Inverse enzyme isotope effects in human purine nucleoside phosphorylase with heavy asparagine labels

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Transition path-sampling calculations with several enzymes have indicated that local catalytic site femtosecond motions are linked to transition state barrier crossing. Experimentally, femtosecond motions can be perturbed by labeling the protein with amino acids containing $^{13}$C, $^{15}$N, and nonexchangeable $^2$H. A slowed chemical step at the catalytic site with variable effects on steady-state kinetics is usually observed for heavy enzymes. Heavy human purine nucleoside phosphorylase (PNP) is slowed significantly ($k_{chem\ light}/k_{chem\ heavy} = 1.36$). An asparagine (Asn243) at the catalytic site is involved in purine leaving-group activation in the PNP catalytic mechanism. In a PNP produced with isotopically heavy asparagines, the chemical step is faster ($k_{chem\ light}/k_{chem\ heavy} = 0.78$). When all amino acids in PNP are heavy except for the asparagines, the chemical step is also faster ($k_{chem\ light}/k_{chem\ heavy} = 0.71$). Substrate-trapping experiments provided independent confirmation of improved catalysis in these constructs. Transition path-sampling analysis of these partially labeled PNPs indicate altered femtosecond catalytic site motions with improved Asn243 interactions to the purine leaving group. Altered transition state barrier recrossing has been proposed as an explanation for heavy-PNP isotope effects but is incompatible with these isotope effects. Rate-limiting product release governs steady-state kinetics in this enzyme, and kinetic constants were unaffected in the labeled PNPs. The study suggests that mass-constrained femtosecond motions at the catalytic site of PNP can improve transition state barrier crossing by more frequent sampling of essential catalytic site contacts.

Heavy-enzyme isotope effects | transition state structure | promoting vibrations | transition path sampling | Born–Oppenheimer enzymes

Purine nucleoside phosphorylase (PNP) provides the sole catalytic pathway for the degradation of 6-oxypurine (deoxy) nucleosides in human cells. Its human genetic deficiency causes 2'-deoxyguanosine accumulation and leads to a T-cell immunodeficiency disorder (1, 2). Conversion of 2'-deoxyguanosine to excess dGTP occurs specifically in activated T cells, and the perturbed deoxynucleotidyl triphosphate pool induces apoptotic cell death specific to activated T cells with no effect on quiescent T cells or B cells. Pharmaceutical inhibition of PNP is therapeutic in T-cell lymphoma and gout (3, 4). For example, Immucillin-H (Mundine) is a transition state analog of PNP, approved for treatment of T-cell lymphoma. Another transition state analog of PNP has completed phase II clinical trials for gout therapy.

The transition state of human PNP is defined by the loss of the bond-restoring mode for the N-riboside bond in its 6-oxypurine riboside substrates (Fig. 14). Transition path sampling (TPS) calculations have shown this lifetime to be ~10 fs and to be dependent on (coupled to) the motions of atoms local to the catalytic site (5–7). Experimental perturbation of the catalytic site local modes can be achieved by substitution of heavy amino acids into enzyme proteins (8–11). Heavy enzymes often show slower chemical rates at the catalytic site, interpreted as slowed dynamic modes that decrease the probability of finding the transition states. In contrast to the fast modes coupled to transition state formation, slower motions (nanosecond to picosecond) are involved in conformational or structural changes during substrate binding or product release (12–15). The slower motion may or may not be affected in the heavy protein (16). Of 12 heavy-enzyme constructs, 8 show slowed chemistry at the catalytic sites and 4 showed no effects. Altered steady-state kinetic constants are found in one-half of the heavy-enzyme constructs (16, 17). As steady-state constants often reflect slower conformational changes related to reactant binding or release, they indicate that protein mass effects can also be linked to slower kinetic conformational changes. These early studies led to the general conclusions that slowing the femtosecond motions throughout enzymes slows transition state barrier crossing and may slow global protein dynamics to affect steady-state kinetics. In cases where parts of enzymes have been segmentally labeled with a peptide fragment, or where histidines have been replaced with heavy histidine, enzyme functions are also slowed (9, 18).

Few reports have indicated that heavy enzymes can have faster chemistry than the light counterpart. With PNP, TPS explored the possibility that a mass-altered catalytic site mutant protein could be designed to exhibit increased chemistry rates relative to its unlabeled counterpart at the catalytic site (19). The analysis indicated that altering phenylalanine 159 to tyrosine (F159Y) would create an enzyme where the femtosecond dynamics of the heavy enzyme were more likely to find the transition state than the same enzyme in its natural isotopic form. This protein design element from TPS was confirmed by demonstrating a $k_{chem\ light}/k_{chem\ heavy}$ of 0.75. However, the F159Y mutation in PNP also caused less efficient catalytic site chemistry, slowing it by a factor of 32 (19). An inverse heavy-enzyme isotope effect was also

Significance

Enzymes achieve catalytic efficiency by optimizing contacts between reactants and catalytic site amino acids. The transition state forms rarely, with a lifetime of a few femtoseconds. Femtosecond motions required for transition state formation are investigated with heavy enzymes containing $^2$H, $^{13}$C, and $^{15}$N amino acids to alter bond vibrational modes. Asparagine is a critical amino acid at the catalytic site of human purine nucleoside phosphorylase (PNP). PNP with heavy asparagine, or with all heavy amino acids except asparagine, yields PNPs more efficient at forming the transition state. Computational chemistry reveals that essential catalytic site contacts become more frequently optimized in the labeled enzymes than in the normal enzyme. Heavy enzymes provide unprecedented detail for understanding enzymatic catalysis.

