Title: A simple model for determining affinity from irreversible thermal shifts

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Supplemental Information

Extended derivations

$\Delta G_L = -RTln(Q)$ (1)

$\because \quad Q = \frac{[F]}{[F]} + \frac{[FL]}{[F]} = 1 + \frac{[L]}{K_D}$

$\Delta G_L = RTln\left(1 + \frac{[L]}{K_D}\right)$ (2)

where T is in Kelvin, and is fixed at the temperature at which the KD was determined (*eg*, 298 K).

$\Delta^L G_u - \Delta G_u = \Delta^u G_L - \Delta G_L$ (3)

$k = Ae^{-Ea_1/RT}$ (4)

$Ea = \Delta G + \Delta G‡$ (5)

$A_1 \cong A_2$ (6)

$\Delta T_M = T_{M,bound} - T_{M,apo}$ (7)

$k_{U1} = A_1 e^{\left(\frac{-Ea_1}{RTm,apo}\right)}$ (8)

$k_{U2} = A_2 e^{\left(\frac{-Ea_2}{R(Tm,apo+\Delta Tm)}\right)}$ (9)

$\because$ k_{U1} at $T_{M,apo}$ is approximately equal to k_{U2} at $T_{M,bound}$ (see equations (25-28), **SI Fig. 1** and **SI Fig 2**)

$$1 = \frac{k_{U1}}{k_{U2}} = \frac{A_1}{A_2} \frac{e^{\left(\frac{-Ea_1}{RTm,apo}\right)}}{e^{\left(\frac{-Ea_2}{R(Tm,apo+\Delta Tm)}\right)}} = e^{\left(\frac{Ea_2}{R(Tm,apo+\Delta Tm)}\right) - \left(\frac{Ea_1}{RTm,apo}\right)} \quad (10)$$

$\because \quad ln(e^{(x)}) = x \quad and \quad ln(1) = 0$

$$\frac{Ea_2}{R(Tm_{,apo}+\Delta Tm)} = \frac{Ea_1}{RTm_{,apo}} \quad (11)$$

$$Ea_2 = \frac{Ea_1 R(Tm_{,apo}+\Delta Tm)}{RTm_{,apo}} = Ea_1\left(1 + \frac{\Delta Tm}{Tm_{,apo}}\right) = Ea_1 + Ea_1\left(\frac{\Delta Tm}{Tm_{,apo}}\right) \quad (12)$$

$$\Delta G_U = Ea_1 - \Delta G_U‡ \quad (13)$$

$$\Delta^L G_U = Ea_2 - \Delta^L G_U‡ \quad (14)$$

Substitute equation (13) and (14) into equation (3):

$$(Ea_2 - \Delta^L G_U‡) - (Ea_1 - \Delta G_U‡) = \Delta^U G_L - \Delta G_L \quad (15)$$

Substitute equation (12) into equation (15) and simplify:

$$Ea_1\left(\frac{\Delta Tm}{Tm_{,apo}}\right) + \Delta G_U‡ - \Delta^L G_U‡ = \Delta^U G_L - \Delta G_L \quad (16)$$

$$\Delta G_U‡ \cong \Delta^L G_U‡ \quad (17)$$

$$Ea_1\left(\frac{\Delta Tm}{Tm_{,apo}}\right) = \Delta^U G_L - \Delta G_L \quad (18)$$

Since ligand does not bind to unfolded protein:

$$Ea_1\left(\frac{\Delta Tm}{Tm_{,apo}}\right) = -\Delta G_L \quad (19)$$

Substitute equation (2) into equation (19), followed by rearrangement gives:

$$-RTln\left(1 + \frac{[L]}{K_D}\right) = RTln\left(\frac{K_D}{K_D+[L]}\right) = -Ea_1\left(\frac{\Delta Tm}{Tm_{,apo}}\right) \quad (20)$$

Experimentally, the apparent ΔTM ($\Delta TM_{,APP}$), will be the sum of the ligand binding to the site of interest (ΔTM), plus any additional extra-site binding (ΔTM_X):

