Supporting Information

Isom et al. 10.1073/pnas.0910421107

SI Text

Fabricated Synthetic Gene Sequences (ORFs). ***SN wild-type.***
CGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAACTTCTACTAAAAAATTACATAAAGAACCAGCAACTTTAATAAAAGCAATCGATGGCGATACCGTCAAACTGATGTACAAAGGCCAGCCGATGACCTTTAGACTGCTTCTGGTCGATACCCCTGAAACCAAACACCCGAAGAAAGGCGTGGAAAAATACGGTCCGGAAGCATCAGCGTTCACCAAGAAGATGGTCGAAAACGCGAAGAAGATCGAGGTAGAATTCGACAAAGGCAACCGCACGGATAAATACGGTCGTGGTCTGGCATACATCTATGCGGACGGCAAAATGGTGAACGAAGCACTGGTACGTCAAGGTCTGGCAAAAGTCGCATACGTGTACAAACCGAACAACACCCACGAACAGCATCTGCGTAAAAGCGAAGCACAGGCGAAAAAGGAGAAGCTGAACATCTGGAGCGAAGATAACGCAGATAGTGGCCAAGGAGGCTCGGACTATAAAGACGACGACGACAAGTAATAAGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGG

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Derivation of ΔG_{bind} for Proteins with Single and Multiple Ligand-Binding Sites. For a macromolecule with a single binding site, the relative concentrations of unliganded and liganded species is described by the binding polynomial

$$P = \left(1 + \frac{[L]}{K_D}\right). \quad \textbf{[S1]}$$

The expression for a single-site binding isotherm is derived from the differentiation of $\ln(P)$ with respect to $\ln(L)$ using the chain-rule

$$\bar{X} = \frac{d\ln P}{d\ln L} = \frac{d\ln P}{dP}\cdot\frac{dP}{dL}\cdot\frac{dL}{d\ln L} = \frac{K_A[L]}{1+K_A[L]} = \frac{\frac{[L]}{K_D}}{1+\frac{[L]}{K_D}}, \quad \textbf{[S2]}$$

where $\bar{X}$ is the number of moles of ligand bound per mole of macromolecule and K_A and K_D are the relevant equilibrium constants for the law of mass action

$$M + L \rightleftarrows ML; \quad \textbf{[S3]}$$

$$K_A = \frac{[ML]}{[M][L]}; \quad \textbf{[S4]}$$

$$K_A = \frac{1}{K_D}. \quad \textbf{[S5]}$$

$\bar{X}$ expressed in differential form provides the conceptual link between a ligand-binding isotherm and the free energy of ligand binding (ΔG_{bind}); the area underneath a plot of $\bar{X}$ versus $\ln(L)$ is proportional to the free energy of binding. ΔG_{bind} is obtained by integrating the area under the binding isotherm (i.e. by integrating Eq. **S2**) and multiplying by the proportionality constant RT

$$\Delta G_{\text{bind}} = RT \int \bar{X} d\ln L = RT \int d\ln P = RT \ln P + \Delta G_{\text{ref}}, \quad \textbf{[S6]}$$

where ΔG_{ref} is the reference free energy of the macromolecule in the absence of ligand. In the specific case of a single binding site, Eq. **S6** is expressed as

$$\Delta G_{\text{bind}} = RT \ln P = RT \ln\left(1 + \frac{[L]}{K_D}\right) + \Delta G_{\text{ref}}. \quad \textbf{[S7]}$$

In cases where a macromolecule binds more than one ligand, the expression for ΔG_{bind} is more complex. Consider for example the binding of Ca^{2+} and pdTP (a nucleotide inhibitor) to the enzyme *Staphylococcal* nuclease (SN). Reaction schemes **S8** thru **S13** describe the formation of the relevant binary and ternary complexes of an enzyme (E) combined with ligands A (Ca^{2+}) and B (pdTP).

$$E + A \rightleftarrows EA; \qquad K_1 = \frac{[EA]}{[E][A]}; \qquad K_1 = 90\ \mu\text{M}. \quad \textbf{[S8]}$$

$$E + B \rightleftarrows EB; \qquad K_2 = \frac{[EB]}{[E][B]}; \qquad K_2 = 500\ \mu\text{M}. \quad \textbf{[S9]}$$

$$EA + B \rightleftarrows EAB; \qquad K_3 = \frac{[EAB]}{[EA][B]}; \qquad K_3 = 20\ \mu\text{M}. \quad \textbf{[S10]}$$

$$EB + A \rightleftarrows EAB; \qquad K_4 = \frac{[EAB]}{[EB][A]}; \qquad K_4 = 2.5\ \mu\text{M}. \quad \textbf{[S11]}$$

$$E + AB \rightleftarrows EAB; \qquad K_5 = \frac{[EAB]}{[E][AB]}; \qquad K_5 = 2.2\ \mu\text{M}. \quad \textbf{[S12]}$$

$$A + B \rightleftarrows AB; \qquad K_6 = \frac{[AB]}{[A][B]}. \quad \textbf{[S13]}$$

