NMR-Based Screening with Competition Water−Ligand Observed via Gradient Spectroscopy Experiments: Detection of High-Affinity Ligands

Claudio Dalvit,* Marina Fasolini, Maria Flocco, Stefan Knapp, Paolo Pevarello, and Marina Veronesi

Department of Chemistry, Pharmacia, Viale Pasteur 10, 20014 Nerviano (MI), Italy

Received December 18, 2001

Water−ligand observed via gradient spectroscopy (WaterLOGSY) represents a powerful method for primary NMR screening in the identification of compounds interacting with macromolecules, including proteins and DNA or RNA fragments. The method is useful for the detection of compounds binding to a receptor with binding affinity in the micromolar range. The Achille's heel of the method, as with all the techniques that detect the ligand resonances, is its inability to identify strong ligands with slow dissociation rates. We show here that the use of a reference compound with a known K_D in the micromolar range together with properly designed competition binding experiments (c-WaterLOGSY) permits the detection of strong binders. A derived mathematical expression is used for the selection of the appropriate setup NMR experimental conditions and for an approximate determination of the binding constant. The experiment requires low ligand concentration, therefore allowing its application in the identification of potential strong inhibitors that are only marginally soluble. The technique is particularly suitable for rapid screening of chemical mixtures and plant or fungi extracts.

Introduction

NMR-based screening has emerged as a potent technique for the identification of small molecules that interact with a protein drug target.[1−9] Although this methodology suffers from its intrinsic low sensitivity and therefore it requires significant more protein material than other screening methods, the results obtained with NMR are more reliable. The method is less prone to the type of artifacts observed with other techniques. Recent improvements in cryogenic NMR probe technology enables one to reduce the amount of protein needed for the screening and therefore permits NMR to be competitive with other screening assays.[10]

NMR-based screening can be performed by monitoring either the protein target signals or the ligand signals. Observation of the protein signals provides useful structural information of the ligand binding mode. In addition, the technique is not restricted by the size of the ligands or by an upper limit in the ligand dissociation binding constant.[1,11,12] However, the method requires large amounts of isotope-labeled protein and its application precludes the observation of large proteins, although relaxation-optimized techniques (transverse relaxation-optimized spectroscopy (TROSY))[13] can extend the molecular sizes amenable to NMR beyond 100 kDa.

Ligand-observed screening is not limited by the size of the protein and does not require isotope-labeled proteins. Several methods based on the ligand observation have been proposed in the literature.[14−22] One of these techniques is the WaterLOGSY (**w**ater-**l**igand **o**bserved via **g**radient **s**pectroscop**y**) experiment[23,24] where the large bulk water magnetization is partially transferred via the protein−ligand complex to the free ligand.[25,26] The Achille's heel of this method, as with all the ligand-observed screening techniques, is its inability to detect strongly binding ligands with slow dissociation rates. In the assumption of a diffusion-limited on-rate[27] of 10^8 M^{-1} s^{-1}, the upper limit of detection is represented by molecules with a dissociation binding constant K_D in the 100 nM range.

Compounds binding more tightly to the protein or compounds that have a slow on-rate will not be detected because the residence time of these compounds within the protein is longer than the window of the mixing time (e.g., 1−2 s) employed in the NMR experiments.

We show here that with proper design of competition binding WaterLOGSY experiments, it is possible to detect strong binders. The method requires very low ligand concentration (2−10 μM), therefore enabling its use in the identification of potential strong inhibitors that are only marginally soluble. It should be pointed out that a similar approach could be applied to many other (ligand-detected) NMR screening methods. The technique could also find useful applications for rapid screening of chemical mixtures and plant or fungi extracts.

Results and Discussion

NMR competition binding experiments performed with known inhibitors have been used in order to determine the specificity of the identified NMR hits. We show here that the same competition binding WaterLOGSY experiments, properly designed, can be used to screen chemical mixtures for the detection of strong ligands to the protein of interest. We refer to these experiments as c (competition)-WaterLOGSY experiments.

* To whom correspondence should be addressed. Phone: +39-0248383238. Fax: +39-0248383965. E.mail: claudio.dalvit@pharmacia.com.

