Evidence for dynamic motion in proteins as a mechanism for ligand dissociation

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Supplementary Methods.

Synthesis of compounds 2 and 4-6. Similarly to 3, compounds 4 and 6 (7- and 5-methyl-5,6,7,8-tetrahydroquinazoline-2,4-diamine, respectively) were prepared from dicyandiamide (10.19 g, 0.12 moles) and 3-methylcyclohexanone (11.33 g, 0.10 moles). Reverse-phase HPLC purification using a water and acetonitrile buffer system was used to separate the two products. A combination of very small volumes of the mixture loaded per run along with a very shallow acetonitrile gradient was useful in accomplishing the separation. To avoid isolation of a TFA-salt of each compound, NaOH base was added to the separate compound pools and each was treated with hot chloroform to extract the compounds free of TFA, which remains in the water layer. Extracts were dried over sodium sulfate and rotary evaporation was used to remove the chloroform to yield white powders for each compound. A standard 2D ¹H-¹H NOESY experiment was used to distinguish between the two methyl products: only in 6 would NOESY cross peaks between the methyl protons and the amine protons be observed. In addition, the benzylic methine proton of 6 would be expected to be shifted further downfield (~2.7 ppm) than the methine of 4 (~1.3 ppm).

Analogously, 2 and 5 (8-methyl- and 5,6,7,8-tetrahydroquinazoline-2,4-diamine, respectively) were prepared from the combination of dicyandiamide (10.19 g, 0.12
moles) and 2-methylcyclohexanone (11.33 g, 0.10 moles) or cyclohexanone (9.91 g, 0.10 moles). The compounds were purified via HPLC and isolated via chloroform extraction to yield white powders.

Spectroscopic data for 2-6:

2. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 5.94 (bs, NH$_2$), 5.46 (bs, NH$_2$), 2.46-2.41 (m, 1H), 2.23-2.13 (m, 2H), 1.83-1.70 (m, 2H), 1.62-1.56 (m, 1H), 1.45-1.39 (m, 1H), 1.16-1.15 (d, 3H); $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 165.5, 162.7, 160.9, 100.4, 34.2, 30.4, 22.4, 20.1, 19.5; MS (m/z): [M]$^+$ calcd. for C$_9$H$_{14}$N$_4$, 178.12, found, 179.0.

3. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 5.97 (bs, NH$_2$), 5.51 (bs, NH$_2$), 2.42-2.34 (m, 3H), 1.77-1.73 (m, 3H), 1.29 (m, 1H), and 1.03-1.01 (d, 3H); $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ 162.6, 161.7, 160.9, 100.4, 31.1, 30.4, 28.7, and 21.8; MS (m/z): [M]$^+$ calcd. for C$_9$H$_{14}$N$_4$, 178.12, found, 179.0.

4. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 6.04 (bs, NH$_2$), 5.55 (bs, NH$_2$), 2.42-2.38 (m, 1H), 2.32-2.27 (m, 1H), 2.18-2.12 (m, 1H), 2.04-1.98 (m, 1H), 1.82-1.72 (m, 2H), 1.28-1.20 (m, 1H), 0.98-0.96 (d, 3H); $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 162.6, 161.1, 160.6, 100.6, 30.5, 28.2, 22.5, 21.6, 21.5; MS (m/z): [M]$^+$ calcd. for C$_9$H$_{14}$N$_4$, 178.12, found, 179.2.

5. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 5.93 (bs, NH$_2$), 5.45 (bs, NH$_2$), 2.35-2.33 (m, 2H), 2.19-2.17 (m, 2H), and 1.66-1.65 (m, 4H); $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 162.6,
161.9, 160.8, 100.9, 31.3, 22.5, 22.3, 21.9; MS (m/z): [M]+ calcd. for C₈H₁₂N₄, 164.11, found, 165.0.

6. ¹H NMR (500 MHz, DMSO-d₆): δ 6.00 (bs, NH₂), 5.49 (bs, NH₂), 2.72-2.69 (m, 1H), 2.35-2.33 (m, 2H), 1.78-1.57 (m, 4H), 1.03-1.02 (d, 3H); ¹³C NMR (125 MHz, DMSO-d₆): δ 162.3, 161.1, 160.5, 106.1, 31.2, 29.9, 24.9, 19.5, 17.3; MS (m/z): [M]+ calcd. for C₉H₁₄N₄, 178.12, found, 179.3.

NMR Methods.

