

1 **The ubiquity of directional and reciprocating motion in enzymes out of equilibrium**

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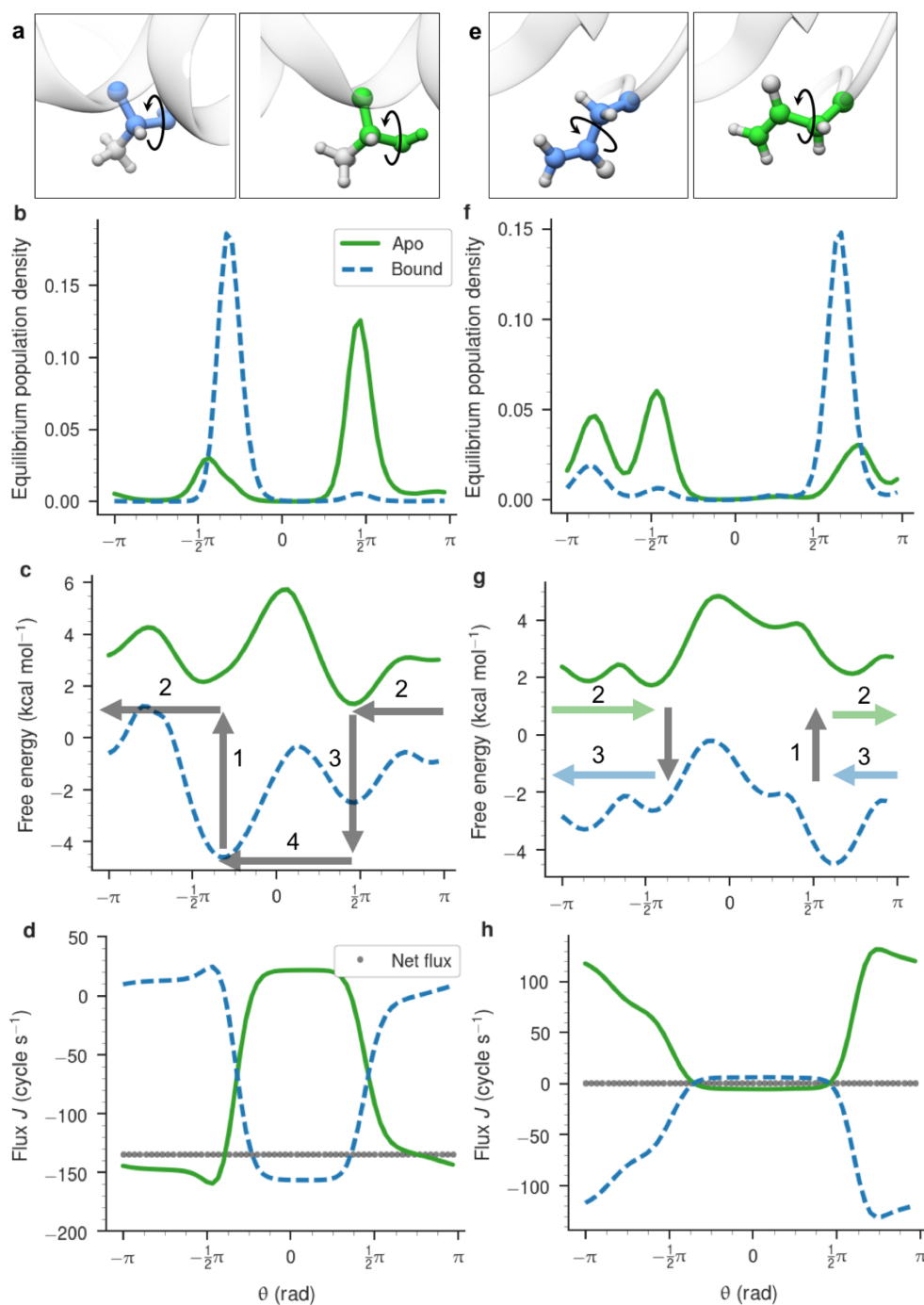
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6 **Summary paragraph**