10.1021/jm011122k CCC: $22.00
Published on Web 05/10/2002

The intensity of the experimental WaterLOGSY signal I_{WL} for a proton i of a reference compound is provided by the expression[24]

$$I_{WL} - [L](\sum_j \sigma_{ij}^{free} + \sum_w \sigma_{iw}^{free}) \propto [EL](\sum_j \sigma_{ij}^{bound} + \sum_k \sigma_{ik} + \sum_w \sigma_{iw}^{bound}) \quad (1)$$

The σ parameters are the different cross-relaxation rates involving proton i in the bound and free states. The index j represents ligand exchangeable protons, k represents protein protons near the ligand, and w is for water molecules near the ligand. The quantities [L] and [EL] correspond to the concentrations of free and bound ligands, respectively. The two concentrations are related to each other via the equation $[L] = [L_{TOT}] - [EL]$ where $[L_{TOT}]$ is the total ligand concentration. Since in our experiments $[L_{TOT}] \gg [E_{TOT}]$ (total protein concentration), we can safely replace [L] in eq 1 with $[L_{TOT}]$. The term

$$[L_{TOT}](\sum_j \sigma_{ij}^{free} + \sum_w \sigma_{iw}^{free})$$

corresponds to the experimental hydration of proton i of the reference compound in the absence of the protein. From now on, we will refer to the term I_{WLOGSY} as the intensity of the measured WaterLOGSY signal I_{WL} plus the correction term obtained from an experiment recorded for the ligand in the absence of the protein.

In the presence of a competitive molecule, the concentration of the protein-bound reference compound diminishes.[27] The WaterLOGSY signal intensity ratio for a reference compound in the presence and absence of a competitor is given by eq 2 in Chart 1 where $I_{WLOGSY}(+)$ and $I_{WLOGSY}(-)$ are the intensity of the reference compound in the presence and absence of the competitor, respectively. The quantities $[E_{TOT}]$, $[L_{TOT}]$, and $[I_{TOT}]$ are the protein, reference compound, and competitor concentration, respectively. The quantities K_D and K_I are the dissociation binding constants for the reference compound and the competitor, respectively. In deriving eq 2, we have assumed the absence of positive or negative cooperativity effects.

Figure 1 shows a simulation of the WaterLOGSY signal of the reference compound as a function of the K_I of a competitor. For the simulation, we have assumed a reference compound and protein concentration of 50 and 2 μM, respectively. Two different concentrations, 5 and 10 μM, have been considered for the competitor.

From this simulation, it is evident that the signal attenuation of the reference compound in the presence of a competitor depends on K_D, K_I, and $[I_{TOT}]$. Therefore, it is possible to detect indirectly the presence of a strong inhibitor in a chemical mixture simply by monitoring the WaterLOGSY signal of a reference compound. The lower limit in affinity strength for the detection can be tuned by properly selecting the reference compound (i.e., different K_D) and/or different $[I_{TOT}]/[L_{TOT}]$ ratios according to eq 2.