Resonance assignments – Standard triple-resonance experiments were used to assign the backbone of the ternary complexes with compounds 3, 4, and 5. E:NADPH:2 and E:NADPH:6 assignments were contingent upon knowledge of the first three ternary complexes. Specifically, HNCACB and CBCA(CO)NH experiments collected at 700 MHz allowed for the assignment of ¹H, ¹⁵N, ¹³Cα, and ¹³Cβ resonances in these complexes. Common residues that could not be assigned include K38, T46, D87, and A145.

Chemical shift perturbations (CSPs) – To demonstrate that the solution conformation of the Met20 loop in each of the five complexes is predominantly closed, the approach described previously was used¹, in which CSPs were calculated for each complex relative to model Met20 loop closed (E:NADP⁺:folate, BMRB access. no. 5470) and occluded (E:5,6-dihydroNADPH:folate, BMRB access. no. 5471) chemical shifts². Of the ~20 resonances considered makers for the conformation of the Met20 loop in ¹H and/or ¹⁵N, all except V13 and I94 have chemical shifts more similar to a Met20 loop.
closed conformation (Supplementary Fig. 2c), consistent with our previously analysis of E:NADPH:1.

Site specific CSPs that result upon binding of each inhibitor (E:NADPH:antifolate – E:NADPH) confirm the ligand orientations observed in the crystal structures. The five inhibitors elicit both similar magnitudes of perturbations and patterns of residues affected (Supplementary Fig. 2d), with mild differences observed due to the specific location of the methyl substituent on the THQ ring. Using a box plot function, outlying CSPs were identified on a per residue basis for each inhibitor (Supplementary Fig. 2e). The majority of outliers are the same amongst the five ternary complexes, with one unique outlier in each complex due to the positioning of the methyl substituent (2 – F31, 3 – I50, and 4 – F31). Additionally, one residue in each complex (light blue spheres in Supplementary Fig. 2e) shows a perturbation in chemical shift, although not considered an outlier (2 – A29, 3 – G51, 4 – T35, and 6 – I94 is split in two). E:NADPH:5 does not possess any unique outliers, which is not surprising as it lacks a methyl substituent. The splitting of I94 in the presence of 6 may be due to switching motions of the protein and/or small molecule, or may also be due to the binding of both enantiomers of 6.

**Identity of sites with \( R_{ex} \)** – The increased number of residues experiencing backbone conformational exchange in the E:NADPH:6 complex are due in part to suspected motion of the Met20 loop. Several residues within the F-G and G-H loops known to undergo reliable changes in chemical shift upon switching of the loop from closed to occluded have been observed to possess slow timescale motion in the presence of 6 (Supplementary Fig. 4i). Although a crystal structure for E:NADPH:6 has not been determined, the structure of E:NADPH:3 serves as an excellent model (Fig. 2a). If one
envisions the methyl group positioned on C5 (instead of C6 in the structure), the methyl group likely comes within steric contact of the nicotinamide of NADPH in the active site. Unlike the switching motion of 1 characterized previously\(^1\), the methyl group on C5 does not sample an alternate binding pose that differs enough to move the group away from nicotinamide. This proposed steric clash between nicotinamide and the methyl group on C5 likely accounts for the significant reduction in binding affinity for 6 relative to the other tetrahydroquinazoline compounds.

As mentioned previously, protonated DHFR was used for relaxation dispersion experiments on E:NADPH:3. At the time these data were collected, protonated protein was deemed sufficient for analysis and not recollected using deuteration. It is possible, however, that additional residues with \(R_{ex}\) would be detected in E:NADPH:3 if highly deuterated protein were to be used in preparation of the complex. This could explain why an increase in the number of sites with slow motion is not seen in E:NADPH:3 relative to E:NADPH:MTX or E:NADPH:TMP\(^3\).

Residues undergoing slow timescale exchange can often move together, or in a concerted fashion, with shared \(k_{ex}\) and \(p_A\) values. Aside from the residues used in group fitting (see Methods in main text), DHFR is known to possess ligand-independent motions at regions containing residues 128-134 and 154-159\(^4\). Exchange at these residues was also identified in the current study, but the sites were excluded from group-based analysis of the data. These C-terminal residues have been group fitted together, and indeed prove to be ligand-independent in the case of bound inhibitors, as \(k_{ex}\) and \(p_A\) are identical for all complexes studied (Supplementary Results, Supplementary Table 3).
A correlation between $k_{\text{conf, forward}}$ and $k_{\text{off}}$ is shown in the main text. A similar exponential relationship also exists between $k_{\text{conf, reverse}}$ and $k_{\text{off}}$ ($R = 0.97$) (Supplementary Fig. 5a), although $k_{\text{conf, reverse}}$ is two orders of magnitude further from $k_{\text{off}}$ relative to $k_{\text{conf, forward}}$ (Supplementary Fig. 5c). The correlation between $k_{\text{ex}}$ and $k_{\text{off}}$ is lower ($R = 0.89$) (Supplementary Fig. 5b).