$\Delta Tm_{,APP} = \Delta Tm + \Delta Tm_X$ (21)

Combining equations (20) and (21) we find:

$$\Delta Tm_{,APP} = -\left(\frac{Tm_{,apo}RT}{Ea_1}\right)ln\left(\frac{K_{D,app}}{K_{D,app}+[L]}\right) = -\left(\frac{Tm_{,apo}RT}{Ea_1}\right)\left(ln\left(\frac{K_D}{K_D+[L]}\right) + ln\left(\frac{K_{Dx}}{K_{Dx}+[L]}\right)\right) \quad (22)$$

where $K_{D,APP}$ is the apparent affinity, and K_{Dx} is the affinity of extra-site binding. After rearrangement and simplification we find:

$$ln\left(\frac{K_D}{K_D+[L]}\right) = -\left(\frac{Ea_1}{RT}\right)\left(\frac{\Delta Tm_{,APP}}{Tm_{,apo}}\right) - ln\left(\frac{K_{Dx}}{K_{Dx}+[L]}\right) \quad (23)$$

where *T* is the temperature at which the K_D was determined (*eg*, 298 K).

$\because \quad ln\left(\frac{K_D}{K_D+[L]}\right) \cong ln\left(\frac{K_D}{[L]}\right)$ when $[L] \gg K_D$

$$ln\left(\frac{K_D}{[L]}\right) = -\left(\frac{Ea_1}{RT}\right)\left(\frac{\Delta Tm_{,APP}}{Tm_{,apo}}\right) - ln\left(\frac{K_{Dx}}{K_{Dx}+[L]}\right) \quad (24)$$

Extended derivations for value of k_{U1} at $Tm_{,apo}$ and k_{U2} at $Tm_{,bound}$

Beginning at equation (10), without assuming the ratio of the unfolding rate constants of apo and bound is unity, $\frac{ku_1}{ku_2}$ =1, substitution with equation (7) will yield:

$$\frac{Ea_2}{RTm_{,bound}} = \frac{Ea_1}{RTm_{,apo}} + ln\left(\frac{ku_1}{ku_2}\right) \quad (25)$$

Rearrangement of equation (25) gives:

$$Ea_2 = \frac{Ea_1 Tm_{,bound}}{Tm_{,apo}} + RTm_{,bound}ln\left(\frac{ku_1}{ku_2}\right) \quad (26)$$

From Sanchez-Ruiz *et al.* (Sanchez-Ruiz et al. 1988) we get the equivalence:

$$\frac{vEa_1}{RTm_{,apo}{}^2} = kU_1 = A_1 e^{-Ea_1/RTm_{,apo}} \quad (27)$$

where v is the heating rate of the irreversible unfolding experiment. If the apo and the bound experiments are collected at the same heating rate, and $A_1 \cong A_2$, the ratio of the rate constants at their TM temperatures is:

$$\frac{ku_1}{ku_2} = \frac{\left(\frac{Ea_1}{RTm_{,apo}{}^2}\right)}{\left(\frac{Ea_2}{RTm_{,bound}{}^2}\right)} = \frac{\left(\frac{Ea_1}{RTm_{,apo}{}^2}\right)}{\left(\frac{\frac{Ea_1 Tm_{,bound}}{Tm_{,apo}} + RTm_{,bound} ln\left(\frac{ku_1}{ku_2}\right)}{RTm_{,bound}{}^2}\right)} = \frac{Ea_1 Tm_{,bound}}{Ea_1 Tm_{,apo} + RTm_{,apo}{}^2 \ln\left(\frac{ku_1}{ku_2}\right)} \quad (28)$$

