Independent saturation of three TrpRS subsites generates a partially assembled state similar to those observed in molecular simulations

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Two new crystal structures of *Bacillus stearothermophilus* tryptophanyl-tRNA synthetase (TrpRS) afford evidence that a closed interdomain hinge angle requires a covalent bond between AMP and an occupant of either pyrophosphate or tryptophan subsite. They also are within experimental error of a cluster of structures observed in a nonequilibrium molecular dynamics simulation showing partial active-site assembly. Further, the highest energy structure in a minimum action pathway computed by using elastic network models for Open and Pretransition state (PreTS) conformations for the fully liganded TrpRS monomer is intermediate between that simulated structure and a partially disassembled structure from a nonequilibrium molecular dynamics trajectory for the unliganded PreTS. These mutual consistencies provide unexpected validation of inferences drawn from molecular simulations.

conformational transition state | domain movement | induced fit | minimum action pathway | nonequilibrium molecular dynamics

Crystal structures of enzymes provide a limited subset of their possible biochemical states and cannot provide details of catalytic conformational changes. Coordinated changes that align catalytic residues properly when substrates or analogs bind are referred to as “induced-fit.” By definition, such changes involve transitions to conformations that are not favored without ligands. It follows that they “store” conformational free energy. Dynamic interactions along pathways between stable crystal structures are therefore of paramount interest if we are to understand molecular details of conformational free-energy transduction.

Linking crystal structures together to form a mechanistic pathway requires procedures that transcend experimental structure determination (1, 2). Molecular simulation techniques to characterize such pathways range from hybrid quantum mechanical/molecular mechanics (QM/MM) (3) and molecular dynamics (MD) (4) to discrete molecular dynamics (DMD) (5, 6) and minimum-action pathway (MAP) (7) computations. Validating these methods relies mostly on comparison with modeling done for known structural states. Only rarely does a new structure compare favorably with predictions based on previous simulations. Such an example is provided by crystal structures of *Bacillus stearothermophilus* tryptophanyl-tRNA synthetase (TrpRS) complexed with fragments bound to its adenosine, tryptophan, and pyrophosphate subsites, solved to test a hypothesis about the structural requirements for induced fit.

Acylation of tRNA^{Trp} proceeds by 2 half-reactions, tryptophan activation by ATP and acyl-transfer to cognate tRNA. Amino acid activation proceeds quite slowly in water (8), whereas mixed anhydrides react rapidly with water and alcohols (9). Thus, the most important catalytic rate acceleration possessed by aminoacyl-tRNA synthetases, by many orders of magnitude, is acyl-group activation by ATP. The structural reaction profile for tryptophan activation by TrpRS has been elucidated by structural, biochemical, and bio-

physical studies. Four structural subclasses have been identified with catalysis of Trp-5'-AMP formation (10–15). Relationships between structures and their catalytic relevance have been extensively documented (13, 15), in particular, by how they rationalize the kinetic behavior of active-site mutations in the closely related tyrosyl-tRNA synthetase (16, 17).

Two TrpRS structures, an Open conformation (12) and a closed, pretransition state (PreTS) conformation (13), represent initial and final states of a substantial conformational change that activates Mg²⁺·ATP before its reaction with tryptophan (13). Conformational free energy is stored during this induced fit (15) and recovered in a subsequent catalytic step (11). Hinge bending and twisting domain movements associated with these conformational changes are correlated with ligands bound to the Open, closed PreTS (13), and Products (11) states (Fig. 1).

The closed-hinge angle appears to depend solely on binding either ATP or Trp-5'-AMP, to both subunits of the dimer, whereas the twist requires Mg²⁺·ATP (18). Both ligands possess a high-energy bond between AMP and either PP_i in the closed PreTS or tryptophan in the closed, Products state, respectively. They induce hinge closure irrespective of whether the third subsite is occupied (Fig. 1) (19). Thus, the covalent linkage seems critical for inducing and/or stabilizing domain closure.