The binding of Ca^{2+} and pdTP to SN is fully described by

$$P = 1 + \frac{[A]}{K_1} + \frac{[B]}{K_2} + \frac{[A][B]}{K_1 K_3} + \frac{[B][A]}{K_2 K_4} + \frac{[AB]}{K_5}, \quad \textbf{[S14]}$$

and ΔG_{bind} is described by

$$\Delta G_{\text{bind}} = RT \ln\left(1 + \frac{[A]}{K_1} + \frac{[B]}{K_2} + \frac{[A][B]}{K_1 K_3} + \frac{[B][A]}{K_2 K_4} + \frac{[AB]}{K_5}\right) + \Delta G_{\text{ref}}. \quad \textbf{[S15]}$$

It has been shown that Ca^{2+} and pdTP bind synergistically to SN. Consequently, the dissociation constant K_5 can be determined independent of the dissociation constants K_1, K_2, K_3, and K_4 by measuring the ligand binding energetics of SN at concentrations of $[AB]$ below K_1, K_2, and K_3 (i.e., 3–24 μM). This approach simplifies Eq. **S15** considerably,

$$\Delta G_{\text{bind}} = RT \ln\left(1 + \frac{[AB]}{K_5}\right). \quad \textbf{[S16]}$$

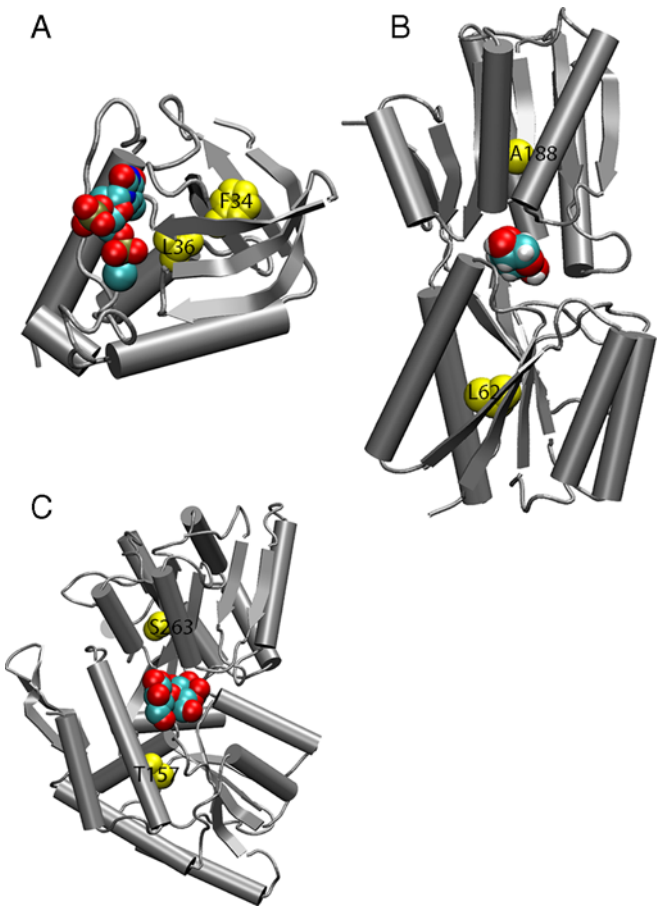

Fig. S1. Cysteine substitution sites in the hydrophobic core of *Staphylococcal* nuclease (*A*; pdb code 1SNC), *E. coli* ribose-binding protein (*B*; pdb code 2DRI), and *E. coli* maltose-binding protein (*C*; pdb code 1ANF).