In order to compare to the average $\Delta \omega$ values fitted for the consensus antifolate residues with $R_{\text{ex}}$, the sign of $\Delta \omega$ was determined for the E:NADPH holoenzyme complex, as it has not been previously reported by Wright and colleagues\(^4\). In Supplementary Figure 4j, we report the sign for three sites in E:NADPH in comparison to our data on the current series of inhibitors. The sign for D11 is not reported for E:NADPH because we have been unable to assign that residue.

**X-ray Crystallography Methods.**

*Crystallization conditions* – For all three complexes, NADPH and inhibitor were present at three-fold molar excess relative to the concentration of DHFR. Briefly, E:NADPH:3 was crystallized via the hanging drop vapor diffusion method under the following conditions: 20 mg/mL DHFR, 20 mM imidazole at pH 8, 300 mM CaCl$_2$, and 30% PEG-6000. E:NADPH:4 was crystallized via the hanging drop vapor diffusion method under the following conditions: 20 mg/mL DHFR, 20 mM imidazole at pH 8, 325 mM CaCl$_2$, and 34% PEG-6000. E:NADPH:5 was crystallized via the sitting drop vapor diffusion method under the following conditions: 10 mg/mL DHFR, 20 mM imidazole at pH 8, 325 mM CaCl$_2$, and 34% PEG-6000. Each hanging or sitting drop was a mixture of 5 $\mu$L mother liquor and 5 $\mu$L of the prepared ternary complex in 20 mM imidazole.
Crystals were allowed to grow at room temperature for several days before harvesting. The mother liquor contained a high concentration of PEG-6000, which was sufficient as a cryoprotectant. To mount the crystals, 10 μL of mother liquor was added to the hanging or sitting drops. Mounted crystals were flash frozen in liquid nitrogen.

**Data collection** – Diffraction data were collected in-house at UNC using a RU300 rotating copper anode (Rigaku/MSC) and Saturn 944+ CCD detector at ~100 K. Data were processed using HKL2000⁵.

**Structure determination** – All three structures were determined using molecular replacement methods. The CCP4 program suite and the MR program Phaser was used⁶. The search model was *E. coli* DHFR bound to NADP⁺ in the C₂ space group (PDB code 1RA9). This search model did not possess a Met20 loop closed conformation. Manual model building was accomplished using Coot⁷. Final rounds of refinement used BUSTER and MOSFLM⁸⁻⁹. The placement of ligands was accomplished via examination of fo-fc difference maps generated in the absence of ligand. Atomic coordinates have been deposited in the PDB under access code 3R33, 3QYL, and 3QYO.

**Assay Methods.**

*Stopped-flow fluorescence measurements* – Briefly, E:NADPH:antifolate (20 μM DHFR, 400 μM NADPH, and 200 μM antifolate in NMR Buffer) was preformed and loaded into the drive syringe of the stopped-flow housing of a Fluorolog spectrofluorometer (Jobin Yvon Horiba, Inc). Methotrexate (MTX) (400 μM in NMR Buffer) was loaded in a second syringe. Intrinsic tryptophan fluorescence was excited at 290 nm while FRET emission (Trp to bound nicotinamide of NADPH) was monitored at
427 nm. Because MTX is a higher affinity inhibitor than 2-6, upon rapid mixing via the stopped-flow apparatus, MTX displaces 2-6, leading to a non-fluorescent ternary E:NADPH:MTX complex. The exponential decrease in fluorescence of E:NADPH:inhibitor to E:NADPH:MTX is fit to determine \( k_{\text{off}} \) for 2-6 (Supplementary Fig. 6)\(^{10}\). The experiments were repeated at a second MTX concentration (600 \( \mu \)M), as \( k_{\text{off}} \) is independent of the concentration of the trapping ligand. The reported \( k_{\text{off}} \) values are the average of multiple runs at both MTX concentrations. Reported errors are the standard deviations of all runs.
Supplementary Results.

Supplementary Table 1. Data collection and refinement statistics for 3R33, 3QYL, and 3QYO.

