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The Michaelis Complex of Arginine Kinase Samples the Transition State at a Frequency That Matches the Catalytic Rate

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Abstract

Arginine kinase (AK), which is a member of the phosphagen kinase family, serves as a model system for studying the structural and dynamic determinants of biomolecular enzyme catalysis of all major states involved of the enzymatic cycle. These states are the apo state (substrate free), the Michaelis complex analogue AK:Arg:Mg-AMPPNP (MCA), a product complex analogue AK:pAIE:Mg-ADP (PCA), and the transition state analogue AK:Arg:Mg-ADP:NO₃[−] (TSA). The conformational dynamics of these states have been studied by NMR relaxation dispersion measurements of the methyl groups of the Ile, Leu, and Val residues at two static magnetic fields. Although all states undergo significant amounts of μ s–ms time scale dynamics, only the MCA samples a dominant excited state that resembles the TSA, as evidenced by the strong correlation between the relaxation dispersion derived chemical shift differences ω and the equilibrium chemical shift differences δ of these states. The average lifetime of the MCA is 36 ms and the free energy difference to the TSA-like form is 8.5 kJ/mol. It is shown that the conformational energy landscape of the Michaelis complex analogue is shaped in a way that at room temperature it channels passage to the transition state, thereby determining the rate-limiting step of the

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b00236. Supplementary tables about sample conditions, dynamics parameters extracted from relaxation dispersion data and supplementary figures of chemical structure of product and its analogue pAIE, 2D NMR spectra, and correlation plots (PDF)

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phosphorylation reaction of arginine. Conversely, relaxation dispersion experiments of the TSA reveal that it samples the structures of the Michaelis complex analogue or the apo state as its dominant excited state. This reciprocal behavior shows that the free energy of the TSA, with all ligands bound, is lower by only about 8.9 kJ/mol than that of the Michaelis or apo complex conformations with the TSA ligands present.

TOC image

1. INTRODUCTION

Enzymes play a critical role in the chemistry of life by catalyzing specific reactions of molecules that otherwise would not proceed efficiently under physiological conditions. Cascades of molecular reactions involving a series of different enzymes lead to biochemical pathways whose up- and down-regulations are critical for health and disease. The reagents of these reactions are often metabolites, which can be comprehensively studied and quantified through the systems biological tools of metabolomics that rely on NMR spectroscopy and mass spectrometry.^{1,2} Each individual catalytic reaction involves a series of events starting from the unbound (apo) state of an enzyme, followed by substrate binding in the Michaelis complex, the formation of the transition state, and the release of the products (Figure 1).

In recent years, NMR spectroscopy, X-ray crystallography, computer simulations, and other biophysical techniques have provided a wealth of new insights for many of these processes at atomic detail. NMR spectroscopy in solution allows the detailed study of structural dynamics of proteins both on the ps–ns as well as the μ s–ms time-scale range, typically by measuring relaxation parameters of the backbone ^{15}N spins or the methylside chain ^{13}C spins. The slower of the two time scale windows can be accessed by relaxation dispersion experiments, such as CPMG and $R_{1\rho}$ experiments.^{3–6} These experiments provide information about interconversion rates (k_{ex}) between different protein conformational substates, such as a state that corresponds to the global free energy minimum and a metastable state, along with their populations and the absolute chemical shift differences ω of those nuclei that experience a change in their chemical environment in different substates. Quantitative comparison of ω with the chemical shift differences δ between different states allows one to infer information about the structure of metastable states. In the following we refer to the global free energy minimum as the “ground state” and the metastable high-energy state(s) as the “excited state(s)”. It should be emphasized that all these states refer to conformations and not to electronic states.

Recent studies of different enzyme systems make it clear that they exist as ensembles of conformational states, rather than single static structures, whereby the character of these ensembles is shaped by the presence of ligands. For many enzymes, substrate recognition follows a conformational selection or population shift mechanism, rather than induced fit.^{7–9} The time scales of interconversion of the various states, such as the apo state, ligand-bound states, and intermediates, is sometimes, but not always, related to the catalytic rates suggesting that the conformational dynamics of the enzymes can be functionally relevant.^{9,10}

The relationship between the transition state and the Michaelis complex and their ensembles plays a key role for enzyme function.^{11,12} Due to its very short lifetime (<1 ps), the structural and dynamic characterization of the transition state is a formidable experimental challenge. For only very few enzymes, it has been possible to stabilize the transition state using a nonreactive assembly of ligands that mimic the true transition state, leading to the formation of a thermodynamically stable transition state analogue (TSA) whose structure and equilibrium dynamics can be studied like any other thermodynamically stable protein state.¹³ For the same reason, it is desirable to stabilize the Michaelis and product complexes by using nonreactive analogues of the ligands that belong to these states. The NMR characterization of the apo state, the Michaelis complex analogue, the TSA, and the product complex analogue in relationship to each other has the goal to uncover changes of the energy landscape in response to the presence and absence of various substrate and product ligands, and their intermediates, and how these changes allow the enzyme to perform its function.