7 A biological molecular motor is an enzyme that uses the free energy of an out-of-equilibrium
8 chemical reaction to drive mechanical motion. This motion must have a specific direction to fulfill
9 the motor's functional role. For example, a corkscrew-shaped flagellum must rotate in the
10 appropriate sense to propel the organism. The generation of directional motion appears to be a
11 complex protein property, and it is not clear how the evolutionary leap from non-motor enzymes
12 to molecular motors could have occurred. Indeed, the existence of biological molecular motors has
13 been held up in the popular press as a mark against the theory of evolution¹. Here, we provide
14 evidence, based on atomistic simulations and kinetic modeling, that conformational switching of
15 non-motor enzymes, induced by out-of-equilibrium substrate binding and catalysis, induces motor-
16 like, directional torsional motions, as well as oar-like, reciprocating motions. Generalizing from
17 these specific results, we provide an argument that virtually any chiral molecule undergoing
18 conformational transitions out of equilibrium should be expected to undergo directional rotations
19 on small and potentially large scales. Thus, the emergence of directional motion did not require an
20 evolutionary leap. Instead, directional motion was present in the earliest enzymes, and only
21 evolutionary optimization was needed for highly adapted motor proteins to emerge. Moreover,
22 because chirality is a *sine qua non* for directional motion, the adaptive value of directional motors
23 means that chirality itself is adaptive, so the need for directional motion may be one reason for the
24 prevalence of chiral molecules in living systems. Finally, the ubiquity of driven molecular motions

- 25 in enzymes catalyzing reactions out of equilibrium might help explain why the diffusion constants
- 26 of some enzymes increase with their catalytic rate²⁻⁴.

27 **Text**



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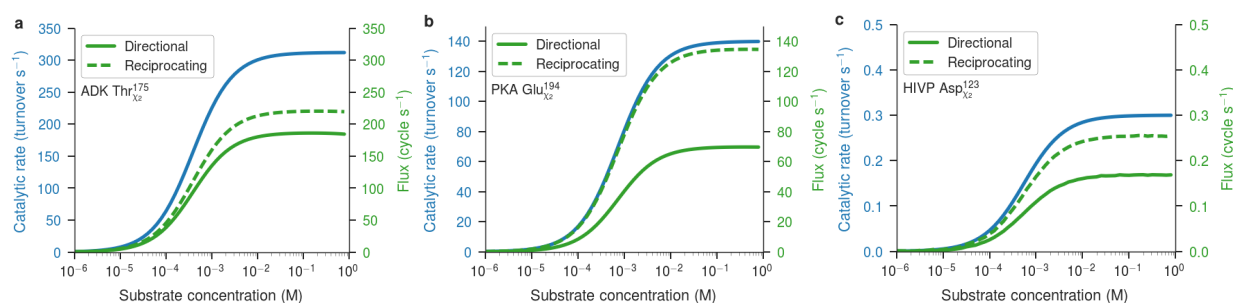
29 Figure 1. Protein torsion angles show directional and reciprocating motion. (a) ADK Thr175 in its crystallographic conformations
 30 for the apo (green) and bound (blue) forms (see Supplementary Methods for PDB accessions) with the χ_2 angle denoted. The
 31 coloring is the same for panels a through d. (b) Equilibrium population densities of this angle from MD simulations (Supplementary
 32 Methods). (c) Free energy surfaces of this angle (Supplementary Methods) derived from the population densities in panel b. Arrows
 33 indicate the direction of probability flux along, and between, the two surfaces. (d) The probability flux drawn separately for each
 34 surface and as a sum (grey points), indicating large directional and reciprocating fluxes. (e-h) Same as a-d for ADK Asn138. In all
 35 cases the substrate concentration is 10^{-3} M.