(A) E:NADPH:3*

| **Data Collection** | **3R33** |
|---------------------|----------|
| Space Group         | P2₁2₁2₁  |
| **Cell Dimensions** |          |
| a, b, c (Å)         | 34.1, 45.1, 97.7 |
| α, β, γ (°)         | 90.0, 90.0, 90.0 |
| Resolution          | 2.09 Å   |
| R_{merge} (%)       | 0.033 (0.044) |
| 1/σI                | 27.9 (17.0) |
| Completeness (%)    | 98.3 (98.9) |
| Redundancy          | 4.8 (3.7) |

| **Refinement**      |          |
| Resolution Range (Å)| 15.32-2.09 |
| No. reflections     | 9261 (2470) |
| R_{work} / R_{free} | .1812/.2447 |
| No. atoms           |            |
| Protein             | 1343      |
| Ligand/ion          | 81        |
| Water               | 177       |
| B-factors           |            |
| Protein             | 9.61      |
| Ligand/ion          | 17.31     |
| Water               | 17.33     |
| R.m.s. deviations   |            |
| Bond lengths (Å)    | 0.009     |
| Bond angles (°)     | 1.15      |
| **Ramanchandran**   |            |
| Residues in most favored regions | 156 |
| Residues in additional allowed regions | 1 |
| Residues in generously allowed regions | 0 |
| Residues in disallowed regions | 0 |

* Dataset was collected from a single crystal.

(B) E:NADPH:4*
### Data Collection

|                | 3QYL | 3QYO |
|----------------|------|------|
| **Space Group**| P2₁2₁2₁ | P2₁2₁2₁ |
| **Cell Dimensions** | | |
| \(a, b, c\) (Å) | 33.9, 44.8, 97.8 | 34.0, 45.1, 97.8 |
| \(\alpha, \beta, \gamma\) (°) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| **Resolution** | 1.79 Å | 2.09 Å |
| **\(R_{merge}\)(%)** | 0.123 (0.260) | 0.050 (0.056) |
| **\(I/\sigma I\)** | 15.5 (2.6) | 26.6 (13.2) |
| **Completeness (%)** | 98.3 (89.3) | |
| **Redundancy** | 9.2 (2.50) | |

### Refinement

|                | 3QYL | 3QYO |
|----------------|------|------|
| **Resolution Range (Å)** | 27.02-1.79 | |
| **No. reflections** | 14401 (728) | |
| **\(R_{work} / R_{free}\)** | .1749/.2088 | |
| **No. atoms** | | |
| Protein | 1314 | |
| Ligand/ion | 77 | |
| Water | 198 | |
| **\(B\)-factors** | | |
| Protein | 17.13 | |
| Ligand/ion | 16.36 | |
| Water | 26.89 | |
| **R.m.s. deviations** | | |
| Bond lengths (Å) | 0.010 | |
| Bond angles (°) | 1.14 | |
| **Ramachandran** | | |
| Residues in most favored region | 156 | |
| Residues in additional allowed regions | 1 | |
| Residues in generously allowed regions | 0 | |
| Residues in disallowed regions | 0 | |

* Dataset was collected from a single crystal.

(C) E:NADPH:5⁺
Completeness (%) 99.6 (99.1)
Redundancy 5.0 (3.9)

**Refinement**
Resolution Range (Å) 15.33-2.09
No. reflections 9409 (1303)
$R_{work} / R_{free}$ .1651/.2359
No. atoms
Protein 1315
Ligand/ion 68
Water 203

*B*-factors
Protein 9.57
Ligand/ion 11.21
Water 18.72

R.m.s. deviations
Bond lengths (Å) 0.010
Bond angles (°) 1.13

Ramanchandran
Residues in most favored regions 156
Residues in additional allowed regions 1
Residues in generously allowed regions 0
Residues in disallowed regions 0

* Dataset was collected from a single crystal.