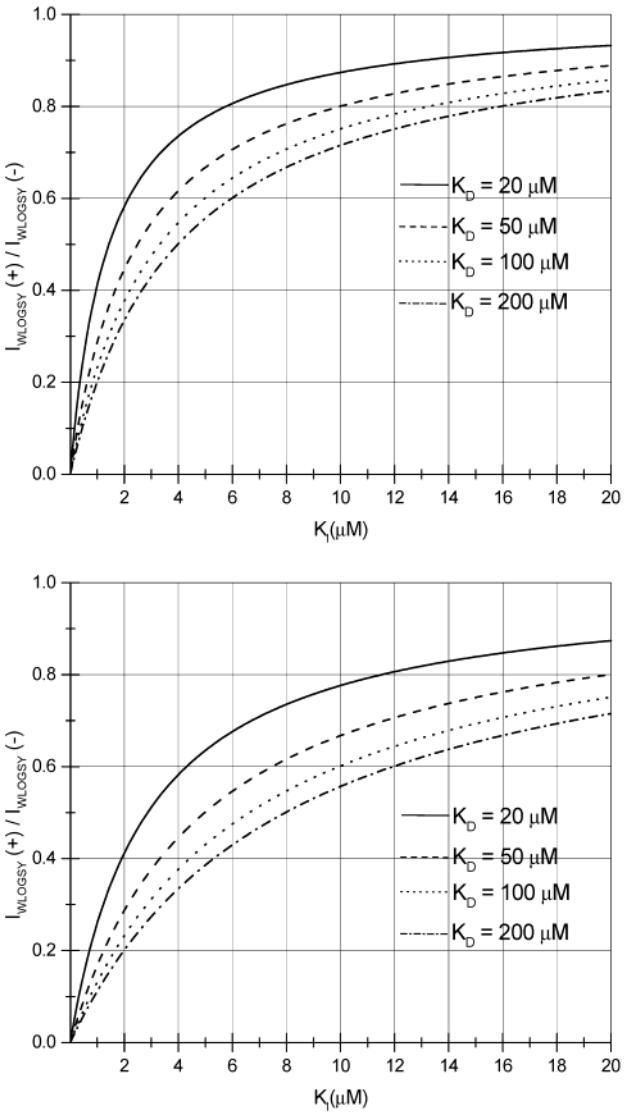

Figure 1. WaterLOGSY signal attenuation of the reference compound as a function of the dissociation binding constant K_I of the competitor. The simulation was performed using eq 2 and with a competitor concentration of 5 μM (upper diagram) and 10 μM (lower diagram). The protein and reference compound concentration was 2 and 50 μM, respectively. The ratio of the WaterLOGSY signal for the reference compound in the presence and absence of the competitor is displayed on the *Y* axis, and the dissociation binding constant (in micromolar) for the competitor is displayed on the *X* axis. The value 1 on the *Y* axis corresponds to the signal of the reference compound observed in the absence of the competitor plus the offset arising from the hydration of the free ligand. The value 0 on the *Y* axis corresponds, in the approximation of only one protein binding site for the reference compound, to the WaterLOGSY signal of the compound in the absence of the protein. Simulations were performed for four different binding constants K_D of the reference compound (values indicated on the graph).

Chart 1

$$\frac{I_{WLOGSY}(+)}{I_{WLOGSY}(-)} = \frac{[E_{TOT}] + [L_{TOT}] + K_D\left(1 + \frac{[I]}{K_I}\right) - \sqrt{\left\{[E_{TOT}] + [L_{TOT}] + K_D\left(1 + \frac{[I]}{K_I}\right)\right\}^2 - 4[E_{TOT}][L_{TOT}]}}{[E_{TOT}] + [L_{TOT}] + K_D - \sqrt{\{[E_{TOT}] + [L_{TOT}] + K_D\}^2 - 4[E_{TOT}][L_{TOT}]}} \quad (2)$$

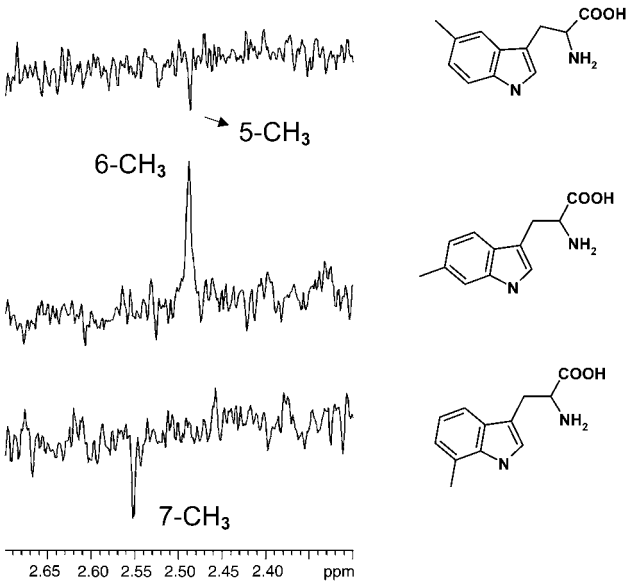

Figure 2. One-dimensional WaterLOGSY spectra recorded for a 5 μM human serum albumin (HSA) solution in the presence of 50 μM 5-CH_3-D,L-Trp (top), 50 μM 6-CH_3-D,L-Trp (center), and 50 μM 7-CH_3-D,L-Trp (bottom). The displayed expanded spectral region contains the methyl group signals. The spectra were recorded with 2048 scans, 2.6 s repetition time, and 1.5 s mixing time. Positive and negative signals identify HSA binding and noninteracting molecules, respectively.

