Supplementary Information

Force Spectroscopy of Single Proteins. All single-molecule force measurements were performed on a custom-built atomic force microscope. Calibration of cantilevers was done in solution by using the equipartition theorem (1,2). This method provides a resolution in force of ≈10%. Gold-coated cantilevers (Bio-Levers, Olympus, Tokyo) with a spring constant of ~7 pN/nm and a resonance frequency of ~2.5 kHz were used. The protein solution (concentration ≈0.5 g/liter) was incubated with the substrates for 5 min at romm temperature. For the measurements, ≈20 µl of the protein solution (PBS buffer, pH 7.4) were applied on a freshly evaporated gold surface. Final substrate concentrations were ~20µM for MTX and ~1mM for NADPH. The force curves on the Ddfilamin-DHFR construct were collected at a pulling speed of 1,000 nm/s. All experiments were conducted at room temperature. For each substrate condition, several experiments were pooled.

Determination of Unfolding Forces. Unfolding forces were determined by picking the maximum in a predefined section around the expected unfolding position of a force-extension curve. A minimum force threshold was set at 30 pN. Unfolding forces are systematically shifted to higher forces due to the thermal noise error when picking the maximum. In order to compensate this effect, forces were shifted towards lower forces by 2σ of the thermal force noise.

To correct for the 10% error in force calibration, the DHFR unfolding forces were rescaled by taking into account the Ddfilamin unfolding forces. The Ddfilamin forces should not be affected by substrate binding. The quotient of the average Ddfilamin force in one experiment divided by the average Ddfilamin force of all experiments was taken as a force correction factor: $F_{DHFR}^{corr} = F_{DHFR} \cdot \frac{\overline{F}_{DdFLN}^{one\,\exp}}{\overline{F}_{DdFLN}^{all\,\exp}}$. Only experiments that yielded more than 20 Ddfilamin force values were included in the evaluation. This method led to slight force corrections.

Force vs. Lifetime. While a determination of the unfolding forces is the appropriate way to analyze the transition from folded DHFR to the intermediate state, the unfolding forces of the intermediate state do not yield information on the mechanical stability of the intermediate. This is due to the fact that the force to which the intermediate is exposed depends on the previous unfolding of the native state (fig.1). Moreover, the force does not increase significantly during the relatively short lifetime of the intermediate. Thus, intermediate state lifetimes at approximately constant force were measured instead of unfolding forces in order to analyze the intermediate state (3).

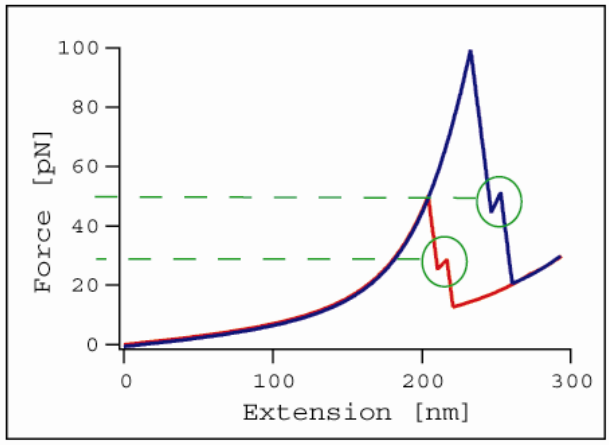

Figure 1. Simulated curves that show unfolding events of the native state at 50 pN (red) and at 100 pN (blue). The intermediate states are exposed to different forces (green circles) that stay approximately constant during the intermediate state lifetime.

Monte Carlo Simulations. Our measurements resulted in unfolding force distributions and lifetime distributions. They were interpreted with a linear two-state model (4). The two parameters that describe mechanical unfolding are the unloaded lifetime τ_0 and the potential width, Δx, of a conformational state. A common method to include exact experimental conditions into an analysis of such distributions is to conduct Monte Carlo simulations. In such a simulation, we stretch a virtual polypeptide of contour length L with pulling speed v_p, starting at extension $d = 0$. This calculation leads to an additional extension $\Delta d = v_p \cdot \Delta t$ in every time step, Δt. After every time step, we calculate the acting force for the current extension d by means of the WLC interpolation introduced by Bustamante *et al.* (5), $F(d) = (k_B T/p)\cdot(d/L + 0.25\cdot(1 - d/L)^{-2} - 0.25)$. To simulate the conformational kinetics of a

contained structure able to transition into another conformation with contour length contribution ΔL, we calculate a transition rate given by $k_{off}(F) = \exp(F \cdot \Delta x / k_B T)/\tau_0$ within the linear two-state model. This rate is calculated at every time step for the actual acting force. The probability for a transition at a certain time step is then given by $\Delta P = k_{off}(F) \cdot \Delta t$. Then we compare this number with a computer-generated random number within the interval [0,1] (2^{32} distinct values; periodicity, $>10^{18}$). If ΔP is greater than this random number, the simulation decides for transition and the parameter contour length, L, is increased by ΔL. The properties of a cantilever of spring constant k_c are simulated by including a relaxation phase with force-extension characteristics of $F_{relax} = -k_c \cdot d$ throughout the next timesteps until F_{relax} equals the force produced by a polypeptide of contour length $L + \Delta L$ at the current extension. Subsequent transitions (corresponding, for instance, to the unfolding of intermediate states) can now be included in the same way into the simulation. In general, we would have to include probabilities for back reactions (by means of $k_{on}(F)$ transition rates) into the simulation. In our experimental situation, these rates are negligible.

Analysis of Intermediate State Stability. In order to analyze the effect of substrate binding on the stability of the intermediate state quantitatively, we conducted Monte Carlo simulations and calculated the differences of experimental and simulated data by means of a chi square analysis. In the simulations, we used the experimental force distribution for the transition from folded DHFR to the intermediate to calculate the forces to which the intermediate is exposed. We then compared these simulated lifetime distributions for the intermediate state with the measured ones and adjusted the parameters Δx and τ_0 in such a way that best matching between the two distributions was observed by chi square analysis. The errors for the lifetimes at zero force were calculated with a 75% confidence interval for the chi square analysis.

UV Detection of Substrate Binding. Binding of MTX to the Ddfilamin-DHFR construct was monitored by the characteristic spectroscopic UV shift of DHFR upon MTX binding (fig.2) (6).

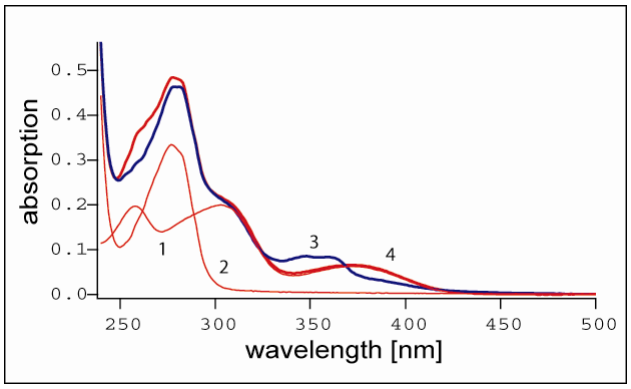

Figure 2. 1. MTX spectrum. 2. Spectrum of the Ddfilamin-DHFR construct. 3. Spectrum of DDfilamin-DHFR with MTX (molar ratio ~ 1:1) 4. Sum of spectra 1 and 2. The absorption shift in the region of 340-400 nm is characteristic for MTX binding.

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