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Notes & Tips

A nondetergent sulfobetaine improves protein unfolding reversibility in microcalorimetric studies

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ABSTRACT

A nondetergent sulfobetaine (NDSB) was found to improve unfolding reversibility of several proteins by inhibiting heat-induced aggregation. As a consequence, $\Delta H_{cal}/\Delta H_{vH}$ ratios were also improved to values close to 1 for a two-state unfolding. NDSB is effective in a wide range of pH values and especially at acidic pH generally used to calculate ΔC_p values by the Kirchhoff relation. The sulfobetaine also allows recording protein refolding by protecting the heat-induced unfolded state against aggregation.

Differential scanning calorimetry (DSC)[1] allows a fine thermodynamic analysis of heat-induced unfolding of macromolecules and has contributed significantly to the elucidation of protein energetics [1–3]. However, recording fully reversible unfolding transitions is essential to calculate reliable and accurate parameters associated with protein stability in the formalism of equilibrium thermodynamics. Unfortunately, unfolding reversibility is restricted to a limited number of small proteins because most proteins aggregate as a result of the entropy-driven, nonspecific association of nonpolar groups exposed to the solvent in the heat-induced unfolded state [4]. Such limitation has impaired the microcalorimetric analysis of many proteins. In a previous study, we showed that a nondetergent sulfobetaine (NDSB) prevents protein aggregation in DSC experiments [5]. Here we report that NDSB greatly improves unfolding reversibility and even allows recording refolding of a large (49.3-kDa) multidomain protein by DSC.

Samples (~4 mg/ml) of the recombinant α-amylase from the gram-negative bacterium *Pseudoalteromonas haloplanktis* [6] were dialyzed overnight against 30 mM Mops [3-(*N*-morpholino)propanesulfonic acid], 50 mM NaCl, and 1 mM $CaCl_2$ (pH 7.2). Before experiments, an equal volume of 2 M NDSB [3-(1-pyridinio)-1-propanesulfonate, Fluka] prepared in the dialysis buffer was added to both the protein sample and the reference buffer. Reference baselines were recorded using the latter solution. Both the reference and sample cells of the microcalorimeter were rinsed thoroughly with 1 M NDSB in the dialysis buffer. Protein concentration was determined by the bicinchoninic acid protein assay reagent (Pierce) before the addition of NDSB and was approximately 2 mg/ml in the calorimeter cell. Measurements were performed using a MicroCal VP-DSC instrument at a scan rate of 60 K h^{-1} unless otherwise stated and under approximately 25 psi positive cell pressure. Thermograms were analyzed using the MicroCal Origin software (version 7).

The ultrasensitive VP-DSC microcalorimeter has a low cooling rate after completion of the unfolding transition (from 50 to 15 °C in ~15 min as compared with, e.g., ~1 min obtained with the water bath of an MCS–DSC microcalorimeter). The unfolded state of the protein, therefore, is exposed to high temperatures for longer periods. Fig. 1 (upper panel) displays 24 consecutive scans without refilling in conditions favoring optimal in vitro stability of the protein. In this experiment, thermograms were interrupted after more than 99% completion of the unfolding transition because the main criterion for reversibility is the lack of kinetically driven aggregation within the transition and a post-transition baseline is required only for progress baseline subtraction. As judged from the transition areas, the native state concentration decreases between scans, with a mean loss of approximately 6% of ΔH_{cal} values per scan. As the native state concentration decreases between scans, the C_p values of the pretransition baselines increase. However, the C_p values of the posttransition baselines decrease, indicating that the unfolded state does not accumulate but rather is converted into another species, presumably in the form of aggregates. An unsuspected result of this experiment is the occurrence of a point at the beginning of the transition at which all scans cross each other, mimicking an isosbestic point in spectrophotometry. This point, which could be tentatively termed an isothermic point, corresponds to the temperature at which all thermograms have an identical C_p value and is obviously independent of the native state concentration in the sample.

