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Rapid communication

Protonation effect on drug affinity

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Abstract

Pharmacologic ligand–macromolecule interactions are commonly characterized by affinity (dissociation) constants such as K_d or K_i without regard to the protonation effect of the buffer used in the measurement. The protonation effect is demonstrated here using isothermal titration microcalorimetry measurements of the competitive inhibitor binding of cytidine 2′-monophosphate (2′-CMP) to RNase-A as a model system in buffers of different ionization ΔH_{buffer}. The results demonstrate the importance of protonation in measures of affinity.

Keywords: Affinity; Protonation; Microcalorimetry

The interaction between a pharmacologic ligand and a macromolecular receptor complex is commonly quantified by a measure of affinity (e.g., the K_d or K_i)—and often characterized as the net attraction resulting from the making and breaking of multiple weak intermolecular bonds between ligand and receptor. However, all such interactions occur in some solvent/buffer environment, whether in vitro or in vivo, and include the rearrangement of bonds between the interacting species and buffer molecules. This affects the measure of affinity, as seen from the relationship between affinity (reciprocal of the equilibrium constant, K_{eq}) and the free energy change of the interaction, $\Delta G = -RT \ln K_{eq} = \Delta H - T\Delta S$, in which the enthalpic (ΔH) and entropic (ΔS) terms include contributions of the buffer interactions. Binding occurs only if the total free energy decreases, thus solvent effects are as important as ligand–receptor bonds to the energy balance (Collins, 1997). To the extent that the measure of affinity ignores the contribution of buffer, it is a poorer estimate of the affinity of ligand and macromolecule.

A common interaction with buffer is exchange of protons (protonation). Modern isothermal titration calorimetry (ITC) devices (Wadsö, 1995) measure an observed enthalpy change, ΔH_{obs}, as a composite of binding enthalpy (ΔH_{bind}) and enthalpy of ionization of the buffer (ΔH_{buffer}) according to (Jelesarov and Bosshard, 1999):

$$\Delta H_{obs} = \Delta H_{bind} + n_{H^+} \cdot \Delta H_{buffer}, \quad (1)$$

from which the binding enthalpy (and, hence, affinity) can be determined by repeating the experiment at the same pH in buffers of different ΔH_{ion} (tabulated in Fukada and Takahashi, 1998). This is demonstrated for the binding of the competitive inhibitor cytidine 2′-monophosphate (2′-CMP) to RNase-A.

RNase-A (EC 3.1.27.5) and 2′-CMP free acid (98% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Buffer chemicals (analytical grade) were purchased from VWR Scientific (Bridgewater, NJ). The RNase-A was dissolved in deionized water and dialyzed at 1.5 °C. The RNase-A concentration (0.04–0.05 mM) was determined by quantitative UV spectrophotometry (277.5 nm, $\varepsilon = 9800$ M^{-1} cm^{-1}). The 2′-CMP (1.2 mM) was prepared in the same buffers as RNase-A and measured spectrophotometrically (260 nm, $\varepsilon = 7400$ M^{-1} cm^{-1}). Solutions were degassed at 36.5 °C under filtered vacuum (about 686 mm Hg). The reference cell of the calorimeter (model VP-ITC; MicroCal, Northampton, MA) contained degassed deionized water. The reaction cell contents were stirred at 400 rpm at 25 °C (pH 5.5) throughout the experiment (the frictional heat of stirring is incorporated into the baseline). 2′-CMP was introduced into the reaction cell in a series of thirty-five 4-μl injections, each delivered over 16 s at 3-min intervals. The

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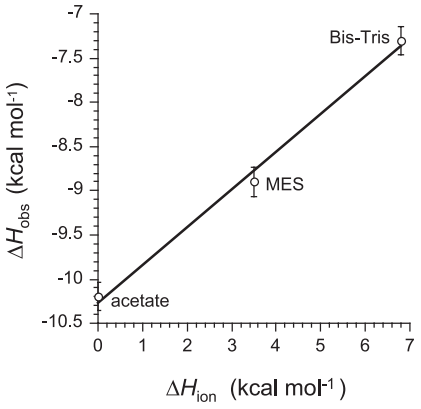

Fig. 1. ΔH_{obs}, measured by isothermal titration microcalorimetry, plotted against the ΔH_{ion} of each buffer (Fukada and Takahashi, 1998). The data are the means ± S.D. of triplicate runs. Linear regression ($r^2 = 0.99$) yielded a slope of 0.01 and a y-intercept of -10.27 kcal mol^{-1} (-42.93 kJ mol^{-1}).

equipment automatically adjusts for the change in volume. The data (sampled at 2 s^{-1}) were evaluated using ORIGIN® (v. 5.0) software (Microcal) as previously described (Raffa et al., 2002; Spencer et al., 2002).

The addition (binding) of 2′-CMP to RNase-A produced consistent isotherms with stable baseline in each of the three buffers tested (50 mM): acetate, Bis(2-hydroxymethyl)iminotris(hydroxymethyl)methane (Bis-Tris) and 2-(*N*-morpholino) ethanesulfonic acid monohydrate (MES). Maximal output was about −1.5 to −2.5 μcal/s, the negative deflection indicative of the exothermic reaction. The transposed data were plotted as the integrated heats (kcal/mol of 2′-CMP) for each injection against the 2′-CMP/RNase-A molar ratio. Fitting parameters for the single-site nonlinear regression computer-fit of the raw data points yielded the K_{eq} and ΔH_{obs} in each buffer. The stoichiometry was close to 1:1 in each buffer, consistent with a 1 to 1 interaction between 2′-CMP and RNase-A. Fig. 1 displays the measured ΔH_{obs} (means ± S.D. of triplicate runs), plotted against the ΔH_{ion} of each buffer. In agreement with Eq. (1), there was a linear relationship between ΔH_{obs} and ΔH_{ion} ($r^2 = 0.99$). The projected ΔH_{bind} (y-intercept) was -10.27 kcal mol^{-1} (-42.93 J mol^{-1}). The corresponding K_d was 1.57×10^{-6} M, an affinity up to 3-fold higher than that obtained without correction for protonation.

The results demonstrate: (1) the importance of the protonation effect on ligand affinity, (2) the ability of isothermal titration microcalorimetry to measure and correct for the effect and (3) the value of measuring the thermodynamic parameters that underlie the affinity of pharmacologic interactions (Raffa, 2001).

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