 600 MHz, ^{15}N T_1 was measured using relaxation delays of 0.02*, 0.05, 0.1*, 0.2, 0.5*, 1.0, 2.0, and 4.0 second delays; ^{15}N T_2 was measured using relaxation delays of 10*, 30*, 50, 70*, and 90 ms; the steady-state ^1H - ^{15}N NOE was measured using a ^1H saturation time of 12 seconds.

Chemical exchange on the μs -ms timescale was quantified at 600 and 800 MHz static fields using a constant-time ^{15}N CPMG relaxation dispersion measurement with TROSY detection (Loria et al., 1999). A 32 ms constant-relaxation time period was utilized in both datasets. At 600 MHz, the CPMG effective field strengths (ν) were 31.25*, 62.5, 93.75*, 125, 187.5, 250, 500, 750, and 1000 Hz. At 800 MHz, ν = 31.25, 62.5, 93.75, 125, 187.5, 281.25, 375, 500, 625, 750, 875, and 1000 Hz.

Fast (ps-ns) Timescale Analysis

^{15}N spin relaxation time constants T_1 and T_2 were calculated from two-parameter single exponential fits of peak intensities vs. delay times. ^1H - ^{15}N NOE values were calculated from the peak intensity ratios recorded with and without ^1H saturation. Errors on all values were determined from duplicate measurements, if available; otherwise errors were estimated from the root-mean-square baseline noise of each spectrum.

Selection of the rotational diffusion model was performed using the *relxn2.2* software with the *rvi* interface (Boyer and Lee, 2008). Both an isotropic and axially symmetric model were tested on residues which did not show evidence of conformational exchange from either T_1/T_2 ratio analysis or CPMG measurements (Tjandra et al., 1995a). The isotropic diffusion model was optimized via least-squares fitting of spectral density functions to T_1/T_2 ratios. The axially symmetric diffusion model utilized the crystal structure of the closed form of arginine kinase, PDB ID 1M15 (Yousef et al., 2002; Zhou et al., 1998).

Lipari-Szabo parameters S^2 , S^2_f , S^2_s , τ_e , τ_s , and R_{ex} were fit to the five standard motional models – model 1 (S^2); model 2 (S^2, τ_e); model 3 (S^2, R_{ex}); model 4 (S^2, τ_e, R_{ex}); model 5 (S^2_s, S^2_f, τ_s) (Clore et al., 1990; Lipari and Szabo, 1982a; Lipari and Szabo, 1982b; Tjandra et al., 1995a). Model selection was performed in *relxn2.2* and was guided by χ^2 statistics. Typically, the model with the lowest χ^2 was selected; however, for residues with no detectable R_{ex} in CPMG experiments, models 3 or 4 were only selected if the fitted R_{ex} value $> 1 \text{ s}^{-1}$ (d'Auvergne and Gooley, 2003).

Slow (μs -ms) Timescale Analysis

R_2^{eff} was calculated at each effective field strength as follows: $R_2^{\text{eff}} = (-1/\tau_{\text{relax}})(\ln I_\nu/I_0)$, where τ_{relax} is the constant relaxation time period, I_ν is the intensity of a peak at effective field ν , and I_0 is the intensity in the reference spectrum. Relaxation dispersion plots of R_2^{eff} vs. ν were fit to a two-site exchange model with a numerical approximation of the Bloch-McConnell equation using software provided by Dimitri Korzhnev and Lewis Kay (Cavanagh et al., 1996). Residues included in the fitting were those with an estimated R_{ex} value greater than 2 s^{-1} .

The timescale of chemical exchange can be determined with R_{ex} measured at multiple static fields with the assistance of parameter α (Millet et al., 2000): $\alpha = ((B_{02}+B_{01})/(B_{02}-B_{01})) * ((R_{ex2}-R_{ex1})/(R_{ex2}+R_{ex1}))$, where B_{01} and B_{02} are the lower and higher static field strengths, respectively, and R_{ex1} and R_{ex2} are the chemical exchange contributions to R_2 determined at those two field strengths. Ideally, values for α lie between 0 and 2, with $\alpha < 1$ indicating slow exchange; $\alpha > 1$ indicating fast exchange; and $\alpha \approx 1$ indicating intermediate exchange. In the slow to intermediate exchange regime, k_{ex} , p_A , p_B and ω can be determined (Millet et al., 2000). However, for residues in fast exchange, p_A , p_B and ω cannot be uniquely deconvoluted from the product $p_A p_B \omega^2$ that is fit together with k_{ex} . In this work, α is used to pre-fit p_B values for residues that appear to be in the slow to intermediate exchange regime, which are then constrained in subsequent collective fits with other residues.