We were interested in whether saturating the active subsites with tryptophan, adenosine, and PP_i fragments would induce a closed conformation. We therefore prepared and solved X-ray structures in which the enzyme was provided with saturating concentrations of these 3 ligands. Posing this experimental question is a structural counterpart to Wolfenden's studies of fragment binding thermodynamics (20, 21).

The new structures extend understanding of TrpRS catalysis three distinct ways. First, they support the hypothesis that a covalently linked adenosine moiety is necessary to stabilize closed TrpRS conformations. Second, they document the high affinity of the KMSKS loop for the β -phosphate, reinforcing earlier suggestions (13, 15) that this affinity helps develop additional binding energy in a dissociative transition state. Finally, they provide unexpected experimental support and rationalization for nonequi-

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3FHJ and 3FI0).

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Fig. 3. Conformational space defined by internal conformational angles of TrpRS crystal structures and simulations. Open, PreTS, and Products complex crystal structures are indicated by blue, green, and red diamonds (7). Because the 3 Open state crystal structures are isomorphous, there are <18 distinct blue squares. Partially closed native and SeMet WMP structures are shown as black circles and amber squares. MD structures (large, filled red squares) and the origins of their trajectories (fine gray dashed arrows; OM25 and P48) are described in the text. Open squares denote structures along a minimum action pathway connecting the Open and PreTS structures. The red filled star represents the highest-energy structure along the simulated path. Dashed (unliganded) and solid (bound to Mg^{2+} -ATP and tryptophan) semiempirical harmonic conformational energy wells were inferred from experimental conformational free-energy changes with and without ATP (15) and from simulations. Circles denote transition states along the forward and reverse induced-fit transition. Vertical dashed lines relate free-energy minima (gray; observed crystal structures) and maxima (simulations; red, blue arrows) to structures below.

2'-OH (Fig. 2), is 6.22 ± 0.51 Å from the D146 carboxylate, compared with 7.18 ± 0.74 Å in the Open ATP complex, 1MAW, consistent with partial closing of the hinge. In the PreTS state, this distance is 2.64 Å denoting a hydrogen bond.

Conformational Effects of Substrate Fragments and Selenomethionine

Substitution. We relate WMP structures to the structural reaction profile by projecting structural differences to internal coordinates defined by 2 interdomain angles (18) ([supporting information \(SI Text, Internal Angles Defining Domain Orientation; Fig. S1\)](#)). As was true of their closest structural relatives, the previously solved Open states [1D2R (12), 1MB2, and 1MAW (13)], variation in crystallographically independent molecules reduces the variance of the mean domain orientations of the WMP complexes. This statistical precision allowed us to identify 2 significant structural differences by regressing the internal coordinates against chemical parameters ([SI Text, Regression Analyses of Domain Positions Against Ligands and Selenomethionine Substitution; Tables S1 and S2](#)). WMP complexes are more closed than other Open structures, and selenomethionine complexes are untwisted, relative to native WMP complexes

Two principal components derived from TetraRa structural scoring (22) span 84% of the variance in all TrpRS crystal structures solved to date. These 2 principal components, PC1 and PC2, are very nearly equivalent ($R^2 = 0.99$) to the hinge and twist angles (in degrees) by transformations in Eq. 1:

Table 2. Mean TrpRS conformational angles

Structural cluster	$\langle \text{Hinge} \rangle$	$\sigma(\text{Hinge})$	$\langle \text{Twist} \rangle$	$\sigma(\text{Twist})$
Crystal structures (number)				
Open, unliganded (6)*	69.3	0.98	-0.51	0.43
Open, singly liganded (12) [†]	69.2	0.61	-0.07	0.33
WMP (6) 3FHJ	68.3	0.6	-0.2	1.0
SeWMP (18) 3FIO	68.0	0.6	-0.9	0.5
PreTS (5) 1MAU, 1M83	62.5	0.24	8.9	0.34
Products 1I6K, 1I6L, 1i6M	62.3	0.23	4.6	0.12
Simulated structures				
OM25; partially assembled, 2.5 ns	68.9	0.57 [‡]	-0.36	0.60 [‡]
OM45, partially assembled, 4.5 ns	70.25	0.57 [‡]	1.31	0.60 [‡]
MinActPath transition state	67.8	NA	1.74	NA
P48; partially disassembled	67.5	0.63 [‡]	2.61	1.07 [‡]

Numbers in parentheses denote the number of crystallographically independent structures.