**Supplementary Table 2.** $^{15}$N Relaxation dispersion fitted parameters for the series.

(A) E:NADPH:2

| Residue | $k_{ex} (s^{-1})$ | $p_B$ (%) | $\pm \Delta \omega$ | $|\Delta \omega|$ (ppm) | $\chi^2$ |
|---------|------------------|-----------|---------------------|----------------------|---------|
| 8       | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 1.787 ± 0.217        | 17.5    |
| 9       | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 2.406 ± 0.384        | 12.8    |
| 10      | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 0.782 ± 0.118        | 15.6    |
| 11      | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 2.734 ± 0.488        | 8.80    |
| 14      | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 1.353 ± 0.167        | 6.93    |
| 22$^a$  | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 1.983 ± 0.334        | 6.50    |
| 36      | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 0.554 ± 0.180        | 23.3    |
| 40      | 4782.4 ± 845.4   | 4.7 ± 1.7 | n.d.                | 1.657 ± 0.558        | 32.2    |
| 44      | 4782.4 ± 845.4   | 4.7 ± 1.7 | n.d.                | 1.761 ± 0.428        | 51.0    |
| 48      | 4782.4 ± 845.4   | 4.7 ± 1.7 | n.d.                | 1.849 ± 0.412        | 39.5    |
| 50      | 4782.4 ± 845.4   | 4.7 ± 1.7 | n.d.                | 1.932 ± 0.586        | 26.9    |
| 54      | 4782.4 ± 845.4   | 4.7 ± 1.7 | n.d.                | 1.735 ± 0.579        | 32.6    |
| 57      | 4782.4 ± 845.4   | 4.7 ± 1.7 | n.d.                | 1.815 ± 0.418        | 60.6    |
| 98      | 4782.4 ± 845.4   | 4.7 ± 1.7 | n.d.                | 1.261 ± 0.284        | 21.5    |
| 111     | 1658.5 ± 167.7   | 4.1 ± 1.0 | n.d.                | 0.835 ± 0.106        | 9.17    |
| Residue | $k_{ex}$ ($s^{-1}$) | $p_B$ (%) | ± Δω | Δω | χ² |
|---------|-------------------|----------|--------|-----|-----|
| 8       | 1041.4 ± 292.3    | 2 ± 0.5  | +      | 1.910 ± 0.500 | 7.94 |
| 9       | 1041.4 ± 292.3    | 2 ± 0.5  | -      | 2.787 ± 0.899 | 14.2 |
| 11      | 1041.4 ± 292.3    | 2 ± 0.5  | -      | 4.529 ± 2.006 | 12.6 |
| 22,a,b  | 2905.3 ± 965.4    | 3.7 ± 2.4 | -      | 1.028 ± 0.388 | 18.8 |
| 31      | 1041.4 ± 292.3    | 2 ± 0.5  | +      | 1.305 ± 0.434 | 11.9 |
| 111     | 1041.4 ± 292.3    | 2 ± 0.5  | +      | 1.276 ± 0.345 | 7.34 |
| 112     | 1041.4 ± 292.3    | 2 ± 0.5  | -      | 1.339 ± 0.307 | 10.1 |
| 113     | 1041.4 ± 292.3    | 2 ± 0.5  | -      | 0.686 ± 0.416 | 4.93 |

aMarker of Met20 loop switching, based on closed-to-occluded $^{15}$N CSP².
bLocal fit reported.

*(D) E:NADPH:5*
| Residue | $k_{ex}$ ($s^{-1}$) | $pB$ (%) | $\pm \Delta \omega$ | $|\Delta \omega|$ (ppm) | $\chi^2$ |
|---------|-----------------|----------|-----------------|------------------|--------|
| 8       | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 1.697 ± 0.350    | 9.48   |
| 9       | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 3.208 ± 1.254    | 5.10   |
| 10      | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 1.031 ± 0.251    | 11.7   |
| 11      | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 3.738 ± 1.467    | 6.86   |
| 22<sup>a</sup> | 1497.4 ± 384.9 | 2.1 ± 0.9| -               | 2.303 ± 0.650    | 8.54   |
| 29      | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 1.645 ± 0.606    | 13.2   |
| 31      | 1497.4 ± 384.9  | 2.1 ± 0.9| +              | 1.180 ± 0.351    | 8.81   |
| 36      | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 0.868 ± 0.446    | 6.39   |
| 40      | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 1.625 ± 0.421    | 17.6   |
| 48      | 1497.4 ± 384.9  | 2.1 ± 0.9| n.d. *         | 1.221 ± 0.282    | 11.9   |
| 50      | 1497.4 ± 384.9  | 2.1 ± 0.9| +              | 1.532 ± 0.507    | 21.8   |
| 54      | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 1.277 ± 0.618    | 17.3   |
| 58      | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 1.110 ± 0.331    | 21.3   |
| 96      | 1497.4 ± 384.9  | 2.1 ± 0.9| +              | 1.426 ± 0.350    | 32.4   |
| 111     | 1497.4 ± 384.9  | 2.1 ± 0.9| +              | 1.612 ± 0.402    | 12.1   |
| 112     | 1497.4 ± 384.9  | 2.1 ± 0.9| -               | 1.444 ± 0.292    | 11.9   |
| 113     | 1497.4 ± 384.9  | 2.1 ± 0.9| n.d.           | 1.205 ± 0.355    | 7.98   |

* n.d. – Sign was not determined for these residues, likely due to spectral overlap.
<sup>a</sup>Marker of Met20 loop switching, based on closed-to-occluded $^{15}$N CSP<sup>2</sup>.