36 When any enzyme binds a molecule of substrate and catalyzes its conversion to product, it switches
37 stochastically between two distinct conformational free energy surfaces, one for the apo state and
38 one for the substrate-bound state. The flashing potential model⁵⁻⁷, which has been used to
39 understand the mechanisms of molecular motors⁸⁻¹², may be used to compute the dynamical
40 consequences of this switching, given basic enzyme kinetic parameters and knowledge of the two
41 energy surfaces. We computed the one-dimensional free energy surfaces of protein main- and side-
42 chain torsions, discretized into bins, from detailed equilibrium molecular dynamics (MD)
43 simulations of enzymes in their apo and substrate-bound states (Supplementary Methods). These
44 data, coupled with literature values for the enzyme kinetic parameters (Supplementary Table 1),
45 enabled us to define first order rate constants for transitions between neighboring bins on and
46 between surfaces (Extended Data Fig. 1). The resulting set of rate equations was solved for the
47 non-equilibrium steady state probability distribution across the surfaces. This, in turn, was used to
48 compute the probability flux on each surface and the net flux, J , along both surfaces. Nonzero net
49 flux implies directional rotation. We furthermore evaluated power output and performance under
50 load by tilting the chemical potential surfaces to generate a torque, τ , opposite to the directional
51 flux, which modifies the intrasurface bin-to-bin rate constants. The power output is the product of
52 imposed torque and flux: $P = \tau J$. Both the maximum power and the stall torque, τ_{stall} , which brings
53 the directional flux to zero, were found by scanning across values of applied torque.

54 We used this method to analyze motions in three enzymes, each with distinctive characteristics:
55 adenylylate kinase (ADK), with 214 residues and a relatively high $k_{\text{cat}} \sim 300 \text{ s}^{-1}$ ^{13,14}, undergoes
56 extensive conformational change on binding substrate, with two domains reorienting to form a
57 compact conformation^{15,16}; protein kinase A (PKA), with 350 residues and $k_{\text{cat}} \sim 140 \text{ s}^{-1}$ ¹⁷, acts as
58 a “dynamic switch”, with long-range allosteric interactions and domain rearrangement upon ligand

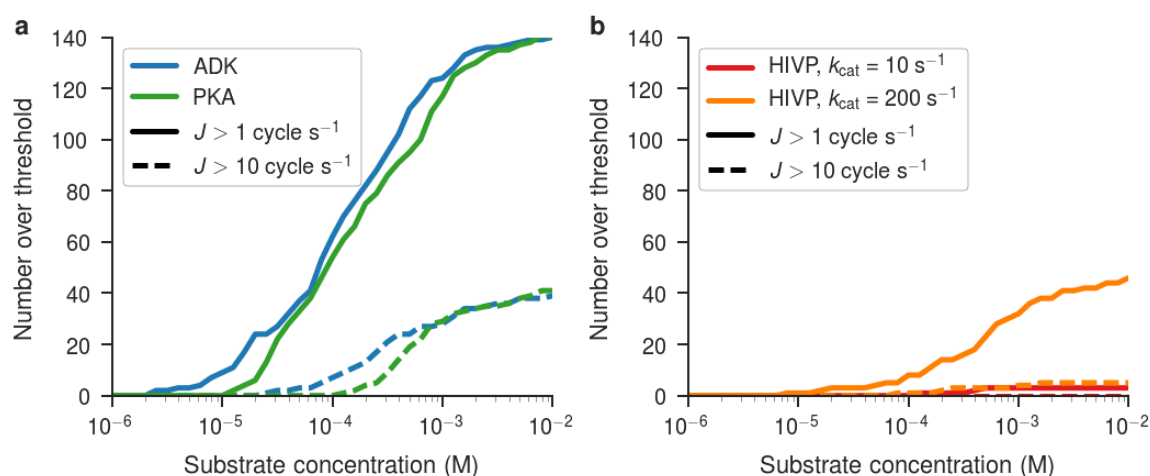
59 binding¹⁸; while HIV-1 protease (HIVP), with 200 residues and lower $k_{\text{cat}} \sim 10 \text{ s}^{-1}$ ¹⁹⁻²¹, contains
60 two flexible flaps that lose mobility in the substrate-bound state^{22,23} (Extended Data Fig. 2).
61 The present analysis indicates that torsions in all three enzymes exhibit directional torsional flux,
62 driven by the catalytic conversion of substrate to product. The general mechanism by which
63 directional flux is generated is illustrated by the χ_2 torsion of ADK Thr175 (Fig. 1a). This angle
64 has a two-peaked probability distribution in both the bound and apo states, but the peak near $+\frac{\pi}{2}$
65 is favored in the apo state, while that near $-\frac{\pi}{2}$ is favored in the bound state (Fig. 1b,c). In the
66 presence of substrate, the bound-state energy minimum near $-\frac{\pi}{2}$ is highly occupied (Fig. 1b,c).
67 Catalytic breakdown of substrate pumps the system to the secondary energy minimum of the apo
68 state at $-\frac{\pi}{2}$ (Fig. 1c, arrow 1). Probability then flows primarily to the left on the apo surface,
69 because this is the lowest-barrier path to the apo state's global energy minimum near $+\frac{\pi}{2}$ (arrow
70 2; this flux goes through the periodic boundary at $\theta = -\pi \equiv +\pi$). Probability pooled in the global
71 energy minimum of the apo state near $+\frac{\pi}{2}$, then flows primarily to the bound state, by binding
72 substrate and landing in the secondary energy minimum of the bound state (arrow 3). It then flows
73 back to the global minimum of the bound state via the lowest-barrier path, which is again leftward
74 (arrow 4). The net effect is a leftward flux of up to $-140 \text{ cycles s}^{-1}$. Fig. 1d shows the steady state
75 flux on each surface: leftward flux predominates overall, but occurs on the apo surface between
76 $-\frac{\pi}{2}$ to $+\frac{\pi}{2}$, and on the bound surface elsewhere, with crossovers between surfaces at the energy
77 minima. This process parallels flashing potential mechanisms previously invoked to explain highly
78 evolved molecular motors^{7,8,11,12,24-27}. In the absence of significant directional flux, a torsion can
79 still be actively driven back and forth between two angular ranges. For example, χ_2 of Asn 138 in