Author contributions: R.K.H. performed all the experimental determinations; I.Z. and D.A. performed computational analysis and TPS calculations; S.D.S. and V.L.S. conceived and supervised the project; R.K.H., I.Z., D.A., S.D.S., and V.L.S. analyzed the results; and R.K.H., I.Z., D.A., S.D.S., and V.L.S. wrote the paper.

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reported for pentaerythritol tetranitrate reductase, which gave a $k_{\text{chem light}}/k_{\text{chem heavy}}$ of 0.89 with flavin mononucleotide (FMN) and NADPH as reactants, but a normal heavy-enzyme isotope effect of 1.29 when using FMN and NADPH (20).

Native, isotopically heavy PNP responds to increased protein mass by a $k_{\text{chem light}}/k_{\text{chem heavy}}$ of 1.36 for full [2H,13C,15N] substitution (8). Partial labeling by [2H] gave $k_{\text{chem light}}/k_{\text{chem heavy}}$ of 1.19, and [13C,15N] substitution gave $k_{\text{chem light}}/k_{\text{chem heavy}}$ of 1.18 (9). These mass-proportional effects on chemistry do not distinguish full protein dynamic collective effects from the local catalytic site vibrations implicated by TPS calculations (10). The catalytic site of PNP contains three contacts between the reactants and histidines. His64 and His86 are in contact with the phosphate nucleophile and His257 contacts the 5′-hydroxyl group of the nucleoside (Fig. 1B) (21). Five additional histidines per subunit are remote (15–32 Å) from the catalytic site. Expression of PNP in a His-auxotroph Escherichia coli supplemented with [2H,13C,15N]His produced labels at the catalytic site. The $k_{\text{chem light}}/k_{\text{chem heavy}}$ of 1.13 for this construct supports local catalytic site dynamics as dominating the heavy-enzyme effects.

Here, we extend heavy-amino acid probes at the catalytic site of PNP by specific labeling of the asparagines. Asn243 plays an essential catalytic role at the transition state of PNP by stabilizing the N7-protonated purine ring, thereby activating the leaving group (Fig. 1B). Mutation of this amino acid (Asn243Ala) decreases the catalytic efficiency by 3 orders of magnitude (22). Expression of PNP in an E. coli asparagine auxotroph in media supplemented with [2H,13C,15N]asparagine introduces heavy Asn243 as well as 10 other Asn amino acids located more distant from the catalytic site with Cα distances of 34.5 Å (Asn3), 39.9 Å (Asn12), 33.8 Å (Asn55), 29.4 Å (Asn74), 12.4 Å (Asn115), 11.4 Å (Asn121), 20.9 Å (Asn137), 27.1 Å (Asn145), 37.9 Å (Asn151), and 8.9 Å (Asn256), respectively. Experimental analysis indicated this enzyme to be more efficient at the chemical step, an unexpected finding. Fully labeled PNP heavy-enzyme [2H] and [13C,15N] mass effects are additive (9). Because Asn labeling alone caused faster chemistry (an inverse isotope effect), it was predicted that a PNP fully labeled except at the asparagines would demonstrate even slower catalytic site chemistry than the fully labeled heavy [2H,13C,15N]PNP effect of $k_{\text{chem light}}/k_{\text{chem heavy}}$ of 1.36. We produced PNP with heavy amino acids throughout except for light

![Fig. 1. Transition state and structure of PNP for guanosine phosphorolysis. (A) The reaction is catalyzed via an S,1-like mechanism with a ribocationic transition state. α-Ribose 1-phosphate and guanine are the products. (B) Stereoview of the PNP catalytic site (green) in complex with DADMe-lmmG (pink) and PO₄ (orange) including the catalytic-site residues (PDB entry 3PHB). Hydrogen-bonding interactions are indicated by dashed lines. Asn243 is hydrogen bonded with N7 and O6 of guanosine. (C) Stereoview of the PNP monomer (PDB entry 3PHB). Ten asparagines (orange) are remote from the active site. Asn243 (yellow) is in the active site, making direct contact with substrates as detailed in B.](image-url)
asparagine. Unexpectedly, this construct also exhibits faster chemistry at the catalytic site than native enzyme.

We applied TPS to understand how these partially labeled enzyme constructs could increase the rate of the chemical step. The promoting vibrations (of femtosecond motions) in PNP cause the transition state to be formed when the distance between catalytic site amino acids and reactants are simultaneously optimized (7, 8). Asn243 and Glu201 are leaving-group interactions, His257 participates in ribocation formation, and Ser33, His64 and His86 activate the phosphate nucleophile sufficiently to change its P=O symmetric stretch mode (23). TPS studies indicated that catalytically optimal distances between Asn243 and the purine leaving group are sampled more frequently in heavy Asn243 light PNP (A1-PNP1′) and in heavy PNP light Asn243 (A1-PNP1) than in native PNP. Thus, TPS analysis reveals the atomic femtosecond–picosecond dynamic mechanism for the unusual inverse heavy-enzyme isotope effects in A1-PNP1′ and A1-PNP1 enzymes. This rare case of catalytic site activation demonstrates that mass of both primary and adjacent amino acids at the catalytic site influence the fitness of catalytic site contacts. The Asn-labeled PNPs provide a unique dimension for investigating enzymatic mechanisms by interpreting heavy-enzyme isotope effects.