(E) E:NADPH:6

| Residue | $k_{ex}$ ($s^{-1}$) | $pB$ (%) | $\pm \Delta \omega$ | $|\Delta \omega|$ (ppm) | $\chi^2$ |
|---------|-----------------|----------|-----------------|------------------|--------|
| 7       | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 1.885 ± 0.382    | 42.8   |
| 8       | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 2.356 ± 0.514    | 14.5   |
| 10      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 1.017 ± 0.443    | 16.8   |
| 11      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 4.676 ± 1.724    | 10.2   |
| 12      | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 1.303 ± 0.274    | 24.8   |
| 19      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 0.814 ± 0.406    | 23.0   |
| 30      | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 0.961 ± 0.562    | 12.5   |
| 36      | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 1.516 ± 0.344    | 14.2   |
| 37      | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 1.218 ± 0.340    | 15.0   |
| 40      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 1.922 ± 0.428    | 18.7   |
| 45      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 1.480 ± 0.320    | 29.0   |
| 49      | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 0.927 ± 0.236    | 22.2   |
| 54      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 1.150 ± 0.313    | 26.2   |
| 57      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 1.447 ± 0.308    | 18.0   |
| 59      | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 1.070 ± 0.360    | 11.9   |
| 98      | 1514.9 ± 206.0  | 2.3 ± 1.1| -               | 1.776 ± 0.453    | 16.1   |
| 104     | 1514.9 ± 206.0  | 2.3 ± 1.1| n.d. *         | 0.559 ± 0.517    | 8.29   |
| 111     | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 1.574 ± 0.347    | 26.1   |
| 112     | 1514.9 ± 206.0  | 2.3 ± 1.1| +              | 1.923 ± 0.408    | 28.0   |
| 115<sup>a</sup> | 1514.9 ± 206.0 | 2.3 ± 1.1| +              | 1.474 ± 0.361    | 12.4   |
| 116<sup>a</sup> | 1514.9 ± 206.0 | 2.3 ± 1.1| +              | 1.198 ± 0.256    | 24.6   |
| Compound | $k_{ex}$ (s$^{-1}$) | $p_A$ (%) | $k_{conf,forward}$ (s$^{-1}$) |
|----------|------------------|-----------|------------------|
| 2        | 585.0 ± 132.2    | 97.5 ± 0.2| 14.6 ± 3.3       |
| 3        | 509.8 ± 51.3     | 97.4 ± 0.2| 13.3 ± 1.4       |
| 4        | 647.4 ± 58.4     | 97.5 ± 0.1| 16.2 ± 1.5       |
| 5        | 542.9 ± 113.8    | 97.3 ± 0.3| 14.7 ± 3.1       |
| 6        | 389.6 ± 47.3     | 96.6 ± 0.3| 13.2 ± 1.7       |

**Supplementary Table 3.** Relaxation dispersion group fitted parameters of the ligand-independent C-terminal residues for all complexes.

| Compound | $k_{ex}$ (s$^{-1}$) | $p_A$ (%) | $k_{conf,forward}$ (s$^{-1}$) |
|----------|------------------|-----------|------------------|
| 2        | 585.0 ± 132.2    | 97.5 ± 0.2| 14.6 ± 3.3       |
| 3        | 509.8 ± 51.3     | 97.4 ± 0.2| 13.3 ± 1.4       |
| 4        | 647.4 ± 58.4     | 97.5 ± 0.1| 16.2 ± 1.5       |
| 5        | 542.9 ± 113.8    | 97.3 ± 0.3| 14.7 ± 3.1       |
| 6        | 389.6 ± 47.3     | 96.6 ± 0.3| 13.2 ± 1.7       |

**Supplementary Table 4.** Visually broadened residues in each complex.

(A) E:NADPH:2

| broadened                         | severely broadened                          |
|-----------------------------------|--------------------------------------------|
| 4, 6, 29, 30, 32, 35, 37, 46, 59, 64, 68, 94, 122, 123 | 7, 15, 17, 18, 23, 70, 97, 124 |