One such enzyme whose TSA structure has been determined by X-ray crystallography is the 42 kDa arginine kinase (AK) of the Atlantic horseshoe crab.^{14,15} AK belongs to the phosphagen kinase protein family, and it catalyzes the reversible transfer of a phosphoryl group between ATP and the substrate arginine, analogous to the ATP-creatine transfer in mammals catalyzed by creatine kinase. AK enables intermediate energy storage in cells through rapid phosphorylation/dephosphorylation of cellular arginine. The reaction has been generally characterized as a rapid equilibrium, random order substrate binding mechanism.^{16,17} The 3D structure of AK consists of two flexibly linked domains: an α -helical N-terminal domain (NTD) and a larger, primarily β -sheet C-terminal domain (CTD). Previous studies identified several loops that are critical for the function of this enzyme, which include the substrate specificity loop (L3, residues 61–68), the substrate binding loop (L8, residues 182–209) and the nucleotide binding loop (L13, residues 309–320).^{14,18,19} An alternative domain assignment was proposed recently, in which five quasi-rigid domains were defined based on the analysis of distance matrices of the available AK crystal structures. The five domains undergo large rotations and small translational displacements from the substrate-free to the transition state form.^{20,21} This monomeric enzyme with its rich loop and domain motions serves as an excellent model for studying the connection between enzyme structure, dynamics, and catalysis.

The substrate-free (apo) form of arginine kinase has been found to undergo differential backbone dynamics in various protein regions. Specifically, backbone ¹⁵N laboratory frame relaxation data indicates that, with the exception of loop L13, AK has very limited backbone flexibility on the fast (nanosecond) time scale. Slow microsecond to millisecond dynamics

are confined to four enzyme regions, especially loop L8 and the NTD–CTD interface.^{21,22} Although the NMR-derived activation energies for closure of the loop L8 and the NTD–CTD interface are consistent with the catalytic turnover energy barriers, the absence of analogues of the reactants in all previous dynamics studies may limit the transferability of these findings to the reacting enzyme. X-ray crystal structures and NMR data suggest large conformational changes upon transitioning from the apo to the TSA state in the presence of substrate.²⁰ In order to gain a more comprehensive understanding of AK and its function, we extend the analysis of slow dynamics to the transition state analogue (TSA), a Michaelis complex analogue (MCA), and a product complex analogue (PCA) using the methyl groups of isoleucines, leucines, and valines (ILV) as NMR probes. We found that the MCA state already probes the structure of the TSA in the form of its dominant excited state and vice versa, shedding new light on how the protein energy landscape is shaped by the presence of ligands to enable catalysis.

2. MATERIALS AND METHODS

2.1. Sample Preparation

The ILV-methyl labeled arginine kinase of *Limulus polyphemus* were expressed following standard methods.²³ *Escherichia coli* BL21 (DE3) harboring the recombinant plasmid pET22b-Arginine kinase was cultured in D₂O-based M9 minimal medium. Each 1 L culture of *E. coli* was grown at 37 °C containing 1.0 g/L ¹⁵NH₄Cl and 2.5 g/L glucose (D-²H, ¹³C glucose for [U-²H, ¹⁵N, ¹³C]; Ileδ1-[¹³CH]; Leu, Val-[¹³CH, ¹²CD₃] AK and D-²H-¹²C glucose for [U-²H, ¹⁵N, ¹²C]; Ileδ1-[¹³CH₃]; Leu, Val-[¹³CH, ¹²CD₃] AK). When OD₆₀₀ of the media reached 0.55, 70 mg of α-ketobutyrate and 120 mg of α-ketoisovalerate were added to the culture. For [U-²H, ¹⁵N, ¹³C]-Ileδ1-[¹³CH₃]-Leu, Val-[¹³CH₃, ¹²CD₃]-labeled AK, 2-keto-3-*d*₂-1,2,3,4-¹³C-butyrate and 2-keto-3-methyl-*d*₃-3-*d*₁-1,2,3,4-¹³C-butyrate were used. For [U-²H, ¹⁵N, ¹²C]-Ileδ1-[¹³CH₃]-Leu, Val-[¹³CH₃, ¹²CD₃]-labeled AK, 2-keto-3-*d*₂-4-¹³C-butyrate and 2-keto-3-methyl-*d*₃-3-*d*₁-4-¹³C-butyrate were used. Upon reaching OD₆₀₀ of 0.7, the cultures were induced with 0.5 mM IPTG at 37 °C for 8 h.

