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This paper is a valuable addition to the literature on ITC studies of protein-ligand binding. It addresses the fact that publications on supposedly repeat experiments often report different results, and draws attention to the lack of critically established experiments and the tendency of many workers to use commercial instruments uncritically, without control and blank experiments to eliminate artefacts. The paper also analyses to way in which statistical errors accumulate. The paper is a welcome practical follow-up to the large ABRF comparison of results for the binding of carboxybenzylesulphonamide (CBS) to carbonic anhydrase by 14 groups, which resulted in a remarkable range of results from the use of several commercial instruments with possibly different software (reference 3 of the paper). Is the CBS of this paper the same as the CBS of ref 3? The names are different and the reviewer was unable to find an Aldrich C11804 in his catalog.

This manuscript gives a good analysis of the effect of experimental details on the statistical outcomes, and recommends control and blank experiments to avoid interfering artefacts and to generate thermodynamic parameters. This reviewer's comments first address the experimental methods, and secondly suggest that a recent publication, not cited, may be supported by aspects of the statistical collaboration not fully allowed for in protein-ligand ITC, with severe consequences.

The dilution of water in to water (or buffer into buffer) is a good blank experiment to start with. – this certainly gives a non-zero heat with an ITC instrument used by the reviewer. This really confuses the results at high ligand binding.

The reference to protein purification is pertinent. It could be made more forceful by noting that a 1% incompletely dialyzed impurity of molecular mass 300 (for example) would comprise three times the number moles of CA in solution, comparable to the ligand concentrations in a titration

The errors in pipetting small quantities are widely ignored, and the authors do well to enlarge on this. The reviewer found it difficult to follow some of the recommended procedures – the down command to correct for a syringe-drive backlash and the correction for diffusive loss. The authors should revisit their account of these corrections for those not familiar with their particular calorimeter. They are surely on the right track, and a clearer explanation would help. Part of the difficulty is the point at zero ligand binding in Figure 1. It is not readily obvious why it is not included in the total heat with a small correction to the protein/ligand ratio

The solubility of the CBS ligand in water at room temperature is given – a rare and welcome piece of information in this literature. Is the solubility the same in the unspecified PBS buffer? Is the buffer the same as that used and fully specified - including 0.15M NaCl - in the ABRF study? The ligand is ionic and its solubility may be much less in the buffer solution. Figure A2 indicates heat pulses from titrating ligand into buffer, which have several possible origins – viscous dissipation in the injection process, pH and ligand ionization changes, direct buffer/ligand interaction, micellization or simply a heat of dilution, which could be tested by a ligand into buffer titration compared to a water in water titration. If the ligand does show a heat of dilution into water and/or buffer, the ligand in solution will definitely not be ideal and equation 3 will not give the thermodynamic equilibrium constant or the derived free energy.

Similarly, the heat of dilution of the protein in solution is worth testing – perhaps never tested in the ITC literature? The fundamental question is whether the ligand and protein solution concentrations are equal to the thermodynamic activities, as are strictly required in Equation 3.

This paper is directed to experimental precision and its effect on the subsequent estimates of free energies etc ., but the authors have an important opportunity with their well-controlled and calibrated ITC system to address the whole basis of the protein/ligand field, its universal assumption of the validity of Equation 3 and the estimates of free energy that follow. A recent paper (Analytical Biochemistry vol 472, 21-29, 2014) has challenged these assumptions on what appears to be firm thermodynamic grounds. The arguments can be tested and exemplified with the authors' present protein/ligand system on two fronts- the ligand and protein thermodynamic activities. It is pointed out that if the apparent equilibrium constant changes when the protein concentration is altered, its concentration is not its activity, equation 3 does not define the equilibrium constant and the first sentence of the authors' manuscript will be quite incorrect. The ligand chosen in this manuscript is a favorable case for studying the potential non-ideality of its solution. A zero heat of dilution is not proof of ideality, but a definite heat of dilution is proof of non – ideality. Other tests for ideality seem practical with CBS - surface tension for example often confirms aggregation in solution. A test of the thermodynamic status of protein and ligand using the new standards is waiting. The attention to experimental and statistical basics in this paper is still valuable even if equation 3 is shown to be invalid for the CA/CBS case. ITC will remain a powerful tool. Well-measured enthalpies are very valuable. This paper raises the experimental standards in the field and deserves publication whether or not the authors wish to add any notes relevant to this reviewer's extended comments made in this last paragraph.