*The 6 monomer structures from PDB ID code 1D2R.

[†]Six independent monomers each from PDB ID codes 1MAW and 1MB2.

[‡]Standard deviations obtained from variances of the mean as described in [SI Text, Statistical Error in Molecular Simulations](#).

$$(\text{hinge twist}) = \begin{bmatrix} -1.63 & -0.49 \\ 1.32 & 1.76 \end{bmatrix} \begin{bmatrix} \text{PC1} \\ \text{PC2} \end{bmatrix} + \begin{bmatrix} 65.73 \\ 3.44 \end{bmatrix} \quad [1]$$

$$[\text{PC1} \text{ PC2}] = \begin{bmatrix} -0.785 & -0.216 \\ 0.720 & 0.580 \end{bmatrix} \cdot \begin{bmatrix} \text{hinge} \\ \text{twist} \end{bmatrix} + \begin{bmatrix} 52.3 \\ -40.60 \end{bmatrix}$$

Thus, the hinge (α) and twist (γ) (14, 18) provide a more sensitive and less ambiguous description of domain movement along the structural reaction coordinate than rmsd values. The conformational cycle constitutes a scalene triangle in this plane. Open structures occupy the upper left, open-hinge, untwisted corner; PreTS structures occupy the lower right, closed, twisted corner; and Products structures constitute an intermediately twisted, closed state (Fig. 3).

WMP complexes differ in detail from Open state structures (Table 2). The W, M, and P ligand fragments favor a partially closed hinge. Approximately 45% of the variance in the hinge angle can be attributed to the WMP ligands, with $P(t) < 0.0001$ for the mean difference of 1.14° between all Open and all WMP states (*SI Text, Regression Analyses of Domain Positions Against Ligands and Selenomethionine Substitution*). This hinge angle closing is $\approx 15\%$ of the total difference between the Open and Closed states. Further, the WMP domain orientations are untwisted relative to the OPEN structures. Thus, the substrate fragments partially close the hinge, but, without a covalent bond to pull the subsites together, these ligands cannot convert any of their binding energies into the ≈ 3 kcal/mol of conformational free energy required to close and twist the domains, creating the PreTS (13) structure.

The average hinge angles (68.3° , 68.0°) of the native and SeMet substituted structures are not significantly different. However, the mean twist angle for SeMet-containing WMP complexes (-0.88) is $\approx 0.6^\circ$ smaller than those of the native structures (-0.23°). When account is taken of the covariation of hinge and twist among different structures, regression analysis shows that the twist angle is negatively correlated with the presence of selenomethionine (*SI Text, Regression Analyses of Domain Positions Against Ligands and Selenomethionine Substitution*; $P(t) = 0.02$). SeMet substitution is therefore a likely cause of the different WMP and SeWMP crystal habits.

Fig. 4. The OM partial assembly trajectory. (A) Time courses of the 2 internal coordinates. The star denotes the initial configuration before energy minimization, which carries the system immediately to a configuration that recurs later in the trajectory (OM25). (B) Three structural clusters based on distances derived from the first 2 principal components (which map to the hinge and twist angles), represented by snapshots at 2,500 (OM25, red), 3,500 (OM35, blue), and 4,500 (OM45, green) ps. Radii of dashed ellipses are σ values from Table 2.

The WMP Structures Help Rationalize Several Nonequilibrium Simulations Related to Induced Fit. Kapustina (18) described both liganded

tions Related to Induced Fit. Kapustina (18) described both liganded and unliganded MD trajectories computed for all known TrpRS states. Two types of trajectories migrated from the region of the original crystal structure toward another state. A partially assembled complex constructed by adding tryptophan and Mg^{2+} -ATP to the Open state structure 1MAW progressed toward the PreTS state. A partially disassembled complex constructed by removing Mg^{2+} -ATP from the PreTS crystal structure 1MAU regressed back almost to the Open conformation. We recently described a possible significance of the latter behavior, noting that ligand-free closed, twisted states derived from Mg^{2+} -ATP (1MAU) and adenosine-3'-tetrphosphate (AQP; 2OV4) complexes evolved in opposite directions along the structural reaction profile, implying the existence of a conformational transition state between the PreTS and PostTS crystal structures (14).

