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Impact of Titratable Groups in Studies with Isothermal Titration Calorimetry

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Isothermal Titration Calorimetry (ITC) is a commonly used technique to determine the stoichiometry, affinity, and enthalpy of binding reactions in solution [1,2]. In many cases, conclusions are made on the basis of titrations performed in one buffer system. In this article, we will demonstrate a potential problem that may lead to incorrect conclusions about the energetics of a given reaction based on data acquired using a single buffer system, which originates from changes in pK_as of titratable groups upon complex formation.

In a binding reaction, pK_as of titratable group on the protein and the ligand may shift upon formation of the complex. As a result, protons are either released or taken up by these groups and the buffer responds to such changes either by releasing or taking up protons to maintain pH. Therefore, the observed enthalpy (ΔH_{obs}), includes contributions from the intrinsic enthalpy of binding (ΔH_{int}) and the heat of ionization of the buffer (ΔH_{ion}). ΔH_{int} can be determined based on the following equation and using different buffers with different ΔH_{ion}. While the y-intercept yields ΔH_{int}, the slope of the line yields the net protonation, Δn. $\Delta H_{obs} = \Delta H_{int} + \Delta n (\Delta H_{ion})$

It is important to note, that the ΔH_{int}, determined in this manner, will still include contributions from the heat of ionization of the titratable groups in the protein–ligand complex. Furthermore, it is also a common practice to include a certain level of salt (50-200 mM) to eliminate the heat of nonspecific binding of the buffer to the protein. The importance of determining the intrinsic enthalpy of a reaction can be seen in the experimental data shown in Figure 1. Titration of the aminoglycoside phosphotransferase(3′)-IIIa (APH) with the aminoglycoside antibiotic netilmicin yielded an endothermic reaction when the titration was performed in Tris-HCl pH 7.4 (Figure 1, right

Buffer	ΔH_{ion} (kcal/mol)	ΔH_{obs} (kcal/mol) [a]	ΔH_{int} (kcal/mol)	Δn
PIPES	2.7	-5.1 ± 0.04	-7.7 ± 0.8	0.82 ± 0.1
HEPES	5.0	-4.45 ± 0.05		
ACES	7.4	-1.3 ± 0.08		
Tris-HCl	11.7	1.6 ± 0.04		

All experiments were performed at 25°C. [a]Given errors were calculated by fits to SEDPHAT [4]. [b]Errors for ΔH_{int} and Δn are derived from the linear regression line of ΔH_{obs} vs. ΔH_{ion} plot

Table 1: Titration of APH with netilmicin in different buffers.

panel), while the same titration in PIPES buffer at the same pH yielded an exothermic reaction (Figure 1, left panel). Tris-HCl, ACES, and PIPES buffers have ΔH_{ion} of 11.7, 7.5, and 2.7 kcal/mol respectively [3]. Analysis of these data (Table 1) as described above clearly shows that this is an exothermic reaction with the ΔH_{int}=-7.7 ± 0.8 kcal/mol.

The ΔH_{int} for this binding event is representative of a reaction with a favorable enthalpy, similar to the previously determined thermodynamic data for the binding of aminoglycosides to several enzymes that modify these antibiotics [4-10]. Data acquired in tris buffer alone would cause a misinterpretation of the binding of netilmicin to APH as an entropy-driven complex formation and exception to observations with other aminoglycosides, which is obviously incorrect. Although binding of all aminoglycosides to aminoglycoside-modifying enzymes are accompanied by protonation/deprotonation of titratable groups (i.e., $\Delta n \neq 0$), large negative enthalpy of binding was almost always larger than the positive contribution of the heat of buffer ionization and therefore all thermograms showed exothermic signal. However, when the binding enthalpy is small, as it was with netilmicin, the importance of the contribution of buffer becomes much more significant and may even lead to potential misinterpretations as demonstrated here.

As a final note, we should also mention that the value of Δn itself can also be misleading as it includes both the protonation and deprotonation of several functional groups and represents only the *net* protonation. In other words, conditions where $\Delta n \approx 0$ do not guarantee the lack of protonation/deprotonation as shown earlier [11].
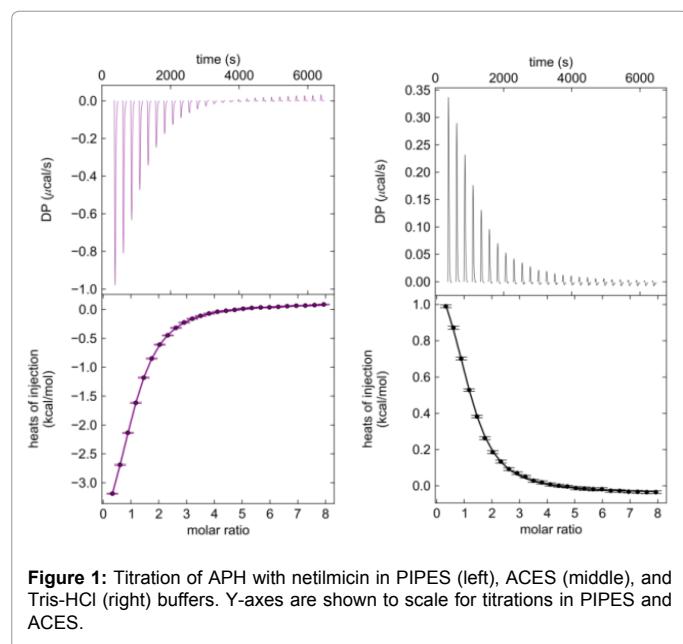
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Figure 1: Titration of APH with netilmicin in PIPES (left), ACES (middle), and Tris-HCl (right) buffers. Y-axes are shown to scale for titrations in PIPES and ACES.

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