Results and Discussion

Fast (ps-ns) Timescale Dynamics

Arginine kinase presents an attractive opportunity to directly compare backbone dynamics (ps-ms) of a large, bimolecular enzyme in both substrate-free and TSA substrate-bound forms. Knowledge of both fast (ps-ns) dynamics in the TSA form of arginine kinase not only complements those of the slower μ s-ms timescale, providing a more complete description of the dynamic landscape of the enzyme, but also allows for comparisons with the substrate-free form of the enzyme.

Fast backbone dynamics were characterized using NMR ^{15}N spin longitudinal (T_1) and transverse (T_2) relaxation rate constants and heteronuclear ^1H - ^{15}N nuclear Overhauser effect (NOE) from two static magnetic field strengths, $\nu_0(^{15}\text{N}) = 50.65$ and 60.78 MHz. Relaxation time constants and NOE values are shown in Supplemental Figure 1 and the mean and trimmed mean values, which were used in the τ_m determination, are summarized in Table 1. Isotropic and axially symmetric rotational diffusion models are described in detail in supplemental information.

Intramolecular ps-ns dynamics of the backbone ^{15}N - ^1H bond of each residue were analyzed following standard Lipari-Szabo model-free methods with the results summarized in Table 2. Order parameters are plotted in Figure 2 and τ_e , τ_s and R_{ex} values are shown in Supplemental Figure 3. Model selection was guided using χ^2 statistics. For those residues in which χ^2 could not be used to distinguish between a subset of models, the simplest model was chosen (Clarkson et al., 2006; d'Auvergne and Gooley, 2003; Mandel et al., 1995). Moreover, models 3 (S^2 , R_{ex}) and 4 (S^2 , τ_e , R_{ex}) were rejected if the fitted R_{ex} value was below 1 s^{-1} . Three residues (T217, S282, and F270) were poorly fit by all models and were thus omitted from subsequent analysis.

The order parameters and fitted R_{ex} indicate that the TSA-bound form of AK is rather rigid on the ps-ns timescale and a larger portion of the enzyme is dynamic on the μ s-ms timescale. The average S^2 value over all residues is 0.91 ± 0.05 . Only two residues have S^2 values more than three standard deviations below the average (D112 and the C-terminal residue A357). Overall, the S^2 values of the substrate-free and TSA bound forms of arginine kinase show a small increase when TSA ligands are bound. For residues measured in both states, the S^2_{apo}/S^2_{TSA} ratio is 0.92, where a value of 1 would reflect that, on average, the S^2 values of the two states are identical. Nearly half (44%) of residues were best fit with models 3 or 4, which include a R_{ex} contribution to R_2 , potentially arising from slow, μ s-ms timescale dynamics. The average fitted R_{ex} value for these residues is 4.3 s^{-1} , with a maximum value of 41.7 s^{-1} fitted for R330. Overall, Lipari-Szabo analysis indicates that arginine kinase in the TSA complex is quite rigid on the ps-ns timescale with a substantial portion of the enzyme exhibiting dynamics on the μ s-ms timescale.

As shown in Supplemental Figure 2, the largest differences in order parameters are observed for residues S318-V322, at the C-terminal end of the flexible substrate-binding loop (L13). In substrate-free arginine kinase, loop L13 (residues V308-V322) was shown to be flexible on the ps-ns timescale, with average S^2 values of 0.53, while the rest of the enzyme is