We previously considered the partially assembled (OM45) and partially disassembled (P48) structures to be similar, because they have similar hinge angles. The new WMP structures, however, have hinge angles intermediate between those of the 2 terminal structures. All 3 sets of structures have quite distinct twist angles. We therefore examined how these nonequilibrium simulations compare with crystal structures in light of quantitative error estimates for the simulations (*SI Text, Statistical Error in Molecular Simulations*) (23). Surprisingly, these are comparable to experimental error estimates (Table 2).

Partial assembly with Mg^{2+} -ATP and tryptophan is both modest and stochastic, oscillating several times between partially closed and partially twisted configurations (Fig. 4A). Using 2 principal components together and error estimates, we identified 3 clusters that occur in approximately equal amounts and approximately in succession (Fig. 4B). The first cluster forms rapidly during the equil-

ibration period, and recurs later as representative OM25. Conformational angles of the 6 native WMP structures are, on average, within one standard deviation of those observed for that structure.

The P48 trajectory regresses from the PreTS state toward the Open state when $\text{Mg}^{2+}\cdot\text{ATP}$ is removed, opening and untwisting rapidly and monotonically (within the first nanosecond of simulation, with little subsequent change in conformation during 4 additional nanoseconds of simulation (18). Although its final hinge angle is similar to those of the WMP complexes and the OM trajectory, its twist angle, 2.61° , differs by 6σ , or $\approx 35\%$ of the full range of the small domain twisting motion. Thus, although chemically identical, the twist angles of the partially assembled and disassembled unliganded TrpRS structures differ sufficiently that they represent distinct states that do not merge from different starting points within 5-ns MD simulations.

Possible resolution of this puzzle (Fig. 3) emerged from a Minimum Action Pathway (MAP) constructed from elastic network models for the fully liganded Open and PreTS conformations to represent the entire forward induced-fit conformational change. The MAP algorithm assumes that the initial and final conformations diffuse within local harmonic potentials (solid parabolas, Fig. 3 *Upper*), centered on the crystallographic coordinates (thin dashed vertical lines). It differs from MD simulations in that it solves analytical expressions for the structure and energy of the conformational transition state where the 2 potentials intersect (7) at the expense of using more approximate potentials.

The relative free energies of free and bound states in both conformations were determined by experiment (15), leaving only the unknown force constants for the Open and PreTS potentials to be adjusted for optimal alignment with structures Fig. 3 *Lower*. Moreover, because crystal structures of unliganded TrpRS and its Open complexes with tryptophan and (substoichiometric) ATP are indistinguishable, binding of these ligands separately induces only minimal changes in the potential energy well of Open unliganded TrpRS. However, together, they favor induced fit by stabilizing the PreTS conformation.

The highest energy structure from the MAP pathway, denoted by the star in Fig. 3, occurs early in the conformational change (blue dashed arrow). Moreover, it is intermediate between the 2 MD structures (Table 2). Consequently, it presents an early kinetic barrier to the fully liganded Open, and a late barrier to opening in the P48 MD simulations. The MD trajectories appear to stall on either side of the high-energy conformation between them, the partially assembled trajectory at a twist angle smaller, and the partially disassembled structure at a twist angle greater, than those of the (similar) transition states.

Dashed parabolas in Fig. 3 represent harmonic approximations for unliganded TrpRS. The remarkably rapid equilibration of the P48 trajectory argues that unliganded TrpRS is less stable in the PreTS state than in the Open conformation and that its minimum energy lies close to where that trajectory stalls during the last 4 ns. Inferred potentials for unliganded TrpRS therefore suggest a transition state for the reversal of induced fit close to that for the forward reaction with liganded TrpRS (circles in Fig. 3).