The transition state for PNP is a near-fully dissociated N-riboseic bond in a classic S,1 mechanism (ref. 24 and Fig. 1A). The crystal structure with phosphate and DADMe-Immucillin-G, a picomolar transition state analog, show His257 hydrogen bonded to the ribosyl 5′-hydroxy group to steer the O5′ toward the O4′ of the purine ring (21). This interaction destabilizes the riboside bond, increases electron density in the purine leaving group, and elevates the pK of N7, allowing N7 protonation, stabilized by the carbonyl oxygen of Asn243. Converting stable reactants toward the transition state is a function of these distances. Both distance and electrostatic interaction forces toward the transition state vary on the femtosecond timescale. The probability of finding the transition state increases as this distance is minimized. The motion of His257–O5′–O4′ follows a similar relationship and is considered to be another of the enzyme-reactant promoting vibrations, facilitating transition state formation (6, 7). The crystal structure of PNP in complex with DADMe-ImmG reflects these interactions (Fig. 1B).

Results and Discussion

Expression, Purification, and MS Analysis of PNPs. Native PNP and PNPs labeled with heavy Asn (A1-PNP) and heavy PNP labeled with light Asn (A1-PNP1) were expressed and purified to homogeneity as explained in Materials and Methods. Intact mass analysis of light and labeled PNPs were made by electrospray ionization (ESI)-MS to determine the mass increase in labeled PNPs (SI Appendix, Figs. S1 and S3). Tryptsin digestion and peptide characterization using nano-liquid chromatography (LC)-ESI-MS/MS confirmed the labeling of PNPs (SI Appendix, Figs. S2 and S3). The observed masses of light PNP, A1-PNP1, and A1-PNP1 were 32,148, 32,280, and 35,442 Da, respectively. The expected mass of heavy A1-PNP1 is 32,247 Da for complete isotopic substitution of the 11 Asn (Fig. 1C). Only Asn243 is in the catalytic site of PNP, and the remaining 10 Asn are remote. The ESI-MS analysis reported 32,280-Da mass for A1-PNP1, suggesting an excess of 33 amu in isotope labeling. Peptide mass analysis by nano-LC-ESI-MS/MS indicated that, in addition to the 11 Asn, four heavy aspartates are also labeled in A1-PNP. Hydrolytic or enzymatic deamidation of labeled Asn accounts for these labels. All Asp are remote from the catalytic site in PNP (3PHB); therefore, we make the limiting assumption that these remote labels are unlikely to contribute to the catalytic site effects of heavy Asn residues (Fig. 1C and SI Appendix, Fig. S4). At present, we have no technical approach to test this assumption.

Steady-State Kinetics of Light and Labeled PNPs. Steady-state Michaelis–Menten kinetics for native PNP were compared with A1-PNP and A1-PNP1 for guanosine arsenolysis (Table 1 and SI Appendix, Fig. S5). Arsenate simplifies the PNP reaction by minimizing enzyme equilibration of reactants and products. It is a close mimic of the plant form of phosphatase, as the nucleophiles form the same transition state with a PNP mutant (25). The and were not significantly different for the three PNPs. Mass-independent steady-state kinetic rates have also been reported for other forms of heavy amino acid-labeled PNPs (8, 9). Product release in human PNP is the rate-limiting step in steady-state kinetic analysis (26). It is governed by slow loop motions where the mass-dependent effects are dwarfed by diffusional and solvent viscosity effects of loop movement (8).

The unchanged kinetic results of light and labeled PNPs indicate that conformational changes related to substrate binding and product release are not significantly affected by heavy Asn labeling. The loop motions during substrate binding and product release in PNPs have been characterized by crystallographic, temperature jump spectroscopy, and NMR studies (26–28).

Stopped-Flow Chemistry. Stopped-flow analysis for native and labeled PNPs used arsenate and guanosine as reactants. Arsenolysis is chemically irreversible. This isolates the chemical step, monitored by the increase in enzyme-bound guanine fluorescence (excitation at 280 nm and detection >305 nm; Fig. 2). Guanosine

<table>
<thead>
<tr>
<th>Table 1. Enzyme kinetic parameters of light and isotopically labeled PNPs for guanosine arsenolysis</th>
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<tbody>
<tr>
<td>PNPs</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Native PNP</td>
</tr>
<tr>
<td>A1-PNP1</td>
</tr>
<tr>
<td>A1-PNP1(^{1})</td>
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*Heavy enzyme KIE (native PNP/heavy PNP) from ref. 8 for guanosine phosphorylation.

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in solution or bound to PNP shows weak fluorescence. Phosphorolysis to guanine at the catalytic site gives rise to more fluorescent, PNP-bound NIH, 6-keto, N7H guanine (28). Guanine binds to PNP with a K_d of 0.4 μM, and the fluorescence intensity of guanine drops twofold when it is released to solution (28). Guanine release is rate-limiting for the reaction. Thus, the fluorescence signal for PNP-bound guanine provides a sensitive approach for measuring single-turnover chemistry rates at the catalytic site in stopped-flow experiments.

Heavy-enzyme mass effects in fully labeled PNP have been shown to be mass proportional. Thus, [\text{H},^{13}\text{C},^{15}\text{N}]PNP slowed the chemical step by 1.56, while [\text{H}][\text{H}]PNP and [^{13}\text{C},^{15}\text{N}]PNP slowed the rate by 1.19 and 1.18, respectively (9). Partial labeling of PNP where only the eight histidines in each subunit were labeled with [\text{H},^{13}\text{N}]histidine gave a 1.13 enzyme kinetic isotope effect (KIE) for guanosine phosphorolysis (9), supporting the enzyme KIEs as originating from local catalytic site dynamic motions, as predicted by TPS analysis (10). Asn243 stabilizes protonation of guanine N7 at the transition state, and we extended single-enzyme acid labeling to explore this interaction.