80 ADK (Fig. 1e) has essentially zero net flux but undergoes cycles of driven, reciprocating flux, with
 81 intrasurface fluxes reaching 130 cycles s^{-1} (Fig. 1d-h).

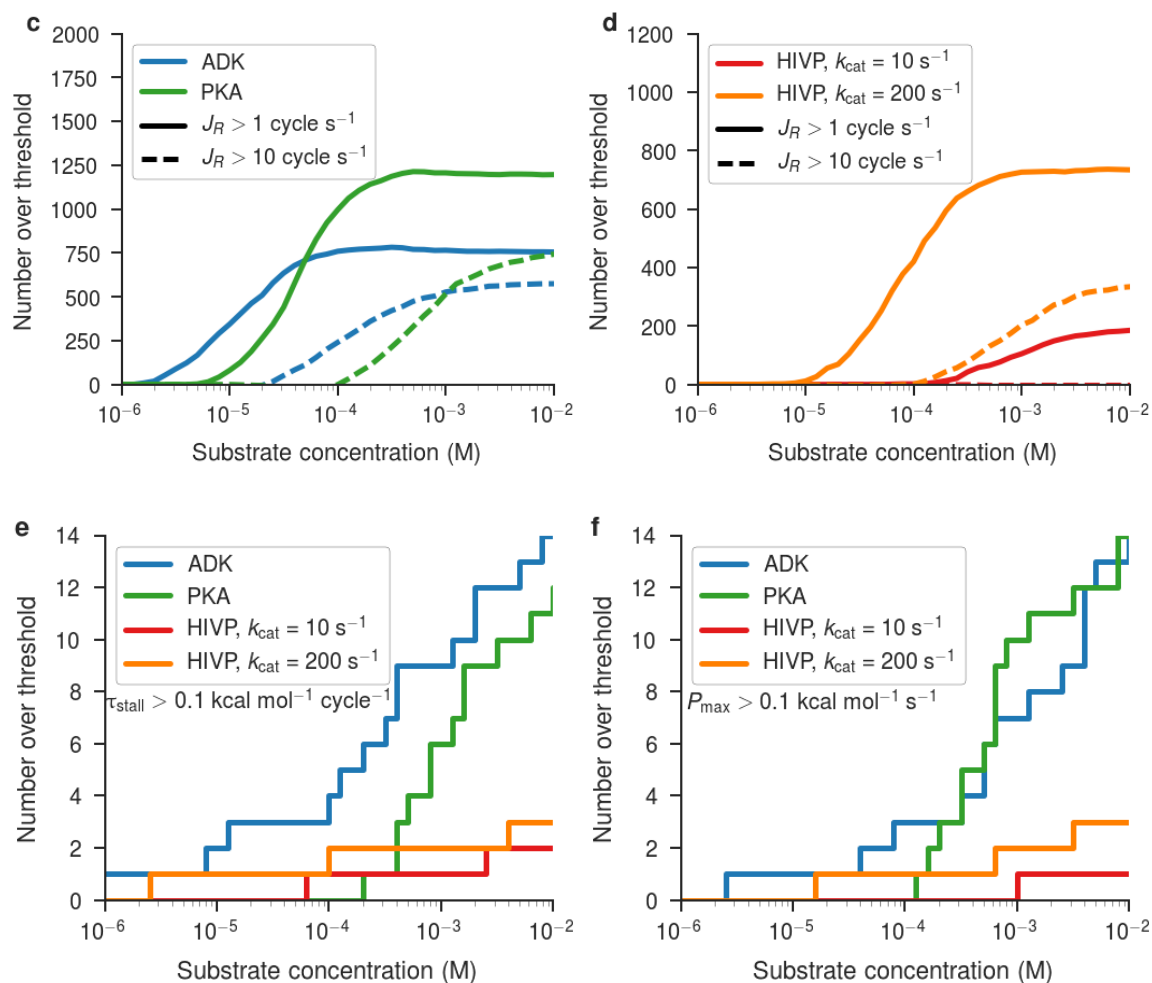


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 83 Figure 2. Dependence of catalytic rates and of the magnitudes of driven and directional fluxes on substrate concentration, for torsion
 84 angles in each enzyme. (a) The χ_2 angle of Thr175 in ADK reaches high levels of both directional and reciprocating flux. (b) The
 85 χ_2 angle of Glu194 in PKA reaches a high level of reciprocating flux and moderate level of directional flux. (c) Although the total
 86 amount of flux in the χ_2 angle of Asp123 in HIVP is low, the ratio of directional and reciprocating flux to the enzyme velocity is
 87 similar to that in ADK and PKA.

88 The fluxes are driven by catalytic breakdown of substrate: they are zero without substrate and rise
 89 sigmoidally with substrate concentration, along with the catalytic rate, as illustrated in Fig. 2 and
 90 Extended Data Fig. 3. Although the maximum directional fluxes in these examples are quite
 91 different for ADK, PKA and HIVP (180, 70 and 0.18 cycles s^{-1} , respectively), the ratios of flux to
 92 catalytic rate are similar, 0.5 – 0.6 cycles/catalytic turnover. This ratio is akin to a 2:1 gearing of
 93 catalysis to torsional rotation.