The protein was purified as described previously.¹⁸ The harvested cells were lysed and purified by anion exchange chromatography with a DEAE column and gel filtration chromatography with an S100 column. Backbone ¹⁵N-labeled samples were prepared for ILV methyl assignments using backbone ¹⁵N-¹H detection. For these samples, exchange of amide deuterons with protons in the core of the protein was performed by urea-based unfolding and refolding.²⁴ The final NMR sample was concentrated between 0.75 to 1.0 mM in the various ligand buffers (see Table S1). Specifically, the buffer of the apo-form contains 10 mM potassium citrate, 50 mM potassium chloride, 0.5 mM dithiothreitol, 50 μM sodium azide, 90% H₂O, and 10% D₂O. For the NMR samples of the different analogues the following ligands were added (see also Supporting Information, Table S1): MCA (100 mM arginine, 20 mM Mg·AMPPNP), TSA (100 mM arginine, 20 mM Mg·ADP, 20 mM NO₃⁻), and PCA (100 mM pAIE, 20 mM Mg·ADP), whereby pAIE is a recently introduced phosphoarginine analogue (see Figure S1).²⁵ The ligand concentrations were chosen to substantially exceed the *K*_d values of the various bound ligands to ensure that the observed slow dynamics are intramolecular and not merely reflective of binding exchange.

2.2. ILV-Methyl Side-Chain Assignments

NMR experiments were performed at 800 and 850 MHz on Avance III HD Bruker instruments equipped with QCI and TCI cryoprobes, respectively. All experiments were recorded at 25 °C. ILV-methyl side-chain assignments were obtained from one set of methyl-HN correlation experiments (3D Ile, Leu-HM(CMCGBCA)NH, 3D Val-HM(CMCBCA)NH) together with one set of out-and-back methyl-detected experiments (3D HMCM[CG]CBCA, Ile, Leu-HMCM(CGBCA)CO, and Val-HMCM(CBCA)CO).²⁶ For apo and TSA, the assignments of Ile as well as nonstereospecific Leu, Val methyl groups were obtained by the above 3D experiments based on previous backbone assignments.^{24,27} Next, the stereospecific assignments of the Leu and Val methyl groups were obtained from a CHD₂-detected CT-[¹H-¹³C]HSQC spectrum, recorded on a sample prepared using 10% U-[¹H,¹³C]-D-glucose and 90% unlabeled glucose as carbon sources.²⁸ The methyl side-chain assignments of the MCA and PCA states were obtained by monitoring cross-peak trajectories of ¹H-¹³C HMQC spectra during titration of apo samples with MCA and PCA stock solution. All NMR spectra were processed using NMRPipe in combination with NMRDraw.²⁹

2.3. CPMG Relaxation Dispersion Experiments and Their Analysis

Single-quantum methyl ¹³C-relaxation dispersion (RD)³⁰ experiments were performed for all ILV-side-chain methyl groups of the apo, MCA, TSA, and PCA samples. Experiments were recorded at two spectrometer frequencies of 600 and 800 MHz (for apo and TSA) or 600 and 850 MHz (for MCA and PCA). For each measurement, the constant-time relaxation period was set to 40 ms and pseudo-3D data sets were collected using 21 CPMG field strengths ranging from 50 to 2000 Hz. The NMR data were processed using NMRpipe and visualized with SPARKY.³¹ Peak intensities were extracted with nlinLS and then analyzed by numerical simulation of the pulse sequences using ChemEx software³² (which is publicly available at <http://github.com/gbouvignies/chemex>). For the CPMG RD data obtained at two static magnetic fields, those residues that exhibit an R_{ex} difference in their effective relaxation rates at low and high CPMG field strength larger than 4 s⁻¹ were fitted simultaneously with a two-state exchange model. The fitting of the dispersion profiles based on the Bloch-McConnell equations yields the conversion rates k_{AB} , k_{BA} between a major state (A) and an excited state (B) as well as the populations of each state (p_{A} and p_{B}). Bootstrap analyses³³ were performed for group fitting of residues to refine their thermodynamic and kinetic parameters.