Pre-steady-state rates for formation of guanine at the catalytic sites of A^{13}-PNP^H and A^{15}-PNP^H were faster than that for native PNP (Fig. 2 and Table 1). Analysis of the burst phase of stopped-flow experiments gave an observed KIE of A^{15}-PNP^H of 0.78 ± 0.04 and A^{13}-PNP^H of 0.71 ± 0.04 (Fig. 2 and Table 1). Thus, both A^{13}-PNP^H and A^{15}-PNP^H demonstrate inverse heavy-enzyme isotope effects.

Placing heavy Asn in the catalytic site of A^{15}-PNP^H makes the heavy enzyme faster by k_chem light/k_chem heavy of 0.78. Since Asn243 is also heavy in the fully labeled enzyme, it is acting in the opposite direction to the slowed chemical step in [\text{H},^{13}\text{C},^{15}\text{N}]PNP. It was anticipated that the chemical step of an A^{15}-PNP^H construct could be 1.36/0.78 (=1.74), a larger heavy-enzyme isotope effect than any reported recently (16). However, a heavy-enzyme effect of this magnitude has a precedent. Fully deuterated E. coli alkaline phosphatase (AP) was characterized kinetically and reported to show a k_cat value of 1.84 for the deuteron-enzyme (29). The chemical step for hydrolysis of p-nitrophenol has been reported to be rate-limiting for this enzyme under their assay conditions (30). This effect of deuterium alone is more than that discussed above for mixed labels with PNP and would be expected to increase with fully labeled enzyme.

Instead of the anticipated normal heavy-enzyme KIE of 1.74 for A^{15}-PNP^H, the effect was k_chem light/k_chem heavy of 0.71 ± 0.04, faster than A^{13}-PNP^H. We performed additional enzyme characterization experiments to assure equivalent catalytic site binding stoichiometry for heavy and light enzymes.

**Catalytic Site Titration to Demonstrate Enzyme Equivalence.** Natural abundance and isotopically labeled PNPs were titrated with DADMe-ImmH, a tight binding transition state analog of PNP (K_d = 9 pM) to assure that each enzyme preparation had the same catalytic site concentration (31). The enzyme concentrations were determined by UV absorbance. Enzymes at a concentration of 1 μM were equilibrated with varied concentrations of DADMe-ImmH, and samples were used to initiate steady-state enzyme assays. The catalytic activity decreased as a linear function of inhibitor concentration as the catalytic sites are saturated. All of the PNPs were completely inhibited at a 1:1 stoichiometry, as expected for an inhibitor with a picomolar dissociation constant (Fig. 3). Light and labeled PNPs catalytic site functions are equivalent from protein and inhibitor binding quantitation.

**Forward Commitment Analysis.** Stopped-flow experiments support an increased rate (lower energy barrier) for the conversion of PNP•Gua•AsO_4 to PNP•Gua•R-1-AsO_4 with the heavy-enzyme Asn constructs. An experimentally independent determination of barrier heights for this step can be determined from forward commitment experiments. Chemical partition of the PNP•Gua•AsO_4 complex to products relative to its dissociation is measured with [\text{H},^{13}\text{C}]guanosine (Gua) (19). Analysis of forward commitment experiments require quantitation of the PNP•Gua•AsO_4 complex by knowing the value of K_d for guanosine with light and heavy enzymes. Steady-state kinetic experiments with varied guanosine and ascorbate concentrations established the K_d values for light PNP, A^{13}-PNP^H, and A^{15}-PNP^H to be 34 ± 4, 35 ± 4, and 28 ± 3 μM, respectively (SI Appendix, Fig. S6).

The forward commitment factors (C = k_chem light/k_chem heavy) for light and heavy PNPs establish the probability that reactants in the Michaelis complex will be converted to products. For lower barrier heights, the probability for the chemical reaction is greater, expressed as the ratio of the rate constants for the chemical step to the rate constant of dissociation of the substrate. The forward commitment factors (C) of bound guanosine committed to ars enolysis were 0.52 ± 0.02, 0.85 ± 0.04 and 0.82 ± 0.04 for native PNP, A^{13}-PNP^H, and A^{15}-PNP^H, respectively (Table 1 and SI Appendix, Fig. S7). The larger forward commitment of labeled PNPs provides independent confirmation that the transition state energy barrier for A^{15}-PNP^H and A^{13}-PNP^H is lower than for native PNP.

**Transition Path Sampling.** We employed TPS (32, 33) to generate and analyze reactive trajectories and the transition state ensemble. We used the CHARMM (34) molecular-dynamics package for all simulations. TPS is a Metropolis Monte Carlo algorithm that generates reactive trajectories in a way that the trajectory space is sampled consistently with the underlying free energy space, without any bias arising from guesses about the reaction coordinate. By comparing the native and heavy Asn PNPs, we investigated the protein dynamics associated with the faster catalysis observed experimentally for both A^{13}-PNP^H and A^{15}-PNP^H. Along each reactive trajectory, one can identify the transition state as the structure with the property that new trajectories initiated from it have equal probability to generate reactants or products. For all systems, native and heavy Asn PNPs, we generated 210 reactive trajectories of length 500 fs. For the native PNP enzyme, we used trajectories from our previous studies (11, 19). Simulations for the labeled PNPs followed the same protocol we employed in previous work (19). Like all Metropolis Monte Carlo schemes, a reactive trajectory is not uncorrelated to the new trajectory that it generates,

![Fig. 3. Stoichiometric catalytic site titration of PNPs by DADMe-ImmH. Catalytic activity remaining following fractional catalytic-site titrations is reported for native isotope abundance PNP (A), A^{13}-PNP^H (B), and A^{15}-PNP^H (C). PNP protein concentrations were determined by UV absorbance and adjusted to 1 μM PNP subunits.](E6212 www.pnas.org/cgi/doi/10.1073/pnas.1805416115 Harijan et al.)
Table 2. Distance and radius of gyration for Asn243 atoms involved in the transition state of PNP