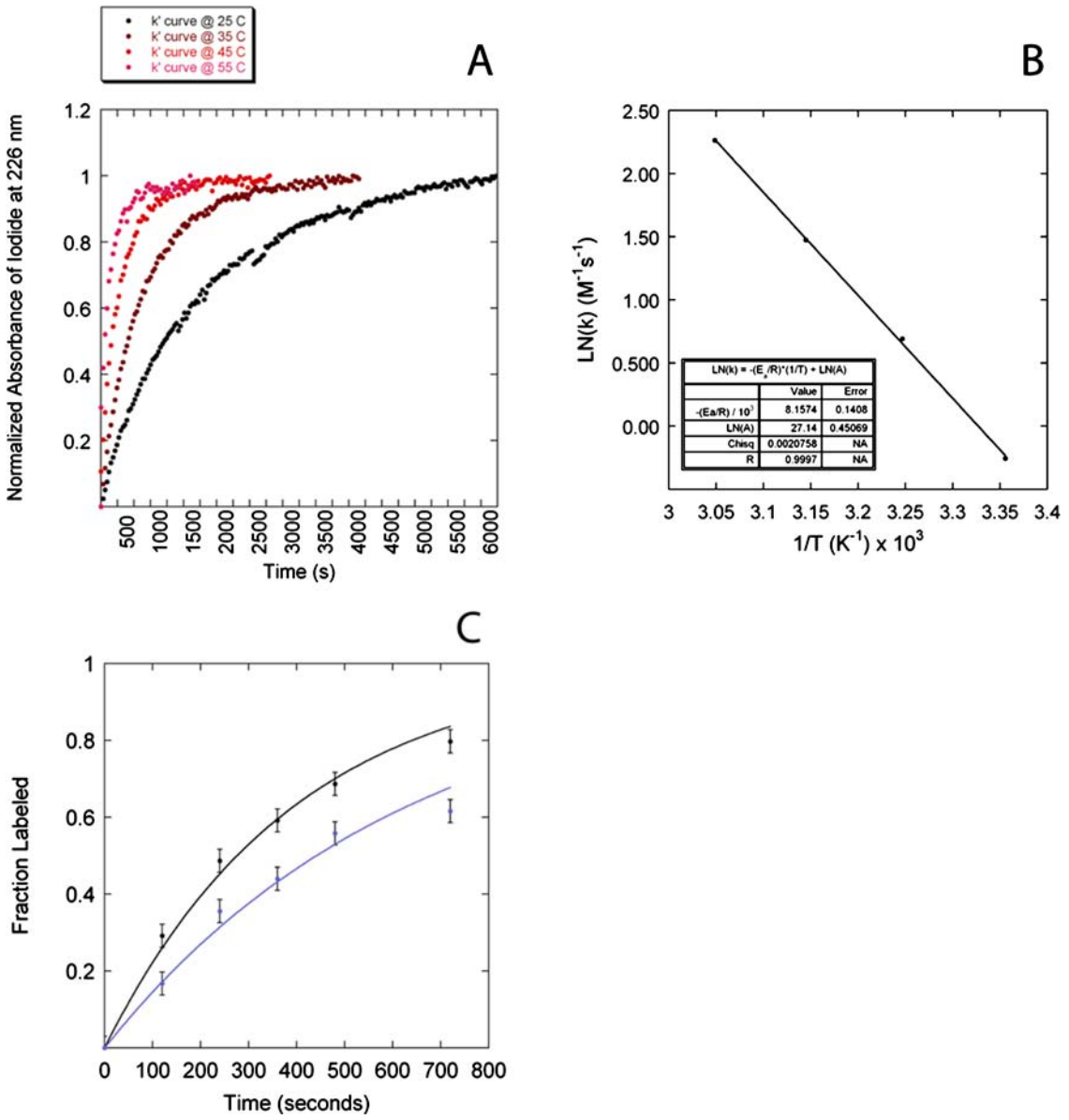

Fig. S2. Determination of k_{int} in model compounds and unfolded SN. (*A*) Progress curves for the reaction of 80 μM IAM-biotin with 800 μM reduced *L*-glutathione (GSH) at different temperatures in 25 mM MOPS, 100 mM KCl, and pH 7.6. Pseudo-first-order rate constants (k_{int}) derived from these data: 6.2×10^{-4} (25 °C), 1.6×10^{-4} (35 °C), 3.5×10^{-3} (45 °C), and 7.7×10^{-3} (55 °C) s^{-1}; second-order rate constants (i.e. $k = k_{int}/[GSH]$) of 7.8×10^{-1}, 2.0, 4.4, and 9.6 $M^{-1}\ s^{-1}$, respectively. (*B*) Arrhenius plot of the second-order rate constants for the bimolecular reaction of IAM-biotin and GSH. (*C*) Representative QCR curves at 50 °C for unfolded SN variants L36C (purple; $k_{int} = 3 \times 10^{-3}\ s^{-1}$) and F34C (black; $k_{int} = 2 \times 10^{-3}\ s^{-1}$) at 438 μM IAM-biotin, ~0.1 μM protein, 25 mM MOPS, 100 mM KCl, and pH 7.6.