3. RESULTS

3.1. ILV-Methyl Side-Chain Chemical Shift Assignments

Selective ¹³C-labeling of methyl groups in amino acids, such as ILV residues, along with a highly deuterated protein background has enabled important studies about the structure, dynamics, as well as the interactions of high-molecular-weight proteins by NMR.³⁴ The relative portion of ILV residues in AK is 19.8% (Ile, 5%; Leu, 9.8%; Val, 5%) and they are quite uniformly distributed throughout the entire protein, providing 124 site-specific methyl probes on 71 residues with which to investigate the dynamics of AK. Stereospecific assignments of Leu and Val methyl groups and Ile $\delta 1$ assignments for all four states (apo,

MCA, TSA, and PCA) are compiled in Table S2. We observed distinct perturbations of the ILV methyl chemical shifts as the different substrates were added to apoenzyme, titrating toward the other three states (Figure S2). As a threshold on the significance of each CSP, we use “10% trimmed mean plus one standard deviation” of the three combined CSP data sets (MCA, TSA, and PCA), which is 0.29 ppm. Comparison of MCA vs apo reveals that most of the peaks either do not move or shift only slightly (Figure 2A) with only three residues (V65, L195, and L331) exhibiting sizable CSPs (>0.29 ppm). These three residues are located at the interface between NTD and CTD (Figure 2B). V65 is part of substrate specificity loop L3 and L195 belongs to active site loop L8. The maximal CSP between MCA and apo is 0.56 ppm, which is much smaller than the largest CSPs of TSA vs apo or PCA vs apo AK, which are 2.07 and 0.97 ppm, respectively. The large CSPs between apo and TSA (Figure 2C) is consistent with backbone ^{15}N - ^1H CSPs²⁰ reflecting relatively large structural changes between these two states. Large methyl CSPs are widely distributed throughout the whole protein (Figure 2D), which includes the CTD, reaching 2.0 ppm. The PCA titration profile also reflects a fast-exchange scenario for most of the peaks, but several peaks (I210, L219, I231, L187, and L331) broadened rapidly and disappeared upon the increase of ligand concentration, which is indicative of intermediate exchange upon ligand binding. No CSPs were found in the NTD of PCA, in contrast to MCA and TSA (Figure 2E, F). Since the chemical structure of the product analogue, pAIE, is shorter by a methylene carbon and it lacks a terminal carboxyl group compared to the actual phosphoarginine product (vide infra), it is possible that these differences reduce the CSP-observable difference between PCA and apo-AK. Overall, the results suggest that the MCA structure is remarkably similar to the apo state and the TSA is the most unique of all four states.

3.2. Distinct μs – ms Dynamics for Apo, TSA, MCA, and PCA States

Side-chain CPMG relaxation dispersion measurements of the ILV methyl groups performed for all four states of Figure 1 show significant differences in their μs – ms dynamics both in terms of the involved residues as well as in terms of the exchange rates (Figure 3). For a two-state exchange system, CPMG fitting, using ChemEx, also gives the absolute values of chemical shift differences (ω_{C}) between the ground state and excited state of each probe, which can be compared to δ_{C} of the different states to obtain structural clues about the nature of the excited state.

3.2.1. Apo State Dynamics—The apo state displays significant dispersion profiles for only 6 residues out of a total of 71 ILV residues, and it exhibits a wide variety of chemical exchange time scales (slow, intermediate, and fast (Table S3)). Large R_{ex} values ($>4 \text{ s}^{-1}$) were observed for L170C $_{\delta 1}$, L187C $_{\delta 1}$, I210C $_{\delta 1}$, L219C $_{\delta 1}$, I231C $_{\delta 1}$, and L331C $_{\delta 2}$ (Figure 3A). In terms of location, the ILV methyl relaxation dispersion results align well with the backbone ^{15}N CPMG profiles reported previously, in which four groups of residues (the NTD–CTD interface, dynamic domain 1 and hinge, loop L8, and a small NTD hinge) were defined based on backbone ^{15}N CPMG RD fitting by two-state exchange processes.²² L219C $_{\delta 1}$ and I231C $_{\delta 1}$ are located in the “dynamic domain 1 and hinge” regions; whereas L170C $_{\delta 1}$, L187C $_{\delta 1}$, and I210C $_{\delta 1}$ belong to, or are close to Loop 8; and L331C $_{\delta 2}$ lies at the “NTD–CTD interface”. The exchange time scales of these apo ILV methyl groups are not completely consistent with their apo ^{15}N backbone counterparts. A collective fit of CPMG