<table>
<thead>
<tr>
<th>PNP construct</th>
<th>Range of OD1–N7, Å</th>
<th>Range of ND2–O6, Å</th>
<th>Radius of gyration CG, Å</th>
<th>Radius of gyration Asn243, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>3.01–2.50</td>
<td>3.55–2.72</td>
<td>0.979</td>
<td>2.02</td>
</tr>
<tr>
<td>A1-PNP</td>
<td>4.31–2.53</td>
<td>6.05–2.36</td>
<td>0.974</td>
<td>2.13</td>
</tr>
<tr>
<td>A2-PNP</td>
<td>4.44–2.37</td>
<td>4.45–2.41</td>
<td>0.971</td>
<td>2.10</td>
</tr>
</tbody>
</table>

Results from 10 simulations, each over 5 ps, were averaged in these analyses.

and a few iterations are needed to lose correlations. For both native and labeled PNP, among the 210 generated trajectories, we identified 25 uncorrelated transition state structures. Details of the computational approach are described in Materials and Methods.

Constrained Molecular-Dynamics Calculations. For each of the three enzymes (native PNP, A1-PNP, and A2-PNP), we identified the transition state structure in the 25 uncorrelated reactive trajectories mentioned above. Among those, we selected randomly 10 for the constrained dynamics study. Starting from the structure in the reactive trajectory that is 10 fs before the TS, we performed a constrained propagation of the system for 5 ps: we restrained motions of the active site with protonated N7-guanosine and HPO42− groups by applying a harmonic force with a constant 1,000 (kcal/mol)/Å2, while the rest of the protein could move freely. During this constrained propagation, we measured the distances between important reaction coordinate residues and the substrate, and tabulated the range of the variation of these distances. We also performed two calculations of the radius of gyration: for atoms of the Asn carboxamide (CG, OD1, and ND2) and for all atoms of residue Asn243. Finally, we averaged these from 10 constrained simulations.

Motion for Reaction Coordinate Atoms at the Transition State of PNP Constructs. TPS makes unbiased selections of transition states for the native and isotopically labeled PNP. Reaction coordinate interactions to reach the transition state involve activation of the leaving group (Asn243 and Glu201), formation of the ribocation (His257 and Tyr88), and activation of the phosphate nucleophile (Ser33, His86, and Ser220). Coincident optimization of these interactions lead to transition state formation. Analysis of distances for each of these residues over 5-ps simulations with the transition state ensemble stabilized at the catalytic site was used to explore the dynamic effect of the isotopic substitutions on the PNP proteins. Experimentally, A1-PNP and A2-PNP are catalytically activated. Interactions that are made more favorable by the heavy-atom substitutions are candidates for promoting vibrations coupled to transition state formation in the heavy PNP. Interactions at the catalytic site included distances from catalytic site atoms to the reactants, their range of motion (radius of gyration), and the angle over which promoting hydrogen bonds will be formed.

The carbonyl oxygen of the Asn243 side chain (OD1) stabilizes protonation of N7, and the amide (ND2) forms a hydrogen bond to the O6 carbonyl oxygen of guanine (Fig. 1B). As Asn243 is the focus of the heavy-atom substitution, its interaction with the transition state ensemble is central to the analysis. One transition state interaction is the Asn243 hydrogen bond (OD1–N7), which periodically reaches a dynamic minimum of ≤2.50 Å in native PNP. It is not improved in A1-PNP+ (2.53 Å) but is improved by 0.13 Å in A2-PNP+ (Table 2). The radius of gyration is not improved for Asn243 in the heavy enzymes (SI Appendix, Fig. S8). However, the ND2–O6 interaction improves significantly in both heavy Asn constructs, by 0.36 Å in A1-PNP+ and by 0.31 Å in A2-PNP+ (Table 2). The angle to form these hydrogen bonds

Table 3. Hydrogen bond angles for Asn243 atoms involved in the transition state of PNP

<table>
<thead>
<tr>
<th>PNP construct</th>
<th>CG–OD1–N7 angle, °</th>
<th>CG–ND2–O6 angle, °</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>144–178</td>
<td>78–105</td>
</tr>
<tr>
<td>A1-PNP</td>
<td>59–178</td>
<td>8–133</td>
</tr>
<tr>
<td>A2-PNP</td>
<td>57–178</td>
<td>51–117</td>
</tr>
</tbody>
</table>

Results from 10 simulations, each over 5 ps, were averaged in these analyses.

Fig. 4. Asn243 interactions to guanine N7 and O6 of the transition state ensemble: (A) Native PNP; (B) A1-PNP+; (C) A2-PNP+. Interactions to form activating hydrogen bonds of ≤2.5 Å occur more frequently in the heavy enzymes.
Table 4. Distances for enzyme to reactant atoms involved in the transition state of PNP

<table>
<thead>
<tr>
<th>Range of</th>
<th>Range of</th>
<th>Range of</th>
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<tbody>
<tr>
<td>ND1–O5′</td>
<td>OE2–N2</td>
<td>OH–O3′</td>
</tr>
<tr>
<td>His257, Å</td>
<td>Glu201, Å</td>
<td>Tyr88, Å</td>
</tr>
<tr>
<td>Native</td>
<td>3.02–2.45</td>
<td>3.69–2.52</td>
</tr>
<tr>
<td>A′-PNP</td>
<td>4.89–3.44</td>
<td>3.74–2.38</td>
</tr>
<tr>
<td>A′-PNP′</td>
<td>4.44–2.48</td>
<td>3.73–2.49</td>
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</table>

Results from 10 simulations, each over 5 ps, were averaged in these analyses.