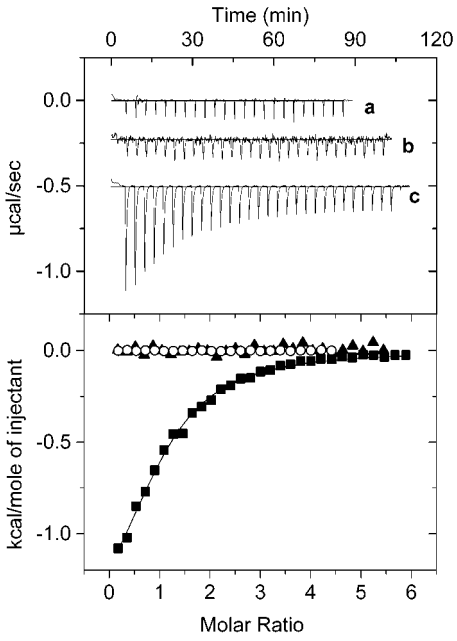

Figure 3. ITC data measured on the binding of tryptophan analogues to HSA. The top panel shows the raw heat data obtained over a series of injections of 7-CH_3Trp (a), 5-CH_3-D,L-Trp (b), and 6-CH_3-D,L-Trp (c) into HSA. The integrated heat signals shown in the top panel of the figure gave rise to the normalized binding isotherms shown in the lower panel (7-CH_3-D,L-Trp (open circles), 6-CH_3-D,L-Trp (solid squares), 5-CH_3-D,L-Trp (solid triangles)). Dilution heats were collected in blank titrations and were subtracted from the data. No net binding heat effects were observed for 5-CH_3-D,L-Trp and 7-CH_3-D,L-Trp, respectively, indicating that these compounds do not interact with HSA, whereas using 6-CH_3-D,L-Trp negative binding heats were observed. The solid line represents a calculated curve using the best-fit parameters obtained by a nonlinear least-squares fit to the measured data. The calculated binding parameters were the following: stoichiometry (N), 0.98; K_B, (2.7 $\pm$ 0.2) $\times$ 10^4 mol^{-1}; ΔH^{obs}, 1.9 $\pm$ 0.1 kcal/mol; ΔS, 13.79 cal/(mol K).

This approach requires first the identification with NMR or other techniques of a weak affinity ligand of the protein target of interest. The binding constant for this compound should be calculated in order to properly design the experiments according to eq 2 and Figure 1. When possible, a compound with a methyl group should be chosen in order to maximize the sensitivity of the experiment. This will allow reduction in protein consumption.

A well-characterized protein, human serum albumin (HSA), was chosen as a test case for demonstrating the application of the c-WaterLOGSY. Drugs such as naproxen, diazepam, and ibuprofen are known to bind to HSA on site II.[28] The endogenous amino acid tryptophan binds also on site II of HSA.[29] Therefore, in our effort to identify a potential reference molecule, we have selected three methyltryptophan derivatives, namely, 5-CH_3-, 6-CH_3-, and 7-CH_3Trp. The spectra of the three derivatives, shown in Figure 2, identified the 6-CH_3Trp as a ligand for HSA. The other two derivatives 5-CH_3- and 7-CH_3Trp do not interact with the protein as indicated by the negative signals in Figure 2. The simple substitution of the proton with a methyl group at position 5 or 7 on the ring abolishes completely the binding to HSA. These findings were confirmed by ITC measurements as shown in Figure 3. In addition, this technique provided us with the association binding constant (K_B) for our selected reference compound (i.e., 6-CH_3Trp), which was determined to be 2.7 $\pm$ 0.2 10^4 mol^{-1}.

In the c-WaterLOGSY approach, a spectrum is first acquired for the selected reference compound in the absence of the protein. This is necessary for extracting the hydration correction term discussed above. Then, an identical spectrum is acquired for the reference compound in the presence of the protein. These two spectra are acquired only once and are then used for the analysis of all the screened chemical mixtures. A small spectral region containing the methyl group of 6-CH_3Trp in the absence and presence of HSA is shown in spectra a and b of Figure 4, respectively. Subsequently, WaterLOGSY spectra are acquired for compound mixtures (sucrose, 7-CH_3Trp, and diazepam in our example) in the presence of the protein and the reference compound as shown in Figure 4c. The WaterLOGSY signals of the reference compound in the absence and presence of the mixture are then compared. A change in sign or substantial signal reduction (as shown in Figure 4c) of the reference compound resonance in the spectrum recorded in the presence of the mixture is an indication that at least one compound comprising the mixture is a potent ligand and displaces the reference compound (6-CH_3Trp) from the protein. Deconvolution of the chemical mixture performed in the presence of the reference compound is shown in Figure 5. No signal intensity change of 6-CH_3Trp was observed in the presence of sucrose (Figure 5b) and 7-CH_3Trp (Figure 5c), whereas a drastic signal reduction was observed in the presence of diazepam (Figure 5d). This deconvolution enables the identification of diazepam as the high-affinity ligand present in the mixture.