RD data of L170C_{δ1} and L187C_{δ1} yields similar parameters ($k_{\text{ex}} = 970 \pm 38 \text{ s}^{-1}$, $p_{\text{B}} = 4.8 \pm 0.3\%$) to those of the backbone. Collective fitting of L219C_{δ1} and I231C_{δ1} provides a k_{ex} of $3890 \pm 29 \text{ s}^{-1}$, p_{B} of $0.56 \pm 0.05\%$, which is faster than observed for the backbone of “dynamic domain 1 and hinge” ($k_{\text{ex}} = 790 \pm 70 \text{ s}^{-1}$).²² Inclusion of I210C_{δ1} in the collective fits of this region results in significantly increased chi-square values, which are also observed for fits of this residue alone, suggesting the existence of exchange involving three or more different states (Table S3). This may be explained by the fact that I210C_{δ1} is in close proximity to the ligand binding site and, hence, probes multiple concurrent dynamic processes.

3.2.2. MCA Dynamics—MCA has the largest number of ILV probes (34) undergoing conformational exchange on the μs –ms time scale. Exchange is confined to the CTD and the flexible linker (Figure 2A,B). These residues are fit well by a two-state global motion model with exchange parameters $k_{\text{ex}} = 914 \pm 15$ and an excited state population $p_{\text{B}} = 3.10 \pm 0.04\%$ and a ground state population of $p_{\text{A}} = 1 - p_{\text{B}} = 96.9\%$. To further characterize the nature of the excited state, ω_{C} was plotted against the chemical shift differences between the MCA and the three other states revealing a high correlation to $\delta_{\text{C,MCA-TSA}}$ with $R = 0.81$ (Figure 4A). This suggests that the excited state of MCA resembles the TSA. Since for two-site exchange $k_{\text{ex}} = k_{\text{AB}} + k_{\text{BA}}$, $k_{\text{AB}}/k_{\text{BA}} = p_{\text{B}}/p_{\text{A}}$ and residence time $\tau_{\text{A}} = 1/k_{\text{AB}}$, it follows that the average residence time of the MCA is 36 ms. Of note, the NMR data provides no evidence that the MCA exchanges with the apo state (Figure S4).

3.2.3. TSA and PCA Dynamics—TSA and PCA have far fewer ILV probes (25 and 15, respectively) with significant relaxation dispersion ($R_{\text{ex}} > 4 \text{ s}^{-1}$) than MCA. They are located primarily in the CTD and they are spread around the substrate binding pocket. The relaxation dispersion data reveals that the TSA and PCA forms have very similar kinetic exchange parameters (4300–4700 s^{-1}), which are more than four times higher than that of the MCA (see also Table 1). Analysis of the ω_{C} values of the TSA reveals that they correlate well ($R = 0.88$) with both $\delta_{\text{C,TSA-MCA}}$ and $\delta_{\text{C,TSA-apo}}$ (Figures 4B, S4). This indicates that the TSA samples an excited state whose conformation resembles that of the MCA and the apo state. Since k_{ex} is relatively large, a stable fit of population p_{A} is not possible. However, with the observed strong correlation, it seems safe to assume that the slope between ω_{C} and $\delta_{\text{C,TSA-MCA}}$ should be one, which yields $p_{\text{B}} = 2.75 \pm 0.05\%$.

The PCA has a similarly high k_{ex} value as the TSA. The data can be well fitted by a two-state process. However, there is no clear correlation of the extracted ω_{C} with the chemical shift differences to any of the other 3 states ($\delta_{\text{C,PCA-TSA}}$, $\delta_{\text{C,PCA-MCA}}$, or $\delta_{\text{C,PCA-apo}}$, Figure S4). Therefore, details about the structure and the population of the excited state of the PCA remain unknown.