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97 Figure 3. The number of torsions above various thresholds of directional flux magnitude, reciprocating flux magnitude, stall torque,
 98 and maximum power, as a function of substrate concentration. (a-b) The number of torsions with directional flux above 1 (solid)
 99 or 10 (dotted) cycle s^{-1} in ADK, PKA, and HIVP. (c-d) The number of torsions with reciprocating flux above 1 (solid) or 10 (dotted)
 100 cycle s^{-1} and, at the same time, directional flux less than 1 cycle s^{-1} . The number of angles with (e) maximum stall force above 0.1
 101 kcal/(mol-cycle) and (f) power above 0.1 kcal/(mol- s^{-1}).

102 In addition to torsions with fluxes that approach the catalytic rate, each enzyme also has many
 103 torsions with a lower but definite directional or reciprocating flux. Thus, at high substrate
 104 concentration, about 40 torsions in ADK are found to rotate faster than 10 cycle s^{-1} , and about 140
 105 are found to rotate faster than 1 cycle s^{-1} (Fig. 3a). The corresponding numbers are lower for HIVP
 106 (Fig. 3b, red). This largely reflects the lower k_{cat} value of HIVP, about 10 s^{-1} ¹⁹⁻²¹ compared with
 107 200-300 s^{-1} for PKA^{17,28} and ADK^{13,14}. Thus, artificially assigning $k_{cat} = 200$ s^{-1} to HIVP leads
 108 to substantial increases in the number of torsions with fluxes of at least 10 s^{-1} and at least 1 s^{-1} (Fig.