mostly rigid on this timescale. It is worth noting that while 15 backbone amides in this loop could be assigned in the substrate-free form, only seven were assigned in the bound form. Unassigned residues in the bound form include the entire stretch from G310-T316, which forms direct interactions with substrates (Yousef et al., 2002; Zhou et al., 1998). While assignment of large proteins is often hampered by high levels of spectral crowding and overlap, it is unlikely that all seven of these residues are unassigned due to resonance degeneracy. It seems more plausible that, on interacting with bound substrates, these residues move from fast exchange to the intermediate exchange regime. This would broaden resonances in a way that often makes them undetectable. Such a dampening of the motions of the L13 loop, on substrate binding, would be consistent with our crystal structures. In the substrate-free form, L13 is mostly disordered, but is fully ordered in the TSA complex structure (Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998). Perhaps, the best indication of the open structure comes from the homolog, lombricine kinase (LK), which substitutes Asp₃₁₈-Asp₃₁₉ for AK's Gly₃₂₀-Gly₃₂₁, at the C-terminal hinge of the L13 substrate-binding loop (Bush et al., 2011). In LK, on substrate dissociation, this loop undergoes a 7° hinged rotation of up to 1.7 Å (backbone), but unlike other phosphagen kinases that have the Gly-Gly hinge, the loop in LK remains ordered. We speculate that the dynamics of loop L13 are reduced on substrate binding, changing the exchange regime, but that the loop retains a fraction of such motion in the bound state, rendering it invisible to crystallographic analysis.

Slow (μs-ms) Timescale Dynamics

Slow backbone dynamics were measured using CPMG ¹⁵N spin transverse relaxation dispersion at two static field strengths, $\nu_0(^{15}\text{N}) = 60.78$ and 81.04 MHz, mapped onto the enzyme structure, and analyzed for exchange parameters. Forty-six residues were identified with $R_{\text{ex}} > 2 \text{ s}^{-1}$ at $\nu_0(^{15}\text{N}) = 60.78$ MHz and 71 at $\nu_0(^{15}\text{N}) = 81.04$ MHz, yielding a total of 77 residues, or roughly 22% of the enzyme (Supplemental tables 3 and 4). Several relaxation dispersion profiles are shown in Figure 3 and residues with R_{ex} are shown with black spheres in Figure 4. Within the context of “dynamic domains 1-5,” they can be clustered into different regions (Figure 4 and Table 3): (1) dynamic domain 4 (orange); (2) loop L8; (3) dynamic domain 5 (cyan); (4) dynamic domain 2 (red); and (5) the hinge and (6) interface between the NTD and CTD. The clustering of R_{ex} by dynamic domain implies that all five undergo motion in the closed state (Table 3). For dynamic domain 1 (NTD), which moves as a large rigid group between open and closed forms, R_{ex} is detected at the hinge and interface between the NTD and CTD.

“Dynamic domains” comprise 3D clusters of residues that are not necessarily contiguous in primary sequence, whose motion can be approximated by a common rotation and translation operation. In AK, motions of the 5 dynamic domains can account for most of the individual coordinate differences between open and closed forms of the enzyme (Figure 1A) (Niu et al., 2011; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998). Collective fitting of the Bloch-McConnell equation (Cavanagh et al., 1996) to dispersion data for the residues in each cluster yields k_{ex} values ranging from approximately 2000 s^{-1} to 3000 s^{-1} and higher-energy (excited) state populations (p_B) ranging from 1.3 to 1.8% (see Materials and Methods for fitting details). Collective fitting of all residues in the CTD yields similar values ($k_{\text{ex}} =$

2300 s⁻¹ and p_B = 1.5%) while fitting of all residues in the enzyme also yields similar values (k_{ex} = 2300 s⁻¹ and p_B = 1.5%).

A potential concern is that R_{ex} observed in the TSA may in part be a result of substrates binding and unbinding and not necessarily conformational exchange, especially in dynamic domain 4 at the center of the active site (Niu et al., 2011). The effect of ligand binding and unbinding on R_{ex} was minimized by using saturating substrate concentrations. Enzyme kinetics has shown that arginine kinase's K_Ms for arginine and ADP are approximately 1.0 mM and 0.1 mM, respectively (Azzi et al., 2004; Davulcu et al., 2011; Gattis et al., 2004; Pruett et al., 2003). Here arginine and ADP concentrations were 60 mM and 6 mM, both 60-fold higher than their respective K_Ms from enzyme kinetics and, extrapolating from creatine kinase measurements, 600-fold higher than the K_Ds for dissociation of individual substrates from the TSA complex (Borders et al., 2002). While it is not feasible to completely exclude contributions of substrate binding to R_{ex}, concentration of substrates well above their respective K_Ds or K_Ms minimize this contribution. We have also estimated the k_{ex} to be about 31,000 s⁻¹ for nucleotide binding/unbinding under our TSA-bound conditions, using k_{on} and k_{off} rate constants of creatine kinase (Hammes and Hurst, 1969) an arginine kinase ortholog. Exchange at that rate constant that would contribute minimally to R_{ex}.