(B) E:NADPH:3

| broadened                         | severely broadened                          |
|-----------------------------------|--------------------------------------------|
| 10, 15, 17, 18, 27, 29, 30, 34, 35, 36, 40, 41, 46, 50, 58, 68, 92, 94, 95, 108, 122, 123 | 6, 7, 14, 23, 37, 59, 70, 87, 124 |

(C) E:NADPH:4

| broadened                         | severely broadened                          |
|-----------------------------------|--------------------------------------------|
| 6, 7, 10, 15, 18, 27, 32, 33, 34, 35, 36, 46, 47, 59, 68, 69, 94, 97, 99, 123 | 23, 30, 70, 124 |
(D) E:NADPH:5

| 6, 7, 14, 15, 18, 30, 35, 46, 47, 97, 103, 109, 123 | 59, 68, 69, 70, 124 |

(E) E:NADPH:6

| 34, 37, 103, 113 | 6, 9, 15, 18, 23, 31, 32, 46, 68, 70, 87, 94, 95, 97, 121, 122, 124, 148 |

**Supplementary Table 5.** Values of $k_{off,B}$ derived from gating model (see eq. 2 in main text).

| Compound | $k_{off,B}$ (s$^{-1}$) | error (s$^{-1}$) |
|----------|-----------------------|-----------------|
| MTX      | 0.0057                | 0.0031          |
| TMP      | 0.17                  | 0.09            |
| 1        | 1.54                  | 0.23            |
| 2        | 18.7                  | 3.3             |
| 3        | 11.6                  | 4.8             |
| 4        | 41.2                  | 7.6             |
| 5        | 179                   | 88              |
| 6        | 1900                  | 1180            |
Supplementary Figure Legends

**Supplementary Figure 1.** Slow timescale dynamics for previously studied antifolate ternary complexes. Sites along the backbone with detectable μs-ms motion are highlighted in colored spheres for (a) E:NADPH:MTX, (b) E:NADPH:TMP, and (c) E:NADPH:1. In (a-b), the black colored sphere indicates G121, at which no slow motion is observed in these complexes, relative to such motions in the holoenzyme. In (c), black colored sphere indicate residues with motions suggestive of a closed to occluded conformational switch of the Met20 loop while green colored spheres indicate residues that report on motion of the flexible thiophenyl ring of the inhibitor while bound to DHFR. Two binding conformations of 1’s thiophenyl ring are also shown in (c).

**Supplementary Figure 2.** Ligand electron density maps and chemical shift perturbations (CSPs) for the series. (a) Divergent stereoview of the electron density map (2fo-2fc) for the binding of 3 in E:NADPH:3. (b) Similar to (a), the electron density map (2fo-2fc) for the binding of 4 in E:NADPH:4. (c) CSPs of each E:NADPH:antifolate complex relative to model closed (E:NADP⁺:folate) and occluded (E:DH2NADPH:folate) complexes. Residues considered chemical shift markers of the conformation of the Met20 loop are highlighted in red. Closed red circles indicate that the E:NADPH:antifolate complex is more similar to a closed Met20 loop in solution, while open red circles indicated that the ternary complex is more similar to an occluded Met20 loop conformation. (d) CSPs for each E:NADPH:antifolate complex relative to the absence of inhibitor (E:NADPH). (h) A box plot function was used to identify outlying CSPs in (e), and these outliers have been highlighted with colored spheres for each complex. Light blue spheres indicate sites with significant CSPs that lie below the outlier threshold, yet are sensitive to small molecule inhibitor binding.

**Supplementary Figure 3.** 15N Relaxation dispersion curves for the series. (a-b) Relaxation dispersion curves generated from 700 (closed circles), 500 (open circles), and in several cases 600 MHz (stars) data are shown for residue 8 (a) and 111 (b), both within the antifolate consensus group. NADPH is abbreviated as NH. Standard errors were determined by peak intensity analysis of duplicate experiments for specific 1/τ_{cp} values. Data at 700 MHz for E:NADPH:2, E:NADPH:3, and E:NADPH:4 used a TROSY version of the relaxation dispersion experiment, hence the smaller R_{2eff} values than for 500 or 600 MHz data. (c-g) R_{2} relaxation dispersion curves of all residues with slow motion in the five ternary complexes studied. In (c) E:NADPH:2, (d)E:NADPH:3 and (f) E:NADPH:5, data collected at 700 (blue) and 500 (red) 1H frequencies are shown. For (e) E:NADPH:4 and (g) E:NADPH:6, 700 (green), 600 (blue), and 500 (red) MHz data are shown.