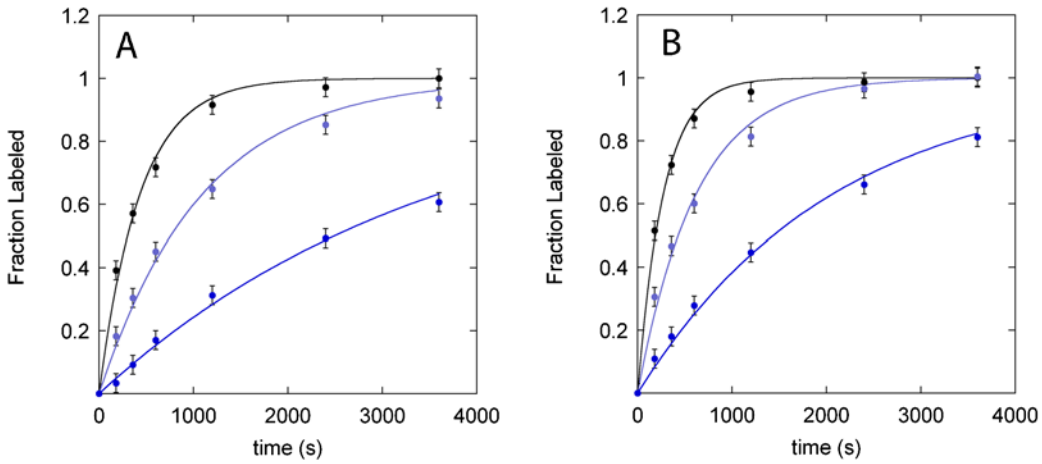

Fig. S3. An illustrative test of EX2 conditions for SN variants F34C and L36C at 38.3 °C. The change in observed rate constant for labeling of these protected cysteines is proportional to the change in concentration of IAM-biotin (black: 3 mM; lighter blue: 1.1 mM; darker blue: 0.3 mM). k_{label} at 3, 1.1, and 0.3 mM IAM-biotin for Cys-34: 2.3×10^{-3} s^{-1}, 9.2×10^{-4} s^{-1}, and 2.8×10^{-4} s^{-1}, respectively; for Cys-36: 3.0×10^{-3} s^{-1}, 1.0×10^{-3} s^{-1}, and 3.5×10^{-4} s^{-1}, respectively.

Table S1. Temperature dependence of ΔG_U for variants of SN and ecRBP

Variant	T °C	[IAM] [M]	k_{int} s^{-1}	k_{ex} s^{-1}	ΔG kcal mol^{-1}
SN.F34C	26.3	3.16E-03	2.82E-03	6.10E-05	2.3
SN.F34C	29.3	3.16E-03	3.69E-03	1.10E-04	2.1
SN.F34C	32.3	1.00E-03	1.53E-03	8.00E-05	1.8
SN.F34C	35.3	1.00E-03	1.98E-03	2.60E-04	1.2
SN.F34C	38.3	1.00E-03	2.55E-03	7.20E-04	0.6
SN.F34C	40.3	1.00E-03	3.02E-03	1.30E-03	0.2
SN.L36C	23.3	3.16E-03	2.14E-03	3.10E-05	2.5
SN.L36C	26.3	3.16E-03	2.82E-03	7.20E-05	2.2
SN.L36C	29.3	3.16E-03	3.70E-03	1.50E-04	1.9
SN.L36C	32.3	1.00E-03	1.53E-03	1.20E-04	1.5
SN.L36C	35.3	1.00E-03	1.98E-03	3.60E-04	0.9
SN.L36C	38.3	1.00E-03	2.55E-03	9.20E-04	0.4
ecRBP.L62C	44.5	1.00E-03	4.26E-03	2.00E-04	1.9
ecRBP.L62C	45.2	1.00E-03	4.50E-03	2.60E-04	1.8
ecRBP.L62C	47.1	1.00E-03	5.25E-03	5.20E-04	1.4
ecRBP.L62C	48.9	1.00E-03	6.05E-03	9.30E-04	1.1
ecRBP.L62C	51.7	1.00E-03	7.53E-03	1.90E-03	EX1, 0.7
ecRBP.L62C	54.6	1.00E-03	9.19E-03	2.60E-03	EX1, 0.6
ecRBP.A188C	45.2	1.00E-03	4.50E-03	1.10E-04	2.3
ecRBP.A188C	47.1	1.00E-03	5.25E-03	1.80E-04	2.1
ecRBP.A188C	48.9	1.00E-03	6.05E-03	5.10E-04	1.5
ecRBP.A188C	51.7	1.00E-03	7.53E-03	1.50E-03	0.9
ecRBP.A188C	54.6	1.00E-03	9.19E-03	3.70E-03	0.3