109 3b, orange and Extended Data Fig. 4). This means that many of the torsional PDFs in HIVP
110 generate low fluxes primarily because k_{cat} is low for this enzyme. The tendency toward lower
111 fluxes in HIVP may also reflect the smaller scale of its conformational changes (Extended Data
112 Fig. 2). In addition to torsions with net directional flux >0.1 cycles s^{-1} , many torsions are predicted
113 to undergo large reciprocating fluxes. Indeed, ADK and PKA are predicted to have ~ 1250 and
114 ~ 750 torsions whose reciprocating motions occur at rates of at least 1 cycle s^{-1} with minimal
115 concomitant directional flux (Fig. 3c,d). These angles are distributed throughout the proteins, with
116 high flux torsions localized near the substrate binding pocket or mobile regions (Extended Data
117 Fig. 2).

118 The directional torsions in these enzymes can do work against small mechanical loads and thus
119 generate power (Fig. 3e,f). At high substrate concentrations, torsions in ADK and PKA are
120 predicted to generate stall torques up to 2.4 and 1.6 kcal mol^{-1} cycle $^{-1}$, respectively, and maximum
121 power outputs per torsion of 70 and 28 kcal mol^{-1} s^{-1} .

122 Directional flux can be generated only by a chiral molecule. Otherwise, the energy surfaces of the
123 torsions would not distinguish between flux to the right (e.g., clockwise) and flux to the left
124 (counterclockwise), so there would be no physical basis for net motion in one direction. Indeed,
125 as illustrated in the Extended Data Fig. 5, artificially symmetrizing the energy surfaces of a torsion
126 angle abolishes directional flux, though substantial reciprocating fluxes can remain.

127 The central result of this study is that enzymes not normally regarded as motor proteins can have
128 motor-like properties: in the presence of excess substrate, they exhibit not only driven
129 reciprocating motions but also directional rotation. Furthermore, the specific findings for ADK,
130 PKA, and HIVP may be generalized by a simple physical argument based on the same flashing
131 potential kinetic model. Consider any chiral molecule with a degree of freedom that is switched

132 back and forth between two energy surfaces. After the energy surface is switched, the asymmetry
133 of the new energy surface means that probability will, in net, flow more in one direction than the
134 other. When the surface is switched back, a second directional probability flow occurs, this time
135 on the other energy surface, and only by coincidence will the two probability flows cancel
136 perfectly, unless a large energy barrier entirely blocks circular motion. The imbalance between the
137 two flows represents directional flux.

138 Although this study focuses on torsional motions, the same physical reasoning applies to larger
139 scale conformational motions, so directional motions in enzymes catalyzing reactions out of
140 equilibrium likely also involve motions through higher-order conformational subspaces. For
141 example, the concerted opening and closing of an active site could occur along two different paths,
142 thus exhibiting hysteretic cycling. Such concerted motions might be stronger, in terms of force and
143 power, than the torsional motions studied here, and their hydrodynamic coupling with solvent
144 might help explain why some enzymes diffuse faster when catalytically active^{2-4,29,30}. We note that,
145 despite the scallop theorem³¹, driven reciprocating motions could also speed enzyme diffusion if
146 they occur on time scales slower than the rotational diffusion of the enzyme, which is typically
147 about 10-100 ns, because then the forward and reverse translations would have different directions.

148 Thus, an enzyme catalyzing a reaction out of equilibrium can undergo directional probability flows
149 through multiple conformational cycles, as well as driven reciprocating motions. Such motions
150 could have been the starting points for the evolution of today's efficient motor proteins, and might
151 also provide footholds for *in vitro* directed evolution of mechanically active enzymes. Additionally,
152 because directional motion requires chirality, the adaptive advantages of directional motor
153 proteins, such as in flagellar propulsion, could be one reason that natural selection led to
154 biomolecules that are chiral.

155 **Methods**

156 Methods, including statements of data availability and any associated accession codes and
157 references, are available in the online version of the paper. The datasets generated and analyzed
158 during the current study are available in the GitHub repository
159 <https://github.com/GilsonLabUCSD/nonequilibrium>.

160

161 Code availability

162 The Python code used to analyze the simulation data and implement the kinetic model is available
163 at the same GitHub repository.

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172 **Author Contributions**

173 M.K.G. conceived and designed the project. D.R.S. implemented the model and performed the
174 simulations. M.K.G. and D.R.S. analyzed the data and wrote the manuscript.

175 **Author Information**

176 MKG has an equity interest in and is a cofounder and scientific advisor of VeraChem, LLC.

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