Supporting information for Tochtrop *et al*. (2002) *Proc. Natl. Acad. Sci. USA* **99** (4), 1847–1852. (10.1073/pnas.012379199)

Supporting Text

Fitting the ITC Data to the Stepwise Binding Model. The stepwise binding model was written in PASCAL, compiled, and then imported as a *.dll file into the Windows-based nonlinear least squares analysis program SCIENTIST (Micromath). The analysis of the fitted curves yielded the stepwise dissociation constants (K_{d1}^{obs}, K_{d2}^{obs}) and, in principle, the stepwise binding enthalpies (ΔH_1^{obs}, ΔH_2^{obs}). However, the calorimetry data did not contain enough information content to define a unique value for ΔH_1^{obs}, presumably because of the positive cooperativity. The value for ΔH_2^{obs} at 20°C was –8.1 kcal/mol.

Initially, the ITC data alone were fit to a two-step binding model as in Fig. 2 of the paper. The unbound ligand concentration (L) was related to the total ligand (L_t) and total protein (M_t) concentrations and the stepwise association constants, K_1^{obs} and K_2^{obs}, as follows. (For clarity, the "obs" superscripts have been omitted from the calorimetry fitting equations below, but note that these K values are observed, stepwise association constants.):

$$K_1K_2 \cdot L^3 + [K_1K_2(2M_t - L_t) + 2K_1] \cdot L^2 + [2K_1(M_t - L_t) + 1]) \cdot L - L_t = 0$$

This cubic equation was solved numerically by using an iterative Newton algorithm. The solution was then used to calculate the concentrations of the unligated (M), singly ligated (ML), and doubly ligated protein (ML_2).

$$M = \frac{M_t}{1 + 2K_1L + K_1K_2L^2} \qquad ML = M \cdot 2K_1L$$

$$ML_2 = M \cdot K_1K_2L^2$$

The concentrations of these species were then used to calculate the overall heat generated since the start of the experiment, where ΔH_1 and ΔH_2 are stepwise binding enthalpies:

$$Q = V_{Cell} \cdot (\Delta H_1 \cdot ML) + (\Delta H_2 \cdot ML_2)$$

This calculation was performed twice, after and before the *i*th injection, to determine the differential heat for the *i*th injection as follows:

$$\Delta Q = Q_{after} + \frac{V_{Injection}}{V_{Cell}} \cdot [(Q_{after} + Q_{before}) / 2] - Q_{before} + Q_{Injection}$$

$V_{\text{injection}}$ and V_{cell} are the volumes of the injection and sample cell, respectively, and $Q_{\text{injection}}$ is the heat of "dilution", resulting from the injection itself. The differential heat is normalized by dividing by the moles of ligand injected.

Fitting the NMR Data to the Site-Specific Binding Model. As with the stepwise model, the equations for the site-specific binding model were encoded and compiled in PASCAL and imported into SCIENTIST as a *.dll file. The cubic equation for calculating the free ligand concentration was similar in form to that for the stepwise model. However, the observed stepwise association constants were replaced by the site-specific intrinsic and cooperativity constants K_1, K_2, and c. The relationship between the stepwise and intrinsic constants is given in the footnote to Table 1 of the paper. The cubic equation was solved numerically

$$cK_1K_2 \cdot L^3 + [cK_1K_2(2M_t - L_t) + K_1 + K_2] \cdot L^2 + [(K_1 + K_2) \cdot (M_t - L_t) + 1] \cdot L - L_t = 0$$

using the iterative Newton procedure. The concentrations of the unligated (M), singly ligated site 1 (ML^1), singly ligated site 2 (ML^2), and doubly ligated protein (ML_2) were calculated as follows:

$$M = \frac{M_t}{1 + (K_1 + K_2)L + cK_1K_2L^2} \qquad ML^1 = M \cdot K_1 \cdot L$$

$$ML^2 = M \cdot K_2 \cdot L$$

$$ML_2 = M \cdot cK_1K_2 \cdot L^2$$

The volumes for the unbound (U), site 1 (1), and site 2 (2) resonances were fitted globally against the total ligand concentration L_t. Peak 1 and 2 each contain contributions from one of the singly ligated states and the doubly ligated state.