4. DISCUSSION

The essential states along the enzymatic cycle of arginine kinase are the substrate-free apo state, the Michaelis complex AK:Arg:Mg·ATP, the transition state AK:Arg:Mg·ADP·PO₃[−], and the product state AK:pArg:Mg·ADP. The NMR dynamics data of the 4 states reported here used nonreactive analogues for all three complexes (Figure 1). Previous X-ray

crystallography and NMR studies demonstrated that AK exhibits large rotations and small translations between the quasi-rigid groups when transitioning from the apo state to the TSA.^{20,21} Three-dimensional X-ray crystal structures of MCA and the PCA have so far remained elusive. We prepared solution samples of these complexes and investigated their conformational dynamics properties by monitoring of their NMR chemical shift changes and relaxation dispersion properties. We used selectively ¹³C-labeled ILV methyl groups as probes, yielding high quality spectra that are well resolved and have good sensitivity for this 42 kDa protein.

Chemical shift perturbation results indicate that the MCA structure most resembles that of the apo structure in solution (see Figures 2 and S2). However, it should be kept in mind that NMR residual dipolar couplings indicate that the apo solution structure is better approximated by a 2:1 weighted average or dynamic equilibrium of apo and TSA crystal structures than by the apo crystal structure alone.²⁰ The number of MCA residues with significant CSPs with respect to apo (>0.29 ppm) is very limited and the maximum CSP is negligible compared with the perturbations found for TSA relative to apo. Significant MCA CSPs are found in Loop 3 (V65) and substrate binding Loop 8 (L195) (Figure 2A), whereas the secondary structures do not show obvious perturbations. Residues 63–68 in Loop 3 have backbone and side-chain hydrogen bonds to the substrate. Conserved residue D62 in this loop does not participate in substrate binding, but forms a salt-bridge to R193 in Loop 8 stabilizing the topology of the catalytic pocket. Substitution of glycine for D62 dramatically reduces enzyme activity.¹⁹ Loop 8, with its residues R193 and L195, switches conformation between open and closed structures to facilitate nucleotide binding.

Modest apo-to-MCA CSPs are consistent with observed small structural differences in the available cocrystallized MCA complexes. The structure of the *Sticopus japonicus* dimeric AK, a homologue with 44% sequence identity to the monomeric *L. polyphemus* AK studied here, has an MCA structure that is intermediate between apo and TSA, whereby the RMSD for MCA vs. apo is 1.3 Å, compared to a difference between apo and TSA of 3.2 Å for *L. polyphemus*.^{20,35} Moreover, X-ray crystallographic studies of dimeric creatine kinase (CK), which is another homologue of AK, also shows a very small RMSD of 0.9 Å between the apo and MCA structures.³⁵ Here, we focused on the characterization of the ternary MCA, TSA, and PCA complexes, which are most relevant for understanding AK's function. Previous studies of binary complexes of phosphagen kinases with nucleotides mostly adopt “closed” conformations.³⁶ Given the spectrum of configurations from “open” to “closed” observed for these kinases, it is conceivable that a role of nucleotide binding is to fine-tune the balance between these states, for the binding of the other substrates, in preparation of the enzyme to perform its function.

From the point of view of the reverse dephosphorylation reaction, the product complex analogue PCA is also a Michaelis complex. The reverse direction has a very similar turnover number (140–180 s⁻¹)^{19,37} as the forward direction (120 s⁻¹). One might expect that the PCA displays a similar dynamic behavior as the MCA. However, we found that the PCA behaves rather differently than MCA, displaying exchange that is 5-fold faster than that of MCA (Table 1, Figure S3). Moreover, we did not observe any correlation between the excited state of the PCA and the TSA (Figure S4). Interestingly, during PCA titration some

peaks disappeared or were severely broadened, which largely coincide with the peaks that exhibit relaxation dispersion in the apo state (Figure 2E, Table S3) and which might reflect conformational exchange between the apo and PCA states. The CPMG relaxation dispersion of PCA did not show any correlation with other states (Figure S4), which is possibly due to differences in the chemical structures between the phosphoarginine and its analogue pAIE used (Figure S1).