At the condition of $T_{M,apo} = T_{M,bound}$, $\Delta T_M = 0$, $\frac{ku_1}{ku_2} = 1$, therefore $\ln\left(\frac{ku_1}{ku_2}\right) = 0$. Starting at $\Delta T_M = 0$, steps can be taken through a range of ΔT_M values using an estimate of $\ln\left(\frac{ku_1}{ku_2}\right)$ from the prior ΔT_M step (*eg*, to approximate $\frac{ku_1}{ku_2}$ at $\Delta T_M = 1$, the $\ln\left(\frac{ku_1}{ku_2}\right)$ value from $\Delta T_M = 0$ is used). In this way it can be demonstrated that under typical protein unfolding conditions (*eg*, TM between 50-70 °C), kU_1 at $T_{M,apo}$ is approximately equal to kU_2 at $T_{M,bound}$ (**SI Fig 1**). Similarly, using a numerical integration approach, a simulation of unfolding can be made which also shows kU_1 at $T_{M,apo}$ is approximately equal to kU_2 at $T_{M,bound}$ (**SI Fig 2**). Please note that since refolding is negligible in this model, the rates constants of folding are not considered; if you have been reading this extension of the derivation as a loop-out from the main or supplemental text assertion of rate equivalence of kU_1 at $T_{M,apo}$ and kU_2 at $T_{M,bound}$, you may now return to the rest of the derivation.

Extended discussion on simulations

In our laboratory experiments, we observed a ligand-dependent ΔTM shift beyond what can be explained by initial occupancy. One possibility is that the effect is due to unfolding through the apo unfolding pathway, which favors net ligand dissociation; thus, the ligand-dependent ΔTM is due to kinetic competition between ligand rebinding to apo protein and unfolding through the apo path. To test this hypothesis, the simulation was adjusted to allows us to predict what ΔTM results for the system when we have high initial occupancy of the bound form (≥ 90%), and then allow the system to evolve over time as the temperature increases. When no interconversion of apo and bound is allowed, we see a ΔTM that is the average of $T_{M,apo}$ and $T_{M,bound}$ values weighted by the initial proportions of apo and bound protein. This model represents the extreme of a very fast rate of rebinding or a very slow rate of dissociation, where no ligand-bound protein is ever lost to the apo unfolding pathway; we see that this system is saturable as the limit of 100-percent $T_{M,bound}$, after which further ligand addition has no effect on ΔTM.

In contrast, a model that allows instantaneous transfer between the bound form to apo, but no transfer of apo to the bound state (unidirectional replacement of every molecule of apo that unfolds with a molecule of bound), represents the limit of very fast dissociation with very slow rebinding; this is the most aggressive example of a system where no ligand rebinding is allowed to protect the protein from unfolding beyond the initial equilibrium distribution of bound protein. As with the prior model, we see a system that is strongly influence by the initial occupancy but even this extreme has only a slightly reduced apparent ΔTM compared to the ΔTM of the fast rebinding model (**SI Fig. 3**).