The transition from the open form of arginine kinase to the closed form is marked by a number of domain and loop reorientations, each discussed below, relative to the domain structure of arginine kinase.

N-terminal domain (NTD)—This domain exhibits the largest displacement between substrate-free and TSA-bound structures: a ~12° rotation about a hinge centered near G91. This rigid body motion also causes a flexing at the interface between the NTD and CTD. Thus residues that are located at the hinge and interface were collectively fit, as they both reflect motion of the NTD (Davulcu et al., 2009; Niu et al., 2011; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998). The fitted k_{ex} for the NTD is 2400 s⁻¹. A reliable estimate of p_B cannot be extracted for the NTD (as well as loop L8 and dynamic domain 2, discussed below), because deconvolution of p_B from the p_{APB} ω² depends on residues in slow to intermediate exchange (see Materials & Methods) (Millet et al., 2000) of which there are none. Loop L8 (I182-G209) undergoes a conformational change between the open and closed structures to shield the nucleotide binding site. Residues in this loop are displaced over 5 Å between the two structures, but there appears to be a significant translational component to this motion (Davulcu et al., 2009; Niu et al., 2011; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998). The collectively fit k_{ex} is 2500 s⁻¹, which is nearly the same for all other clusters fit for the closed form of the enzyme. *Limulus polyphemus* arginine kinase is unique among the phosphagen kinases in that a salt bridge is observed between R193 and D62 in the crystal structure of the closed form, thus connecting the NTD to L8 (Azzi et al., 2004; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998). Collectively fitting residues that reflect the NTD motion with those in loop L8 yields a k_{ex} of 2530 s⁻¹. This is indistinguishable from k_{ex} fit to each subset, as would be expected if motions of the NTD and loop L8 are concerted in the closed enzyme.

CTD (β -sheet) and flanking helices—Residues with R_{ex} are found throughout the entirety of the β -sheet CTD and flanking helices, composed of dynamic domains 2, 4, and 5 (Niu et al., 2011). These residues are near the substrate binding site but others are located within distal helices at the edges of the CTD (Figure 4). The CTD undergoes a complex conformational transition upon binding of substrates, with dynamic domains 2 and 5 both rotating inwards, over the bound substrates. Overlaid on top of this motion is a subtle twisting of the β -sheet, a result of dynamic domains 2 and 5 rotating in opposite directions as they close over the active site (Davulcu et al., 2009; Niu et al., 2011; Yousef et al., 2002; Yousef et al., 2003; Zhou et al., 1998). R_{ex} observed in domain 2 may also reflect motions that are required for the large motion of loop L8.

Loop L13 and dynamic domain 5—A similar pattern may be present in loop L13 and dynamic domain 5. No quantitative comments can be made about loop L13 as many of its residues remain unassigned due to extensive line broadening. However, the large number of residues with measurable R_{ex} indicates motion in dynamic domain 5, which may be coupled to motions in loop L13 that is book-ended by dynamic domain 5. Interestingly, evidence of such motions in dynamic domain 5 is not seen in the R_{ex} of substrate-free arginine kinase (Davulcu et al., 2009).

Dynamic domains 2, 4, and 5—Overall, the collectively fit k_{ex} values for dynamic domains 2, 4, and 5, are similar: 2690 s^{-1} , 2300 s^{-1} , and 2470 s^{-1} , respectively (summarized in Table 3). The differences in these values are within error, and furthermore, a single collective fit of all three of these rigid groups yields a similar k_{ex} of 2300 s^{-1} . Fitting of p_B is possible for dynamic domains 4 and 5 and these values are also quite similar at 1.3% and 1.8%, respectively, with the collective fit yielding a value of 1.5%. Based on the close similarity of the k_{ex} for dynamic domains 2, 4, and 5, it is likely that these motions are collective in nature.