NDSB, a nondetergent sulfobetaine, has been reported to prevent efficiently heat-induced protein aggregation [5,7], and the above-mentioned experiments were performed in the presence

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[1] *Abbreviations used:* DSC, differential scanning calorimetry; NDSB, nondetergent sulfobetaine [3-(1-pyridinio)-1-propanesulfonate]; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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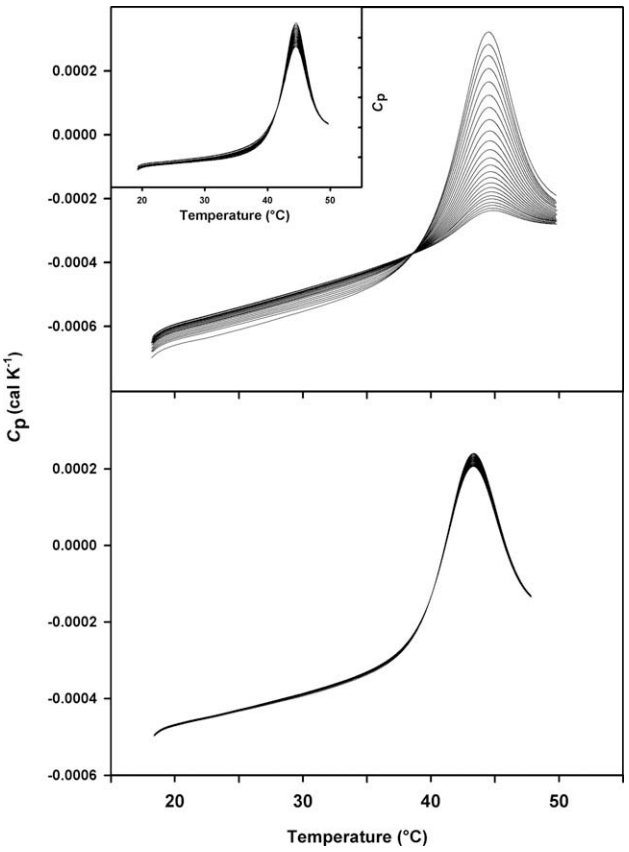

Fig. 1. Raw DSC data showing 24 consecutive scans without refilling of the bacterial α-amylase. Upper panel: 30 mM Mops containing 50 mM NaCl and 1 mM $CaCl_2$ (pH 7.2). Inset: same conditions with the addition of 1 M NDSB. Lower panel: 30 mM Mops containing 5 mM ethyleneglycoltetraacetic acid (EGTA) and 1 M NDSB (pH 7.2).

of this compound. NDSB greatly improved the unfolding reversibility of the bacterial α-amylase, with a mean loss of approximately 0.7% of ΔH_{cal} values per scan (Fig. 1, inset). Calcium ions promote the heat-induced aggregation of the bacterial α-amylase [6]; accordingly, a similar experiment was performed without added calcium. Calcium removal results in a slight decrease of the melting point (~1 °C). Fig. 1 (lower panel) shows that, under these conditions, the sulfobetaine promotes the optimal unfolding reversibility of the protein with an estimated loss of approximately 0.3% of ΔH_{cal} values between scans. It is worth mentioning that the unfolding cycles can be performed sequentially 24 times over 2 days with only a marginal decrease in the native state concentration. We have also noted that another multidomain protein, a bacterial cellulase [8] composed of a catalytic domain and a cellulose-binding domain connected by a long linker, unfolded irreversibly in DSC, whereas the addition of 0.5 M NDSB provided full reversibility of the heat-induced unfolding transition.

Similar experiments were performed with bacteriophage lambda lysozyme (19 kDa) in 40 mM acetate buffer at pH values ranging from 4.0 to 5.6 (data not shown). Here also, 1 M NDSB provided full heat-induced unfolding reversibility at acidic pH levels and improved $\Delta H_{cal}/\Delta H_{vH}$ ratios to values close to 1 for this two-state unfolding as compared with thermograms recorded in the absence of the sulfobetaine. This indicates that NDSB is effective at the acidic pH generally required for accurate determination of ΔC_p values on ΔH_{cal} versus T_m plots using the Kirchhoff relation.