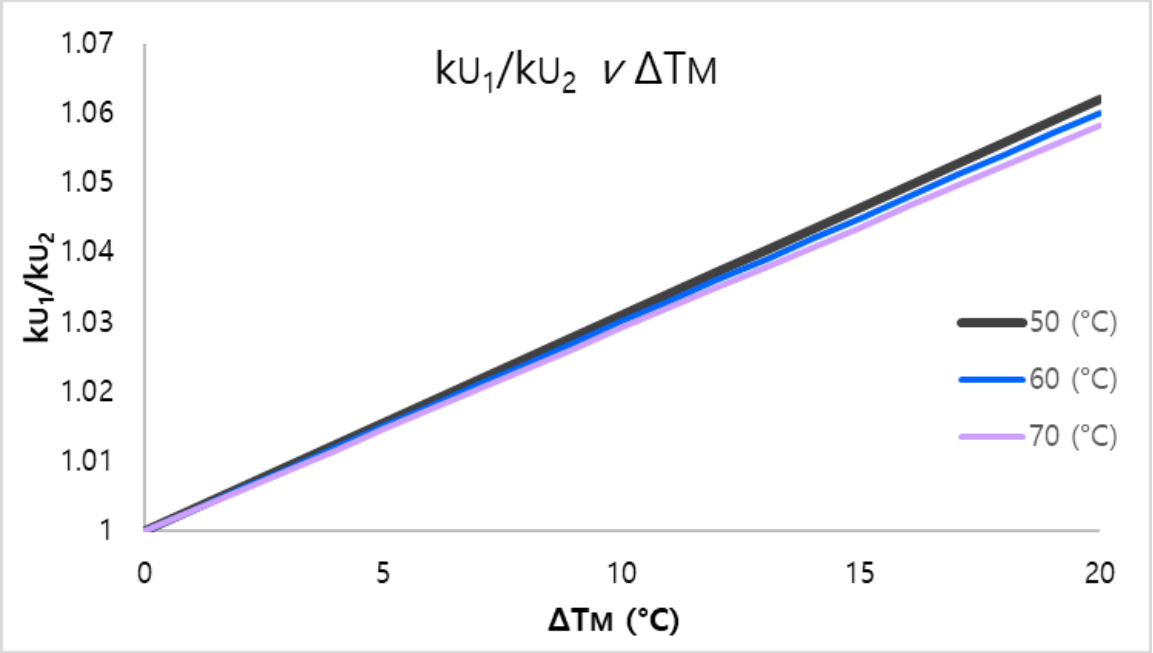
As can be seen in **SI Fig 3**, slow rebinding predicts a ΔTM that is very close to the first model as the initial occupancy approaches 100-percent bound. Therefore, both these models lack the non-saturable effect seen in experiments for increasing ligand concentration, suggesting this

phenomenon is not due to rate competition between ligand binding and the apo unfolding pathway for irreversible unfolding.

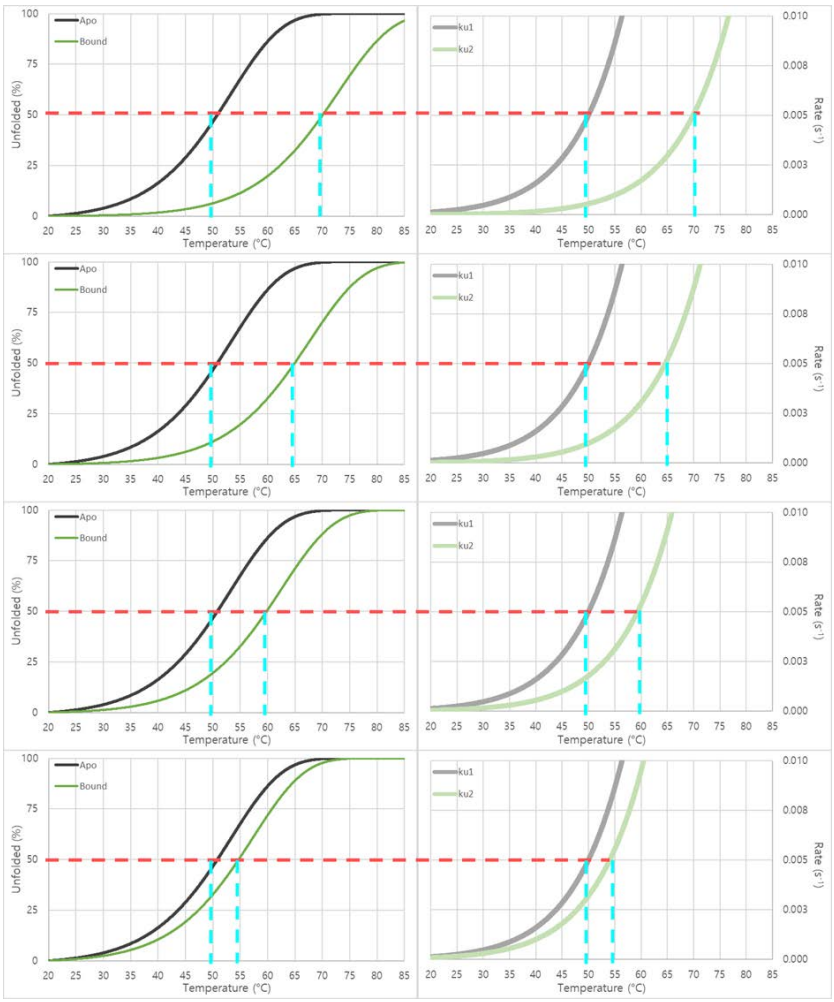
Lastly, these models begins with the equilibrium state, which then evolves with time and temperature. Based on these models, the initial equilibrium occupancy of the system seems to have a dominant influence on the final ΔT_M. Inspection shows this is because ΔT_M is equally dependent on the rate constants and the ligand occupancy (*eg*, $k_{U2} \times [FL]$); since the fold-change between the rate constants k_{U1} and k_{U2} at the same temperatures is small (*ca* 4-fold) (**SI Fig. 2**) compared to the fold-change in the concentration of apo and bound at 95-percent occupancy (*ca* 19-fold), most of the information for the resultant ΔT_M is contained in the initial occupancy term.