The MAP conformational transition state structure should not be confused with the conformational transition state described recently in ref. 14 between the PreTS Mg^{2+} -ATP complex and the (posttransition state) complex with adenosine 5'-tetrphosphate, AQP shown in Fig. 3 *Lower Right*.

Discussion

Domain motion during induced fit closes and twists the 2 TrpRS domains. The anticodon-binding domain moves as a rigid body with both HIGH and KMSKS catalytic signatures to deliver ATP to the tryptophan carboxyl group in the Rossmann fold (RF). Crystal structures of the WMP complexes with 3 subsite-specific fragments show that TrpRS retains an open conformation when bound simultaneously to AMP, tryptophan, and inorganic phosphate.

Table 3. Crystallographic data

Crystal type	Native $\lambda_0 = 1.0 \text{ \AA}$	Se derivative $\lambda_0 = 0.97934 \text{ \AA}$	Se derivative $\lambda_0 = 0.97945 \text{ \AA}$	Se derivative $\lambda_0 = 0.97177 \text{ \AA}$
Space group	C2	P1	P1	P1
Unit-cell parameters	$a = 223.60 \text{ \AA}$ $b = 91.98 \text{ \AA}$ $c = 158.32 \text{ \AA}$ $\beta = 134.01^\circ$	$a = 122.03 \text{ \AA}$ $b = 122.40 \text{ \AA}$ $c = 122.34 \text{ \AA}$ $\alpha = 79.91^\circ$ $\beta = 80.52^\circ$ $\gamma = 79.81^\circ$	$a = 122.41 \text{ \AA}$ $b = 122.74 \text{ \AA}$ $c = 122.71 \text{ \AA}$ $\alpha = 79.91^\circ$ $\beta = 80.59^\circ$ $\gamma = 79.87^\circ$	$a = 122.31 \text{ \AA}$ $b = 122.68 \text{ \AA}$ $c = 122.69 \text{ \AA}$ $\alpha = 79.91^\circ$ $\beta = 80.60^\circ$ $\gamma = 79.88^\circ$
Observations	549,810	811,830	709,775	663,814
Unique reflections	157,769	203,226	178,215	166,163
Resolution limit, $\text{\AA}$	25–2.5	25–2.7	25–2.7	25–2.7
% Completeness	97.6(77.5)	97.7(97.2)	97.8(97.2)	97.9(97.4)
$\langle I/\sigma(I) \rangle$	19.4(1.68)	11.0 (2.4)	10.7(2.3)	11.0(2.8)
Reflections with $I > 3\sigma(I)$, %	65.7	87.3	85.9	82.1
Overall R_{merge}	0.081	0.069	0.079	0.074
Redundancy	3.48	3.96	3.98	3.99
Mosaicity, degrees	0.25	0.54	0.54	0.54
R factor/ R_{free} NCS constrained	0.23/0.24			0.24/0.26
R factor/ R_{free} unrestrained	0.21/0.22			0.22/0.23

Values in parentheses represent the highest resolution shell.

Interconverting Binding and Conformational Free Energy. Lacking covalent links between AMP and either P_i or Trp, the bound WMP ligands do not represent any intermediate state in the tryptophan activation reaction. Bound nucleotide and amino acid subsites in both monomers in WMP dimers and phosphate bound to the KMSKS loop induce only minimal domain closure (Fig. 1D). It is therefore likely that a covalent bond between the nucleotide and the occupant of another subsite is necessary to induce the closed structures. The respective binding subsites are far enough apart that without a covalent bond to pull sites together, there is no need for TrpRS to overcome the unfavorable free energy (+3.0 kcal/mol) (15) associated with ligand-free induced fit to retain optimal binding interactions to the separate fragments.

We failed to capture the structure that would establish this point more conclusively, because crystal growth requires 2.0 M phosphate, which competes effectively for PP_i. Stabilization of pregrown crystals in (NH₄)₂SO₄, which might allow soaking of PP_i into preformed crystals, would convolute the question of conformational stability with the unknown influence of crystal packing forces. It therefore may be difficult to resolve this issue fully.