Table S2. Ligand dependence of ΔG_U for variants of SN, ecRBP, and ecMBP

Variant	[Ligand] μM	T °C	[IAM] [M]	k_{int} s^{-1}	k_{ex} s^{-1}	ΔG kcal mol^{-1}	$\Delta\Delta G$ kcal mol^{-1}
SN.F34C	0	35.3	1.00E-03	1.98E-03	2.62E-04	1.2	0
SN.F34C	3	35.3	1.00E-03	1.98E-03	1.82E-04	1.4	0.3
SN.F34C	6	35.3	1.00E-03	1.98E-03	1.35E-04	1.6	0.5
SN.F34C	12	35.3	1.00E-03	1.98E-03	8.60E-05	1.9	0.7
SN.F34C	24	35.3	1.00E-03	1.98E-03	4.60E-05	2.3	1.1
SN.L36C	0	35.3	1.00E-03	1.98E-03	3.60E-04	0.9	0
SN.L36C	3	35.3	1.00E-03	1.98E-03	1.80E-04	1.4	0.5
SN.L36C	6	35.3	1.00E-03	1.98E-03	1.30E-04	1.6	0.7
SN.L36C	12	35.3	1.00E-03	1.98E-03	6.10E-05	2.1	1.2
SN.L36C	24	35.3	1.00E-03	1.98E-03	3.40E-05	2.5	1.6
ecRBP.L62C	0	48.9	1.00E-03	6.05E-03	9.90E-04	1.0	0
ecRBP.L62C	1.5	48.9	1.00E-03	6.05E-03	5.10E-04	1.5	0.5
ecRBP.L62C	3	48.9	1.00E-03	6.05E-03	3.60E-04	1.8	0.7
ecRBP.L62C	6	48.9	1.00E-03	6.05E-03	1.90E-04	2.2	1.2
ecRBP.L62C	12	48.9	1.00E-03	6.05E-03	1.10E-04	2.6	1.5
ecRBP.L62C	24	48.9	1.00E-03	6.05E-03	9.10E-05	2.7	1.6
ecRBP.A188C	0	54.6	1.00E-03	9.40E-03	2.17E-03	0.8	0
ecRBP.A188C	1.5	54.6	1.00E-03	9.40E-03	1.41E-03	1.1	0.3
ecRBP.A188C	3	54.6	1.00E-03	9.40E-03	9.70E-04	1.4	0.6
ecRBP.A188C	6	54.6	1.00E-03	9.40E-03	7.30E-04	1.6	0.8
ecRBP.A188C	12	54.6	1.00E-03	9.40E-03	3.20E-04	2.2	1.4
ecRBP.A188C	24	54.6	1.00E-03	9.40E-03	1.90E-04	2.5	1.7
ecMBP.T157C	0	63.3	1.00E-03	1.79E-02	5.10E-04	2.4	0
ecMBP.T157C	6	63.3	1.00E-03	1.79E-02	2.50E-04	2.9	0.5
ecMBP.T157C	12	63.3	1.00E-03	1.79E-02	1.80E-04	3.1	0.7
ecMBP.T157C	24	63.3	1.00E-03	1.79E-02	1.30E-04	3.3	0.9
ecMBP.T157C	48	63.3	1.00E-03	1.79E-02	9.10E-05	3.5	1.2
ecMBP.S263C	0	63.3	1.00E-03	1.79E-02	5.30E-04	2.3	0
ecMBP.S263C	6	63.3	1.00E-03	1.79E-02	3.50E-04	2.6	0.3
ecMBP.S263C	12	63.3	1.00E-03	1.79E-02	2.60E-04	2.8	0.5
ecMBP.S263C	24	63.3	1.00E-03	1.79E-02	1.60E-04	3.2	0.8
ecMBP.S263C	48	63.3	1.00E-03	1.79E-02	1.20E-04	3.4	1.0