References

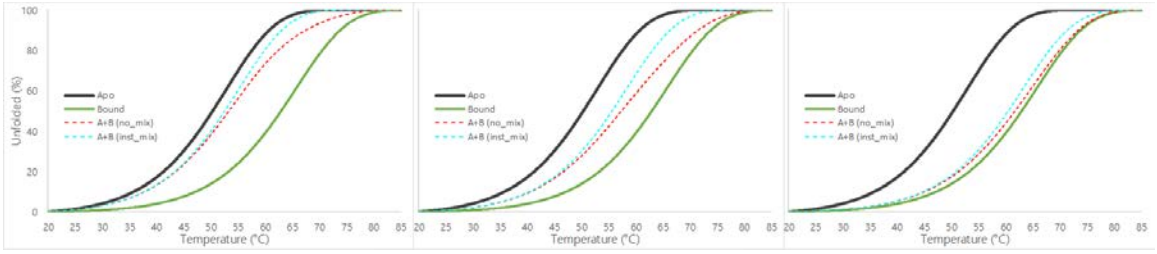
Sanchez-Ruiz, J. M., J. L. Lopez-Lacomba, M. Cortijo, and P. L. Mateo. 1988. 'Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin', *Biochemistry*, 27: 1648-52.



SI Fig 1. The value of the apo unfolding rate constant, k_{U1}, at $T_{M,apo}$ divided by the value of the bound unfolding rate constant, k_{U2}, at $T_{M,bound}$. For a two-state unfolding process, with unfolding rates for apo (k_{U1}) and bound (k_{U2}) that are Arrhenius functions, where $A_1 \cong A_2$, the value of k_{U1} at T_{Mapo} is approximately the same value as k_{U2} at $T_{M,bound}$. Data were generated using equation (28), with T_{Mapo} values of 50, 60 and 70 °C (vantablack, ultramarine and porphyry).



SI Fig 2. Simulated data showing unfolding and rate of unfolding. These data show that for a two-state unfolding process, with unfolding rates for apo (kU_1) and bound (kU_2) that are Arrhenius functions, where $A_1 \cong A_2$, the value of kU_1 at $T_{M,apo}$ is approximately the same value as kU_2 at $T_{M,bound}$. Apo (black) and bound (green) unfolding profiles (left) or rate constant traces (right) are shown; red and cyan dashed lines have been added to guide the eye. Unfolding data were modeled using the methods described in equations (20-22) of the main text, with a data integration steps of 0.1 s, a ligand concentration of 100 µM, and K_D values of 0.005, 0.05, 0.5 and 5 µM (top to bottom).



SI Fig 3. Simulations showing the TM expected for 30 (left), 60 (middle) or 90-percent (right) initial occupancy when there is very fast rebinding (red line) or very slow rebinding (cyan line) relative to the apo unfolding rate. 100-percent apo (black) or bound (green) unfolding traces are shown as reference. Data are for a heating rate of 4 °C/min. An excel file programming these simulations is available to downloaded.