Effects of Reducing Agents on the ITC and DSC Baselines

NOTE: The guidelines below are based on MicroCal guidelines.

The addition of a reducing agent in the solution is often necessary for some proteins to maintain their activity and stability, or to prevent the formation of aggregates. Currently, the most popular reducing agent for proteins is dithiothreitol (DTT). However, DTT is unstable when it comes in contact with air and tends to undergo oxidation in solution in the presence of air. Our in-house study and client samples have shown that even at 0.5 mM concentration, DTT had an adverse effect on DSC and ITC baselines, even when the solution was de-aerated under a mild vacuum (27 mm Hg) immediately before the ITC or DSC experiment. Although we found that purging air from the solution with either nitrogen or argon could minimize the effect of DTT on the calorimetry baseline, this method appears to be impractical for a solution containing detergent or protein, both of which start to foam upon purging with nitrogen or argon.

Even though DTT is a popular reducing agent in biochemical research, there are other reducing agents available commercially that are as effective as DTT. One of these is β-mercaptoethanol, which had been widely used earlier (i.e., before 1970) before it was replaced by DTT, partially due to its "smelly" odor. More recently another reducing agent, TCEP (tris(2-carboxyl)phosphine) has become increasingly popular, not only because it is odorless, but also because it has a stronger reducing capacity and is less prone to oxidation[1,2]. We performed several studies to evaluate the effect of these two reducing agents on the calorimetry baseline. We found, in general, that these two reducing agents gave less DSC and ITC baseline artifacts than DTT. We should emphasize that our conclusions are based on limited experiments in one buffer solution only (i.e., 40 mM Hepes, pH 7.5). The reducing agent solutions for our experiments were freshly prepared and were used for testing within a week. Frequently, we also found that the baseline artifacts caused by these reducing agents were not reproducible. The magnitude of artifacts usually decreased as the solution aged. This result could depend on the air content of the solution, its pH, its state of oxidation, and its temperature. The following are some suggestions we think may be useful for calorimetry users:

1. Reducing agents, particularly DTT, should be avoided in the solution for ITC and DSC experiments when its presence in the solution is deemed unnecessary. If a reducing agent has to be present in the solution, we suggest that users try β-mercaptoethanol first and then TCEP. However, if DTT in the solution is indispensable, we suggest trying a buffer vs. buffer (containing DTT) experiment first, and keeping the DTT concentration as low as possible. If baseline abnormality is observed, try purging the solution with argon or nitrogen to remove oxygen (de-aeration under a moderate vacuum doesn't work). This method might not work for some solutions (such as those containing detergents or proteins, which start to foam upon purging with gas). For a lab that is equipped with a dry box, air-free DTT containing solutions should be prepared, and the calorimeter cells should be purged with nitrogen or argon immediately before the solution loading to prevent the solution's contact with air.
2. We have tried both β-mercaptoethanol and TCEP. We found that both of these reducing agents gave much less baseline artifacts than DTT. Our experiments have shown that β-mercaptoethanol up to 5 mM in the solution does not have much of an adverse effect on the ITC baseline. The ITC baseline showed a slope only in the presence of 5 mM β-mercaptoethanol. However, β-mercaptoethanol did cause some abnormalities in the DSC

baseline. The size of the artifact for 5 mM~ β-mercaptoethanol was rather small compared to that of DTT at 2 mM. We also found that the ITC baseline shows a slope only in the presence of 2 mM TCEP. However, TCEP did cause some artifacts on the DSC baseline. The size of these artifacts was small compared to that of DTT. Because no extensive investigation has been performed on β-mercaptoethanol and TCEP, we can only advise our clients to try these agents to determine if they work for their own buffers and samples. It has been reported that TCEP is not stable in phosphate buffer1. It is prudent for the user to do his/her own control experiment first to ensure that the baseline artifacts are minimized.

We have also noted that, in some of the literature, no anomalies in the DSC or ITC baseline have been reported, even though DTT was used in calorimetric experiments (e.g., Arold et al., *Biochemistry* 37: 14683, 1998; Popovic et al., *in Biocalorimetry*, Ladbury JE, and Chowdhry BZ, eds, John Wiley, p. 277, 1998). This seems to suggest the possibility that under certain conditions, DTT could have no adverse effect on ITC and DSC data.

[1]Han JC, and Han GY. *Anal. Biochem*. 220: 5-10, 1994.

[2]*Preview* 2(4): 16, 1999, a technical publication by Pierce Chemical Co.