The calculation of the signal reduction (with the

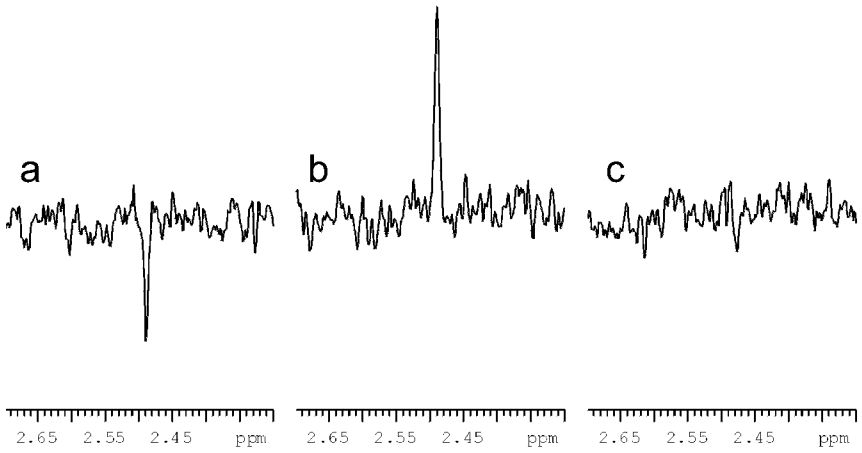

Figure 4. One-dimensional WaterLOGSY spectra recorded for 50 μM 6-CH_3-D,L-Trp (a) and for 50 μM 6-CH_3-D,L-Trp with 5 μM HSA in the absence (b) and in the presence (c) of the three-compound mixture (10 μM sucrose, 10 μM 7-CH_3-D,L-Trp, and 10 μM diazepam). The displayed spectral region contains the 6-CH_3 signal of the tryptophan derivative. The spectra were acquired with 4096 scans, 2.6 s repetition time, and 1.5 s mixing time. The length of the double spin−echo was 25.2 ms in order to destroy most of the protein signals, therefore obtaining a flat baseline.

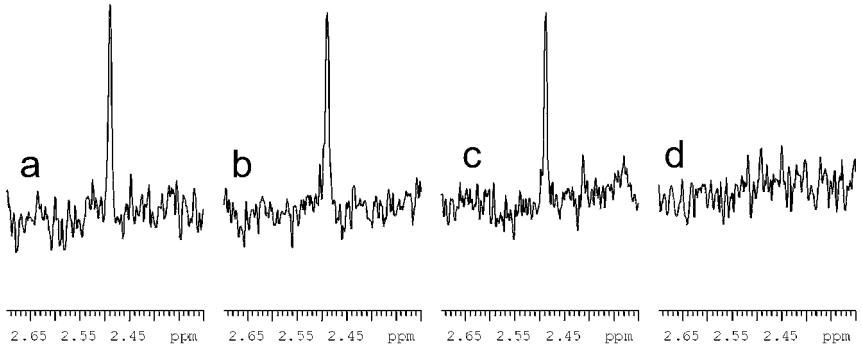

Figure 5. One-dimensional WaterLOGSY spectra recorded for a 5 μM HSA solution with 50 μM 6-CH_3-D,L-Trp in the absence (a) and in the presence of 10 μM sucrose (b), 10 μM 7-CH_3-D,L-Trp (c), and 10 μM diazepam (d). The displayed spectral region contains the 6-CH_3 signal of the tryptophan derivative. The other experimental conditions are the same as in Figure 4.

proper correction) and the knowledge of K_D of the reference compound (6-CH_3Trp) provide an approximate estimation or a lower limit, according to eq 2, of the dissociation binding constant of the identified ligand (diazepam). This is achieved with a single-point measurement, since eq 2 considers also the effect of the protein concentration term. This contribution is neglected in the IC_{50} equation derived in the literature[30] and used in NMR studies[22] where the concentration of both ligands is considered much larger than the protein concentration and K_D. This approximation is not valid in our experiments and in many other NMR experiments because the concentration of the competitive inhibitor is in many cases similar to the protein concentration or is less and the concentration of the ligand can be comparable to K_D. Equation 2 is a general expression and should be applicable to other NMR parameters investigated in competition binding experiments. With a signal reduction of 65% and a K_D of 37 μM for 6-CH_3Trp, we have estimated a binding constant for diazepam of 2 $\pm$ 1 μM that is close to the value of 2.6 μM reported in the literature.[31] Note that with eq 2 it is possible to measure very strong ligands with binding constants in the nanomolar range. For this purpose, it is necessary to use even a lower competitive inhibitor concentration.