The CPMG data of the MCA is well approximated by a global 2-state exchange process, involving 34 methyl groups, between the ground state and an excited state that is 8.5 kJ/mol higher in free energy. The excited state has methyl chemical shifts that closely resemble the ones of the TSA as is manifested by a high correlation ($R = 0.81$, Figure 4). The average lifetime of the MCA is 36 ms, whereas the lifetime of the excited state is 1.1 ms. This corresponds to a kinetic rate constant between the MCA and the TSA of $k_{AB} = 28 \text{ s}^{-1}$. This is commensurate with a k_{cat} of 120 s^{-1} at the pH optimum,²¹ and pH-profiles showing 7-fold reduction at pH 6.5.³⁸ This level of consistency between k_{AB} (from the MCA CPMG relaxation dispersion data) and k_{cat} suggests that the transition from the MCA to TSA is likely to be rate limiting for this enzyme.

Notably, the high correlation between ω_C from TSA and $\delta_{C,TSA-MCA}$ indicates that the TSA has an MCA-like conformation as its excited state (Figure 4B). Hence, on the ms time scale, both the MCA and TSA sample excited states whose conformations resemble the ground state structures of each other (Figure 5). Since for both states the only detectable excited state resembles each other, the enzyme unveils an innate ability to efficiently channel the MCA to a TSA-like conformation and vice versa. The transitions between these states involve relatively large conformational changes, requiring the crossing of a free energy barrier depicted in Figure 5, which determine the kinetic rate constants k_{AB} observed in the CPMG experiments (Table 1). Such channeling from MCA to TSA is likely to be of direct functional importance for the active enzyme. On the other hand, the observed exchange between the TSA and the MCA-like state seems to be much too slow to be relevant for enzyme turnover, since the lifetime of its transition state is many orders of magnitude shorter than the 36 ms measured here for the TSA by NMR. For dihydrofolate reductase (DHFR), which catalyzes the transfer of a proton, it was found that intermediates along the enzymatic cycle sample as an excited state a previous or a successive intermediate. In contrast to the present study, none of the intermediates corresponded to a transition state.³⁹ For ribonuclease A, reciprocal conformational sampling behavior was observed between the apo state and the substrate-bound state.⁴⁰ A study of FKBP12, a peptidyl-prolyl cis-trans isomerase, indicated that the transition state ensemble involves multiple conformations, but no relationship was found between the excited state and other structures.¹³ By contrast, for AK studied here the excited states of the stabilized Michaelis complex and the transition state sample each other at thermal equilibrium, which expedites the turnover of this enzyme.

5. CONCLUSION

Arginine kinase is one of only very few enzymes whose Michaelis complex and transition state analogues can be stabilized and studied at atomic resolution. NMR experiments of ILV-methyl labeled protein provided direct access to thermodynamic, kinetic and structural

information on these states and their excited substates. The finding that the Michaelis complex selectively samples the transition state analogue as its only detectable excited state provides strong support for the critical role of specific protein dynamics in enzyme catalysis. The fact that the kinetic rate constant of the Michaelis complex analogue is comparable to the turnover rate of the enzyme further corroborates this conclusion. We also find that the TSA samples the MCA as its dominant excited state on the millisecond time scale suggesting a reciprocal relationship between these two critical states for enzyme catalysis: the MCA and the TSA populate corresponding sites in the protein landscape whose relative free energies are modulated as the phosphorylation reaction gets under way, alternating which has the lower free energy minimum. The present study suggests that the dynamic interconversion between consecutive states along an enzymatic reaction cycle, originally observed for various intermediates of DHFR, also applies to the Michaelis complex and the transition state of arginine kinase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Catalytic cycle of arginine kinase enzyme (AK), which catalyzes (de)phosphorylation of (phospho)arginine: $\text{Arg} + \text{Mg} \cdot \text{ATP} \xrightleftharpoons{\text{AK}} \text{pArg} + \text{Mg} \cdot \text{ADP}$. Dynamic equilibria between the substrate-free form (apo) three bound states (MCA, TSA, PCA) are indicated by double arrows. The Michaelis complex, transition state, and product complex are represented by analogues AK:Arg:Mg·AMPPNP, AK:Arg:Mg·ADP:NO₃⁻, and AK:pAIE:Mg·ADP, respectively, where NO₃⁻ mimics the phosphate group that is transferred and pAIE acts as phosphoarginine analogue.²⁵ The turnover rate k_{cat} in the forward direction corresponds to the synthesis of phosphoarginine at pH 8.0, 25 °C.²¹

Figure 2.