**Supplementary Figure 4.** Dynamic chemical shift analyses. (a-h) Attempts to correlate the loss of drug in the excited state of the antifolate consensus residues in the presence of each studied inhibitor are shown, where Δω is the dynamic 15N chemical shift change fitted from relaxation dispersion and Δδ is the steady-state difference (E:NADPH - E:NADPH:antifolate): (a) E:NADPH:MTX, (b) E:NADPH:TMP, (c) E:NADPH:1, (d) E:NADPH:2, (e) E:NADPH:3, (f) E:NADPH:4, (g) E:NADPH:5, and (h) E:NADPH:6.
A line with a slope of 1 is drawn on each plot, demonstrating the lack of correlation. At most, two residues out of the twelve could suggest loss of drug in the excited state. (i) Plot of $\Delta \omega$ vs. $\Delta \delta$ (occluded – closed) for Met20 loop marker residues in E:NADPH:6. A linear correlation is seen for the points in black ($R = 0.99$). Points in cyan do not lie along this line, but would be expected to if the correlation were very strong. (j) Comparison of average fitted E:NADPH:antifolate $\Delta \omega$ value to those fitted in physiological complexes. For the twelve consensus antifolate sites observed for the eight inhibitors characterized here, an average fitted $\Delta \omega$ value was calculated along with the standard deviation in that fitted value. Complexes that do not follow the pattern of sign for $\Delta \omega$ were excluded from the average. This average and standard deviation are plotted per residue against $\Delta \omega$ values fitted for physiological complexes studied previously. An asterisk indicates that the sign of $\Delta \omega$ was not determined. An absence of relaxation dispersion or broadening in a particular residues is denoted by “x”, and the presence of peak broadening (but no fitted dispersion curve) is denoted by “b”. Note that the pattern of sign and values of $\Delta \omega$ are distinct in the presence of the antifolates relative to endogenous, folate-derived ligands.

**Supplementary Figure 5.** The correlation of $k_{\text{off}}$ to $k_{\text{conf,reverse}}$ and $k_{\text{ex}}$. Red circles for TMP and 1 indicate $k_{\text{off}}$ values calculated based on $k_{\text{on}}$ for the series. Log-linear plots are shown for $k_{\text{off}}$ vs. $k_{\text{conf,reverse}}$ (a) and $k_{\text{ex}}$ (b). In these two panels, the open circles indicate the best fit for E:NADPH:4 despite a difference in the method of group fitting. In panel (c), the data from (a) and (b) are replotted together on a log-log plot.

**Supplementary Figure 6.** Sample exponential decay curve (compound 3) from stopped-flow fluorescence experiments.
Supplementary References

1. Carroll, M. J. et al. Direct detection of structurally resolved dynamics in a multi-conformation receptor-ligand complex. J Am Chem Soc, 133, 6422-6428 (2011).

2. Osborne, M. J., Venkitakrishnan, R. P., Dyson, H. J. & Wright, P. E. Diagnostic chemical shift markers for loop conformation and substrate and cofactor binding in dihydrofolate reductase complexes. Protein Sci, 12, 2230-2238 (2003).

3. Mauldin, R. V., Carroll, M. J. & Lee, A. L. Dynamic Dysfunction in Dihydrofolate Reductase Results from Antifolate Drug Binding: Modulations of Dynamics within a Structural State. Structure, 17, 386-394 (2009).

4. Boehr, D. D., McElheny, D., Dyson, H. J. & Wright, P. E. The dynamic energy landscape of dihydrofolate reductase catalysis. Science, 313, 1638-1642 (2006).

5. Otwinowski, Z. & Minor, W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. Methods Enzymol, 276, 307-326 (1997).

6. The CCP4 suite: programs for protein crystallography, 1994.

7. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr, 60, 2126-2132 (2004).

8. Leslie, A. G. The integration of macromolecular diffraction data. Acta Crystallogr D Biol Crystallogr, 62, 48-57 (2006).

9. Bricogne, G. et al.; 2.8.0. ed. Cambridge, United Kingdom: Global Phasing Ltd., 2009.

10. Fierke, C. A., Johnson, K. A. & Benkovic, S. J. Construction and evaluation of the kinetic scheme associated with dihydrofolate reductase from Escherichia coli. Biochemistry, 26, 4085-4092 (1987).
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3 (1/3)
Supplementary Figure 4
Supplementary Figure 5