Comparison of apo and TSA-bound slow dynamics—Relaxation dispersion analysis of the substrate-free enzyme showed that residues undergoing motion on the slower μs -ms timescale were located throughout the enzyme. They could be classified into three types: (1) movement of the NTD relative to the CTD; (2) motion within dynamic domain 1; and (3) motion of the substrate binding loop L8 (residues I182-G209). The TSA-bound form of arginine kinase, on the other hand, is much more dynamic on the μs -ms timescale, with 77 residues that have $R_{ex} > 2\text{ s}^{-1}$ - more than double the 35 observed in substrate-free enzyme. All of the clusters that were collectively fit in substrate-free enzyme are represented in the transition state bound enzyme, with its clusters of R_{ex} that were described above. About half of the residues with R_{ex} in the substrate-free enzyme also exhibit R_{ex} in the transition state bound enzyme (Davulcu et al., 2009). Not only do additional residues exhibit R_{ex} in the TSA complex, but the collectively fit k_{ex} values indicate that the backbone motions are faster in the closed form. In substrate-free enzyme, loop L8 exhibits among the slowest intrinsic motions, but in the closed form, the k_{ex} of this loop is indistinguishable from the other dynamic regions of the enzyme (Davulcu et al., 2011; Davulcu et al., 2009; Niu et al., 2011).

Recently, a pair of crystal structures of the arginine kinase TSA complex has been solved at ambient temperature. These two structures, from different crystal forms, are very similar

overall, with a backbone RMSD of 0.3 Å. The largest domain level rotations between the two crystal forms involve domains 3 and 5, which differ by 1.2 and 1.0 degrees, respectively (Godsey et al., 2016). These rotations may reflect locations of flexibility within the TSA complex and may be the origin of the R_{ex} observed in these rigid groups. R_{ex} is also detected in dynamic domains that do not differ between crystal forms, perhaps because of lattice constraints.

Traditionally, the transition state of an enzyme-substrate complex has been viewed as a structurally well-ordered system. Recently, this view has been brought into question by Nussinov and others, who have made theoretical arguments that the energy surface of the protein as a TSA complex would likely be complex, and not a simple saddle point: passage through a rigid transition state form would present the enzyme with a large entropic barrier (Ma et al., 2000). With this perspective, the transition state, and indeed all states of an enzyme, would be viewed as dynamic. Connecting the proposed multi-conformer states would be redundant pathways termed “catalytic networks” (Ma et al., 2000; Wei et al., 2016).

Experimental evidence for enzymes remaining dynamic after substrate binding and through the reaction cycle is building. Dihydrofolate reductase exhibits dynamics through five different intermediates along the reaction pathway and each intermediate has been shown to sample higher energy conformations that resemble adjacent intermediates (Boehr et al., 2006). In ribonuclease A, similar exchange parameters were obtained for dynamic residues in the substrate-free form of the enzyme along with stable mimics of the enzyme-substrate and enzyme-product complexes. Moreover, the distributions of dynamic residues were comparable, leading to the suggestion that these different states of the enzyme share similar modes of motion (Loria et al., 2008). Adenylate kinase was shown to exhibit backbone dynamics in both a substrate-free form and when bound to Ap5A, a covalently fused bi-substrate mimic of the natural substrates ATP and AMP (Henzler-Wildman et al., 2007). Finally, side-chain methyl groups in FKBP12 were shown to increase both in total number and rates of motion upon binding of FK506, a transition state analog of FKBP12's catalyzed peptidyl-prolyl cis-trans isomerization reaction (Brath and Akke, 2009).

Arginine kinase had long been thought to be an induced fit enzyme, with conformational changes being driven by substrate binding. Subsequent work showing that substrate-free enzyme sampled states that were similar in structure to the substrate-bound form suggested that conformational selection plays a role (Davulcu et al., 2011; Davulcu et al., 2009; Niu et al., 2011). As noted in the previous paragraph, others have now shown that conformational dynamics can persist through substrate and product-bound intermediate states of various enzymes. The current work has taken a rare opportunity to map experimentally the conformational dynamics of a transition state analog complex throughout an enzyme. Arginine kinase has shown, perhaps surprisingly, that the μ s-ms timescale dynamics of the transition state are not reduced. Indeed, in qualitative agreement with postulates of catalytic networks, the protein conformational dynamics are elevated relative to that of the substrate-free form.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NMR	nuclear magnetic resonance
AK	arginine kinase
TSA	transition state analog
NTD	N-terminal domain
CTD	C-terminal domain
CPMG	Carr-Purcell-Meiboom-Gill
NOE	nuclear Overhauser effect
RMSD	root mean square deviation
TROSY	transverse relaxation optimized spectroscopy

Figure 1.