Protein refolding is usually not recorded by DSC because the long equilibration time before down-scans induces aggregation of the unfolded state exposed to high temperatures. Considering the protective effect of NDSB on aggregation, we explored the feasibility of recording down-scans in the presence of this compound. Fig. 2 displays five consecutive unfolding/refolding cycles of the bacterial α-amylase without refilling in the presence of 1 M NDSB. Whereas up-scans were performed at a constant heating rate (60 K h^{-1}), down-scans were recorded at decreasing cooling rates (90–7.5 K h^{-1}). Up-scans do not superimpose perfectly as in Fig. 1 because the thermal history of the calorimeter is altered by the varying down-scan rates. In this experiment, up-scan thermograms were recorded at temperatures much higher than the unfolding transition so as to (i) record a posttransition baseline, (ii) equilibrate the calorimeter at the final temperature (~4 min at 60 °C), and (iii) record a pretransition baseline for the subsequent down-scan. Fig. 2 illustrates that, despite the drastic conditions imposed, NDSB allows measurement of refolding thermograms with a moderate loss of protein by aggregation. Furthermore, this loss can be quantified by the ΔH_{cal} value of a subsequent up-scan so as to correct the protein concentration of the preceding down-scan. In the absence of NDSB (Fig. 2, inset), a flat thermogram was recorded for the first down-scan, indicating that all of the protein was already aggregated after the first unfolding transition, thereby demonstrating the efficiency of the sulfobetaine. It should also be noted in Fig. 2 that the apparent T_m values for the down-scans display noticeable scan rate dependence. This suggests that refolding is considerably slower than unfolding in the conditions used [9].

In conclusion, NDSB has been found to improve unfolding reversibility of several proteins by inhibiting heat-induced aggregation. As a consequence, $\Delta H_{cal}/\Delta H_{vH}$ ratios were also improved. NDSB is effective in a wide range of pH values and especially at acidic pH generally used to calculate ΔC_p values by the Kirchhoff relation. The sulfobetaine also allows recording protein refolding by protecting the heat-induced unfolded state against aggregation. It was proposed that the NDSB efficiency in preventing heat-induced protein aggregation arises from both a hydrophobic shield and a charge-screening effect provided by the sulfobetaine chemical functions, thereby avoiding aggregation of unfolded or partly unfolded polypeptides [5,7].

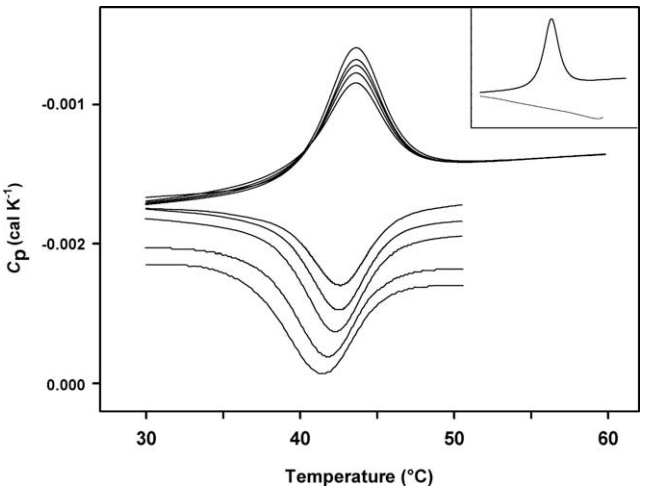

Fig. 2. Raw DSC data showing five consecutive scan cycles of unfolding–refolding without refilling of the bacterial α-amylase in 30 mM Mops containing 50 mM NaCl, 1 mM $CaCl_2$, and 1 M NDSB (pH 7.2). Up-scans (upper curves) were performed at 60 K h^{-1} up to 60 °C. Microcalorimeter equilibration at this temperature before down-scans was approximately 4 min. Down-scans (lower curves) were performed at 90 (lower curve), 60, 30, 15, and 7.5 K h^{-1}. Inset: first up-scan (upper curve) and down-scan (lower curve) in the absence of NDSB, showing the lack of refolding transition.

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