NMR chemical shift perturbations (CSP) observed for three different states of arginine kinase (AK) along the catalytic cycle relative to apo AK: (A) MCA, (C) TSA, and (E) PCA. Horizontal black lines indicate the sum of the 10% trimmed mean CSP plus one standard deviation of the three combined data sets, green dashed lines indicate residues with HMQC peaks that disappeared during titration from the apo to the PCA state. Panels (B), (D), and (F) show the 3D structure of AK (PDB: 1M15¹⁵) with those methyl groups displaying above average ¹³C CSPs indicated as colored spheres. The NTD, flexible linker and CTD are shown in black, dark gray and light gray, respectively. Orange sticks depict the reactants of the TSA as found in the X-ray crystal structure.¹⁵ CSPs were determined using the

expression:⁴¹ $\sqrt{(\Delta\delta_{\text{H}}/\alpha)^2 + (\Delta\delta_{\text{C}}/\beta)^2}$, where δ_{X} is the chemical shift change of nucleus X (H or C) between the free form and the bound state, and $\alpha = 0.26$ ($V_{\gamma 1}$), 0.28 ($L_{\delta 1/2}$, $V_{\gamma 2}$), 0.29 ($I_{\delta 1}$) ppm and $\beta = 1.66$ ($I_{\delta 1}$), 1.59 ($L_{\delta 1}$), 1.70 ($L_{\delta 2}$), 1.37 ($V_{\gamma 1}$), 1.54 (V_2) ppm are the standard deviations of the methyl ¹H and ¹³C chemical shifts from the Biological Magnetic Resonance Data Bank.

Figure 3.

(A) Methyl-¹³C CPMG relaxation dispersion data of the apo, MCA, TSA, and PCA of AK. All ILV methyl side chains are represented as spheres on the apo and TSA crystal structures. ILV-methyl groups that undergo two-site exchange are colored in red and ligands are indicated in magenta color. The 3D structures displayed are 3M10²⁰ (apo) and 1M15¹⁵ (MCA, TSA, PCA). (B) Representative CPMG relaxation dispersion profiles are shown of the substrate specific loop L3 (V65), the flexible linker (L110), the substrate binding loop

L8 (I210), and the nucleotide binding loop L13 (V308). The red, blue, purple, and green curves belong to the apo, MCA, TSA, and PCA states, respectively.

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Figure 4.

Correlation plots of the CPMG-derived ^{13}C chemical shift differences ($\Delta\omega_{\text{C}}$) vs the equilibrium ^{13}C chemical shift differences ($\Delta\delta_{\text{C}}$) between the MCA and TSA states where R is the Pearson correlation coefficient. (A) Plot of $\Delta\omega_{\text{C}}$ for MCA vs $\Delta\delta_{\text{C}}$ between the MCA and TSA states. (B) Plot of $\Delta\omega_{\text{C}}$ for TSA vs $\Delta\delta_{\text{C}}$ between the MCA and TSA states. The blue, green, and red circles belong to residues that are part of the NTD, the NTD-CTD linker, and the CTD, respectively. In Panel A, the outlier ($\Delta\delta \approx 2.5$ ppm, $\Delta\omega \approx 1.5$ ppm) belongs to L331 in the large domain (see Figure 2B).

Figure 5.

Schematic depiction of energy landscape of the Michaelis complex analogue (MCA, blue profile) and the transition state analogue (TSA, red profile). The small symbols reflect the ligands used to stabilize these states (legend on the right). The MCA samples as its excited state a TSA-like conformation, whereas the TSA samples as its excited a MCA or apo-like conformation. The barriers reflect the relatively large conformational changes between these states.

Table 1

Globally Fitted Two-Site Exchange Parameters from CPMG Relaxation Dispersion Data of All Four Different States of AK

	no. of methyl probes for fitting	k_{ex} (s^{-1})	p_{B} (%)	k_{AB} (s^{-1})	k_{BA} (s^{-1})
APO	6	$(860 \pm 120)^a$	$(4.9 \pm 0.5)^a$	$(42 \pm 7)^a$	$(818 \pm 141)^a$
MCA	34	914 ± 15	3.10 ± 0.04	28 ± 0.6	886 ± 15
TSA	25	4247 ± 72	2.75 ± 0.05	117 ± 3	4130 ± 72
PCA	15	4690 ± 119	NA	NA	NA

^aThe methyl data of the apo state is insufficient for a global analysis. Values in parentheses were determined from backbone ^{15}N CPMG results.²¹