The procedure described here can also be applied to the identification of high-affinity ligands present in plant or fungi extracts. The composition and concentration of the different components present in the extracts are not known. Nevertheless, the knowledge of the presence of a strong ligand in the extract can guide the chemist in the separation and isolation of the active compound.

We also recommend the use of a weak reference compound in all the WaterLOGSY experiments. In the search for weak and medium strength inhibitors, the concentration of the mixture constituents should be the same as for the reference compound (e.g., 50 μM). The characteristic appearance of the positive signals for a compound of the mixture will identify that molecule as a ligand of the target of interest. If this is associated with no signal reduction for the reference compound, it is possible to conclude, according to eq 2, that the compound does not compete with the reference molecule and binds on a different site of the protein. However, the absence or strong reduction of the positive signals for the reference compound is an indication that one of the molecules comprising the mixture is a high-affinity ligand. Deconvolution of the mixture will then allow the identification of the molecule. When this approach is used, both weak and strong inhibitors will be detected. The c-WaterLOGSY technique was successfully applied in our laboratory in the search of strong kinase inhibitors that bind in the ATP binding site. Protein and ligand concentrations as low as 2 and 5 μM, respectively,

were employed in these studies, therefore allowing the identification of strong inhibitors that are only marginally soluble (data not shown).

Conclusion

We have shown that the use of a medium- to low-affinity reference compound together with properly designed c-WaterLOGSY experiments permit the indirect detection of high-affinity ligands. In addition, an approximate value or a lower limit of the dissociation binding constant of the identified molecule can be extracted with a single point measurement. The technique is particularly suitable for rapid screening of chemical mixtures and natural product extracts. Finally, the experiment is not limited to the interactions of small molecules with proteins but can be used efficiently also in the identification of molecules interacting with DNA or RNA fragments.

Experimental Section

Fatty acid free human serum albumin (A-3782) was purchased from Sigma and used without further purification. Sucrose (S7903) and 7-CH_3-D,L-Trp (M8379) were purchased from Sigma, and 5-CH_3-D,L-Trp (69560) and 6-CH_3-D,L-Trp (69570) were purchased from Fluka. Diazepam was purchased from Carlo Erba.

The NMR samples were in phosphate-buffered saline (PBS) solution (Sigma), pH 7.4. D_2O was added to the solutions (8% final concentration) for the lock signal.

NMR Experiments. All spectra were recorded at 293 K with a Varian Inova 600 MHz NMR spectrometer equipped with a 5 mm triple-resonance inverse probe and an autosampler. For each sample, a reference spectrum and a 1D WaterLOGSY spectrum were recorded. The details of the pulse sequence version used for the WaterLOGSY experiment reported here can be found in the literature.[23,24] The first water-selective 180° pulse was 25 ms long. A weak rectangular PFG is applied during the entire length of the mixing time (1.5 s). A short gradient recovery time of 2 ms was introduced at the end of the mixing time before the detection pulse. The two water-selective 180° square pulses of the double spin−echo scheme[32] were 2.6 ms long. The gradient recovery time was 0.2 s. The data were collected with a sweep width of 7407 Hz, an acquisition time of 0.648 s, and a relaxation delay of 2.648 s. Prior to Fourier transformation, the data were multiplied with an exponential function with a line broadening of 1 Hz.

ITC Experiments. Calorimetric measurements were carried out at 298 K in PBS buffer (Sigma) at a protein concentration of 30 μM and a ligand concentration of 1.5 mM using a VP-ITC titration calorimeter (MicroCal). Heats of dilution were measured in blank titrations by injecting the protein into the buffer used in the particular experiment and were subtracted from the binding heats. Thermodynamic parameters were determined by nonlinear least-squares methods using routines included in the Origin software package (MicroCal).

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JM011122K