Concluding Remarks. In fully or partially His-labeled heavy PNP, it has been shown that increased mass increases the barrier to TS formation to decrease the rate of the chemical step. In contrast, A′-PNP and A′-PNP′ PNP gave rare inverse-isotope effects, to increase the probability of locating the transition state. The barrier crossing probability is increased by an altered dynamic architecture of the labeled PNP to increase the protein–reactant interactions, most likely at the interaction of the Asn243 carbonyl group with O6 of the guanine leaving group. The results with partially labeled PNP add additional support for the concept of femtosecond protein dynamics creating the geometry to form the electrostatic environment for transition state formation.

Materials and Methods

Materials. Deuterium oxide (D2O, 99.8%), [13C6]-[1H3]glucose, [15N]ammonium chloride, and [1-15C]-[1-15N]asparagine (98%) were purchased from Cambridge Isotope Laboratories. [1-15C]Ribose purchased from Moravek Biochemicals. Pyruvate kinase (PK), myokinase (MK), guanylate kinase (GK), nucleoside diphosphate kinase (NDK), and AP were from Sigma-Aldrich. The ribokinase (RK), hypoxanthine-guanine phosphoribosyltransferase (HGPRT), and phosphoribosyl-1-lypophosphosphate synthetase (PPPS) were expressed and purified in our laboratory. All other chemicals and reagents were purchased from commercially available sources and were used without further purification.

Expression and Purification of Light PNP. Native PNP was expressed in E. coli (DE3) strain plamy5 using a pCR-T7-CTOP vector containing the encoding sequence of human PNP without affinity tag. The purification of native PNP was as described previously with some modifications (8). One colony from an overnight plate was inoculated into 20 mL of LB medium containing carbenicillin (100 μg/mL), and cells were grown at 37 °C with shaking (200 rpm) for 6–8 h. The inoculum was added to 1 L of LB medium containing carbenicillin (100 μg/mL) shaking at 37 °C for 3–4 h until the OD600 reached 0.7–1.0. Isopropyl β-D-thiogalactopyranoside (IPTG) (1.0 mM) was added for PNP induction with overnight growth at 37 °C at 200 rpm. Cells were suspended in 50 mM Tris Cl, pH 7.6, containing 200 μg/mL lysozyme and incubated for 30 min at 4 °C. Cell lysis was assisted by brief sonication followed by centrifugation at 30,000 × g for 30 min at 4 °C. The supernatant was mixed with streptomycin [1% (w/v), incubated at 4 °C for 1 h, centrifuged at 30,000 × g for 30 min at 4 °C, and dialyzed against 50 mM Tris Cl, pH 7.6. The dialysate was resolved on an anion exchange Q-FF column (GE Healthcare), equilibrated with 50 mM Tris Cl, pH 7.6, and eluted with a gradient to 50 mM Tris Cl, 200 mM NaCl, pH 7.6. The eluted PNP was concentrated and dialyzed against 50 mM Tris Cl, 1.0 M (NH4)2SO4, pH 7.6. The sample was resolved on a phenyl Sepharose hydrophobic interaction column (HiQ) (GE Healthcare), equilibrated with 50 mM Tris Cl, 1.0 M (NH4)2SO4, pH 7.6, and eluted with a gradient to 50 mM Tris Cl with a step to 500 mM Tris Cl. The concentrated and dialyzed against 50 mM phosphate buffer, pH 7.6 (1 L), containing 0.5% activated charcoal at room temperature for 36 h to remove tightly bound purines. Chromatography in 50 mM Tris Cl, pH 7.6, on a Superdex 200 (16/60) provided high-purity PNP. PNP concentration was determined by absorbance at 280 nm using the calculated molar extinction coefficient of 29.9 mM−1 cm−1 (Protparam; https://web.expasy.org/protparam). Protein was used directly or frozen rapidly in 50 mM Tris Cl, pH 7.6, for later use.

Expression and Purification of A′-PNP. A′-PNP was expressed using the asparagine auxotroph of E. coli strain ER, obtained from the E. coli Genetic Stock Center at Yale University transformed with a pCR-T7-CTOP vector encoding human PNP without an affinity tag. Cells were grown in M63 minimum medium (prepared in water) supplemented with glucose (3 g/L), [1-15C]-[1-15N]asparagine (0.1 mg/mL), 34 μg/mL chloramphenicol, and 100 μg/mL carbenicillin at 37 °C. IPTG (1 mM final) was added at OD600 of 0.8–1.0 for the induction of heavy Asn-PNP. Cells were grown overnight and harvested by centrifugation. A′-PNP was purified as described for native PNP.

Expression and Purification of A′-PNP′. A′-PNP′ was expressed using the asparagine auxotroph of E. coli strain ER, obtained from the E. coli Genetic Stock Center at Yale University transformed with a pCR-T7-CTOP vector encoding human PNP without affinity tag. Cells were grown in M63
minimum medium prepared in 99.8% D2O at 37 °C. The growth media was supplemented with [13C6,15N2]glucose (3 g/L), [15N]Nammonium chloride (1 g/L), asparagine (0.1 mg/mL), 34 µg/mL chloramphenicol, and 100 µg/mL carbenicillin. IPTG at 1 mM was added at OD600 of 0.8–1.0 for PNP induction. Purification was as described for native PNP.