WMP Complex Is Functionally Relevant. The adenosine moiety binds preferentially to the HIGH sequence even when it cannot use the binding of the triphosphate moiety to the neighboring KMSKS loop (13). Two interactions—between D146 and the ribose and between K111 and the γ -phosphate across the domain boundary—appear to drive induced-fit active-site assembly. The K111 ϵ -amino group is 13.5–19 Å from the bound phosphate in the WMP structures and therefore unable to interact. The D146 carboxylate is reproducibly somewhat closer (6.22 Å vs. 7.18 Å) to the adenosine ribose 2'-OH in the WMP structures than it is in the Open ATP complex. Adenosine, by itself, does not induce a closed hinge to create that contact.

The distance between the α -phosphate position and the P_i moiety bound to the KMSKS loop is consistent with that expected for a dissociative transition state for phosphoryl transfer. The low variance of the P_i position, its coincidence with β -phosphate in crystals containing a PP_i leaving group (1MAU, 1M83, 2OV4), and the high variance of the α -phosphate position are likewise consistent with a mechanism that stabilizes the PP_i leaving group while allowing the α -phosphate to migrate toward the tryptophan carboxylate nucleophile.

Corroborating Molecular Simulations. The partially closed WMP structures are similar to structures observed previously only during

MD simulations of fully liganded, Open TrpRS. The relationship of that and the partially opened PreTS simulated structure to the induced-fit pathway was clarified by the early conformational transition state in the MAP pathway based on analytical solution of the structure and energy of the crossing point between elastic network models for the fully liganded Open and PreTS states. These observations afford semiquantitative agreement between the WMP structures, several types of molecular simulations, and experimental free-energy changes for the overall conformational changes and ATP binding (Fig. 3).

Experimental relative free energies of the 4 different structures (15), together with the evidence in all 3 simulations for a barrier with a relatively open hinge and small twist angles, afford a semiempirical calibration of the force constants for the different potentials. In particular, ATP binding appears from Fig. 3 to broaden the PreTS harmonic well, relative to that of the Open state. However, the large numbers of monomers in Open state crystal asymmetric units reflect a range of domain motion in Open TrpRS states, whereas closed-state dimers crystallize with monomers in their crystallographic asymmetric units and diffract to higher resolution (10, 11, 13, 15). These results suggest the opposite conclusion that Open states have relaxed, and closed states have tighter force constants. The puzzling inconsistency between the semiempirical energy surface described in Fig. 3 and that inferred from crystallogensis suggests that we can further validate or reject the 3 interrelated simulations by comparing the MAP force constants with those obtained by using potentials of mean force (18).

In related work (V. Weinreb, unpublished data), we have used multimutant thermodynamic cycles to confirm active-site lysine-metal interactions whose relative strengths were also first predicted by nonequilibrium MD trajectories (18). These confirmatory results therefore provide important justification for using relatively short, nonequilibrium MD simulations as a way to investigate dynamic aspects of ligand-dependent conformational changes in proteins.

Experimental

Crystals and Diffraction Data. Native and SeMet TrpRS from *B. stearotheophilus* were expressed in *E. coli* BL-21 and purified as described in ref. 13. *B. stearotheophilus* crystals were grown by microdialysis in the presence of tryptophan (0.5 mM), AMP (0.01 M), PP_i (0.01 M), and magnesium (0.01M). Response-surface analysis (24) revealed a crystal growth optimum in $\approx$ 2.28 M potassium phosphate at pH 6.58 with 0.6% vol/vol PEG400 as an additive at 315 K.

Unit-cell parameters for a cryoprotected crystal were $a = 122.45$ Å, $b = 122.86$ Å, $c = 122.81$ Å, $\alpha = 79.91^\circ$, $\beta = 80.62^\circ$ and $\gamma = 79.87^\circ$. Strong suggestions of higher hexagonal (R3) or possibly monoclinic (C2) space group symmetry were