Structures and NMR spectra of the transition state analog (TSA) complex of arginine kinase. (A) The crystal structure of the TSA complex is overlaid on the substrate-free form, shown with transparent hues. Substrates are shown in stick model. The protein model is colored by dynamic domain, with blue, red, green, orange, and cyan corresponding to domains 1-5 (Niu et al., 2011). Residues which cannot be assigned to a dynamic domain are colored grey. (B) Chemical structure of the transition state analog bound to arginine kinase: an unreactive nitrate is held with non-covalent enzyme interactions between ADP and arginine in the active site, closely mimicking calculated structures with a γ -phosphoryl group bridging between nucleotide and guanidinium with the partial covalent bonding of the transition state (Zhou et al., 1998). The underlying enzyme structure is shown with high transparency. A stereo version is available as supplemental figure 4. (C) 2D [^{15}N , ^1H]-TROSY of arginine kinase bound to the transition state analog. (D) Boxed-out area of panel C shown as an overlay of 2D [^{15}N , ^1H]-TROSY of arginine kinase in the substrate-free form (blue) on that of the TSA form (red). An overlay of the full spectra is shown in supplemental figure 5.

Figure 2. Generalized order parameters obtained for transition state analog bound arginine kinase. Secondary structure is noted above each plot with cylinders and arrows representing α -helices and β -strands, respectively.

Figure 3.

Arginine kinase TSA complex ^{15}N spin relaxation dispersion curves. For selected residues with μs - ms timescale dynamics, R_2^{eff} is plotted as a function of ν_{CPMG} for $\nu_0(^{15}\text{N}) = 60.78$ MHz (red squares) and $\nu_0(^{15}\text{N}) = 81.04$ MHz (blue circles). Lines represent two-state collective fits of each cluster of residues with R_{ex} : D71 (NTD motion), H185 (loop L8), F211 (dynamic domain 2), I230 (dynamic domain 4), and D293 (dynamic domain 5).

Figure 4.

Conformational dynamics of transition state analog bound arginine kinase. The crystal structure of the closed (TSA) form of arginine kinase, with substrates shown in stick model, is colored by dynamic domain: domain 1 (blue), domain 2 (red), domain 3 (green), domain 4 (orange), and domain 5 (cyan) and shown as stereoscopic pairs. In grey are residues that are not assigned to a dynamic domain. Mapped onto the model are residues with R_{ex} measured by ^{15}N spin relaxation dispersion, shown as spheres. They are located primarily at the NTD-CTD interface (approximated as a dashed line) and throughout the CTD. Shown are views looking down into the active site (top), with loop L8 and dynamic domains 2 and 3 in front (middle), and with dynamic domains 4 and 5 in front (bottom).

Table 1
Mean and trimmed mean ^{15}N T_1 , T_2 and ^1H - ^{15}N NOE values for backbone amides of arginine kinase bound to the transition state analog

	500 MHz	600 MHz
T_1 (s)	1.172 ± 0.086	1.602 ± 0.122
Trimmed T_1 (s)	1.172 ± 0.044	1.610 ± 0.065
T_2 (s)	0.039 ± 0.005	0.032 ± 0.004
Trimmed T_2 (s)	0.039 ± 0.002	0.032 ± 0.002
^1H - ^{15}N NOE	0.768 ± 0.125	0.749 ± 0.106
Trimmed ^1H - ^{15}N NOE	0.799 ± 0.090	0.776 ± 0.064

Reported are the all-residue and trimmed mean values $\pm$ one standard deviation. Trimmed means were determined using criteria described in (Tjandra et al., 1995b).

Table 2
Summary of Lipari-Szabo model selection parameters for arginine kinase bound to the transition state analog

Model	Number of Residues	Percentage
1 (S^2)	48	17.8
2 (S^2 , τ_e)	64	23.7
3 (S^2 , R_{ex})	29	10.7
4 (S^2 , τ_e , R_{ex})	90	33.3
5 (S^2_s , S^2_f , τ_s)	39	14.4
Total	270	100.0

Table 3
Summary of collectively-fit two-state exchange parameters for arginine kinase bound to the transition state analog

Cluster	Number of Residues	k_{ex} (s^{-1})	p_{B} (%)
Loop L8	12	2750 ± 180	*
NTD motion (NTD hinge + interface)	10	2410 ± 260	*
Dynamic domain 2	10	2700 ± 180	*
Dynamic domain 4	31	2300 ± 170	1.3 ± 0.1
Dynamic domain 5	12	2470 ± 210	1.8 ± 0.1

Asterisks denote clusters for which p_{B} could not reliably be fit (see Materials and Methods).