MS Analysis. For intact mass analysis, PNP proteins were desalted off-line using C4 ZipTip (Millipore) and analyzed by ESI-MS using a TriVersa NanoMate nanoelectrospray source (Advion) connected to a linear ion trap LTQ-XL mass spectrometer (Thermo Scientific). For peptide and protein identification, PNP proteins (20 µg) in 50 mM NH4HCO3 (Sigma) and 0.01% Protease Max (Promega) were reduced using 5 mM Tris(2-carboxyethyl)phosphine (Thermo Scientific) for 30 min, and then alkylated with 25 mM iodoacetamide (Sigma) for 30 min at room temperature, in the dark. Samples were digested with trypsin (Trypsin Gold; Promega) (1:100), at 37 °C for 3 h. An aliquot of the peptide digest was diluted 50-fold with 2% (v/v) vololol acetonitrile (Fisher) in 0.1% trifluoroacetic acid (Pierce) and analyzed by nano LC-EASI-MS/MS. For the analysis of A1-PNP, C18 reversed-phase nano-LC-EASI-MS/MS analysis was performed using a Dionex Ultimate 3000 RSLCnano System (Thermo Scientific) connected to a TriVersa NanoMate nanoelectrospray source (Advion) and a linear ion trap LTQ-XL mass spectrometer (Thermo Scientific). Automated protein identification was performed by Mascot search engine, version 2.5.1 (Matrix Science), against Swissprot AC and PNP complexes. 3KBQAPDB database with the following search parameters: trypsin; two missed cleavages; peptide charge of +2 and +3; peptide tolerance of 2.0 Da; MS/MS tolerance of 0.6 Da; carbamidomethylation (Cys) for fixed modification; deamidation (Asn and Gin), oxidation (Met), PNP Asn label (Asn), and PNP Asp label (Asp) for variable modifications. Scaffold software, version 4.5.1 (Proteome Software), was used to validate the MS/MS peptide and protein identification. For the analysis of A1-PNP, C18 reversed-phase nano-LC-EASI-MS/MS analysis was performed using a Dionex Ultimate 3000 RSLCnano System (Thermo Scientific) connected to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Automated protein identification was performed by Mascot search engine, version 2.5.1 (Matrix Science), against PNP_human_3KBQAPDB database, and A1-PNP peptides were further assigned manually for confirmation.

Steady-State Kinetics. Kinetic assays used a CARRY-100 spectrophotometer (VARIAN) at room temperature (25 °C) for guanosine arsenolysis as described previously (24). Assay mixtures contained 50 mM Tris HCl, pH 7.4, 50 mM arsenate, pH 7.4, and varying concentrations of guanosine (10–160 µM). Absorbance spectra were recorded at 258 nm after addition of 10 nM light or labeled PNP. The rate of absorbance change was calculated from the associated software, and a molar extinction coefficient of $-5,500$ M$^{-1}$ cm$^{-1}$ was used for the conversion of guanosine to guanine. Kinetic parameters were analyzed by data fitting to the Michaelis constant for the variable substrate.

Pre-Steady-State Kinetics. Pre-steady-state rate constants for guanosine arsenolysis were determined with a stopped-flow spectrofluorometer (Applied Photophysics; dead time, ≤1.25 ms) at 25 °C. The increase in the fluorescence signal was measured upon the formation of enzyme-bound guanine. The reaction mixture was excited at 280 nm with slit width of 1 mm, and fluorescence signal above 305 nm was collected using WGG305 Scod filter positioned between the photomultiplier and the sample cell. The fluorescence spectra were monitored for 250 ms and 1,000 points were collected for individual rate curves. Syringe 1 contained 50 mM Tris HCl (pH 7.4), 50 mM arsenate (pH 7.4), and 30 µM PNP. Syringe 2 contained 50 mM Tris HCl (pH 7.4), 50 mM arsenate (pH 7.4), and 40 µM guanine.

Dissociation Constant ($K_0$) Calculation. The dissociation constants for PNP-guanosine for the arsenolysis reaction were estimated from kinetic assays with varying concentrations of both guanosine (10–160 µM) and asenate (0.5–10 mM). The experimental data were fitted to Eq. 1, where $v$ is the initial velocity, $V$ is the maximal velocity, $S$ is the concentration of variable substrate, and $K_0$ is the Michaelis constant for the variable substrate. Extrapolation of the $K_0$ values for guanosine to zero concentration of arsenate was used to calculate the $K_0$ for guanine:

$$v = \frac{V}{K_0}(S/V) + (1/V).$$

Titration of DADMe-ImmH to PNP. DADMe-ImmH, a transition state analog of PNP with a $K_d$ of 9 µM, was used to titrate defined protein concentrations of PNP to validate catalytic site concentrations. Incubation mixtures (50 µL) containing 50 mM Tris HCl, pH 7.4, 50 mM inorganic phosphate (Pi), pH 7.4, 1 µM PNP, and varying concentration of DADMe-ImmH (0–1 µM) were incubated at room temperature for 30 min. Samples (5 µL) were assayed in 50 mM Tris HCl, pH 7.4, 50 mM inorganic phosphate (Pi), pH 7.4, 2 mM imino, and 60 mM of xanthine oxidase, and measured with a CARY-100 spectrophotometer (VARIAN) at room temperature (25 °C) at 303 nm. The remaining activity of each incubation mixture was determined.

Synthesis of 1-[14C]Guanosine. 1-[14C]Guanosine was synthesized from 1-[14C]ribose and guanine in coupled enzymatic reactions with some modifications (19, 31). A reaction (1 mL) containing 50 µCi of 1-[14C]ribose, 20 mM phosphoenolpyruvate, 2 mM ATP, 2.2 mM guanine, 50 mM MgCl2, 100 mM phosphate buffer, pH 8.0, 50 mM KCl, 1 mM DTT, 0.2 µL PRPPS, 4 µL PNP, 2 µL MK, 2 µL HgAaPT, 2 µL RK, 2 µL GK, and 2 µL NDK was incubated for 3 h at room temperature. After that, 1 mM additional cold ribose was added to the reaction as carrier and was incubated overnight for the purification of 1-[14C]GTP. The purification of 1-[14C]GTP was done by C-18 reverse-phase HPLC column using 50 mM triethyline, 50 mM acetic acid, and 5% methanol, pH 5.3. The purified 1-[14C]GTP was treated with AP to remove the triphosphate group. A reaction (1 mL) containing purified GTP with 50 mM Tris- HCl, pH 7.8, 100 mM NaCl, 10 mM MgSO4, 1 mM DTT, and 2 U/mL AP was incubated at room temperature for 3 h. 1-[14C]Guanosine was purified using C-18 reverse-phase HPLC and a gradient of 0.1% formic acid to 0.05% formic acid in 50% acetonitrile. 1-[14C]Guanosine was purified on Dowex-1 (chloride) to remove phosphate. Purified 1-[14C]Guanosine was used to determine the forward commitments.

Forward Commitment Analysis. Forward commitment factors (C) were determined for guanosine arsenolysis with PNPs by isotope-trapping methods (37, 38). Reaction mixtures (20 µL) containing 25 µM PNPs, 160 µM 1-[14C]guanosine, and 50 mM Tris HCl, pH 7.4, at 25 °C, were diluted with 980 µL of 2 mM guanosine, 50 mM Tris HCl, pH 7.4, and 50 mM inorganic arsenate, pH 7.4. Samples (100 µL) were quenched with 50 µL of 1 N HCl at 15, 30, 45, and 60 s and neutralized with 50 µL of 1 M NaOH. Samples were purified using charcoal columns (200-mg Carlograph Extract-Clean columns; Vertical Chromatography). The product 1-[14C]ribose was eluted into scintillation vials with 6 mL of 20 mM ribose in 10% ethanol. Solvents were removed by vacuum centrifugation, and samples were redissolved in 500 µL of water, mixed with 10 mL of scintillation liquid (PerkinElmer), and counted for radioactivity in a Tricarb 2910 TR scintillation counter (PerkinElmer). The values obtained in the absence of arsenate in the reaction were used as controls. Forward commitment factors were calculated according to Eq. 2, where $Y$ is the molar ratio of 1-[14C]ribose formed from bound 1-[14C]guanosine. The enzyme–guanosine concentration [ES] was calculated using Eq. 3, in which [E] and [S] are the concentrations of enzyme and guanosine, respectively, at equilibrium in the initial mixture, and $K_a$ is the dissociation constant of enzyme-substrate complex:

$$[ES] = [E]([S]/K_a).$$

Computational Methods. The starting point for the simulations was crystal structure 1R86 from the Protein Data Bank (PDB). It is a homotrimeric human PNP structure in complex with Immuclillin-H and phosphate. To build our model, we modified the transition state inhibitor (Immuclillin-H) to the guanine substrate by replacing atoms N4 with O4 and C9 with N9. His257 was modeled in the neutral form with the proton at NE leaving ND1 to interact with S-hydryl of the ribose ring. The catalytic-site residue Glu201 was deprotonated at neutral pH to stabilize the purine base and atom N7 of the purine base was protonated. In the native heavy PNP enzyme, we manually modified the atomic masses of C, N, and non-exchangeable H of the amino acids with heavy isotopes. In A1-PNP enzyme, we followed the same mass modification for all amino acids except for the 11 asparagines. Finally, in the A1-PNP, we modified the atomic masses of C, N, and nonexchangeable H of the 11 asparagines only. All crystallographic waters were retained for all structures.

The PNP system was partitioned into quantum-mechanics (QM) and molecular-mechanics (MM) regions, where the QM region consisted of 40 atoms including the protonated N-guanosine and HPO₄⁻ mieties and was modeled using the PM3 method. All remaining atoms of the protein, including ions and solvent water molecules form the MM region. No link atoms were used since there are no covalent bonds between the atoms of the QM and the MM regions.
The system was neutralized by placing 18 chloride and 29 sodium ions in the simulation system. After the setup, the protein was minimized using 150 steps of steepest descent followed by 6,000 steps of adopted basis Newton-Raphson minimization. The system was heated slowly from 0 to 300 K for 70 ps, and any harmonic constraints applied to the system were gradually removed. For the last 10 ps, we switched the QMMM on. Following the heating process, the system was equilibrated at 300 K with the QMMM on for 150 ps. The final structure was used as a starting configuration for the TS study.

TPS was used to harvest trajectories of the chemical event, according to the following steps. First, we defined the order parameters to be the C1–N9 bond-breaking and C1–Op bond-forming distances, such that the reactive region contained all configurations with the C1–N9 bond length <1.70 Å and the C1–Op bond length >1.65 Å. As the product region contains all configurations with C1–N9 bond length >1.70 Å and C1–Op bond length <1.65 Å. Then we perturbed the momenta of a randomly chosen slice of this reactive trajectory and propagated using the new momenta. Finally, we generated a new reactive trajectory. The initial reactive trajectory is generated by applying a harmonic force with constant £65 (kcal/mol)Å² on both the bond-breaking and bond-forming distances followed by propagation for 250 fs. Using this first biased trajectory as a seed, we iterated the TPS reactive trajectory generation procedure until a sufficient set of uncorrelated reactive trajectories was generated. Then, we performed committor analysis to locate the transition state, defined as the time slice with the property that new trajectories initiated from that slice with random momenta have probability 0.5 to reach reactants or products. The set of these configurations known as isocommittor structures, forms the transition state ensemble (TSE).

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