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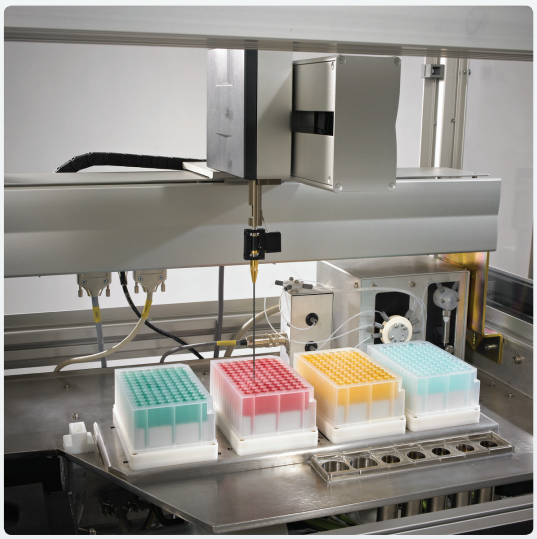

CHIRASCAN SERIES APPLICATION NOTE

Quantification of Subtle Changes in Protein Stability by Automated CD Spectroscopy

Abstract: This application note demonstrates the use of automated circular dichroism spectroscopy in detecting subtle changes in protein stability. A protein is gradually destabilised by increasing denaturant concentration, and this is related to the ΔG° of unfolding. Due to the reproducibility and number of samples that can be run on the Chirascan-plus ACD instrument, extremely accurate and consistent data can be generated that can easily discriminate changes in ΔG° of unfolding of significantly less than 2 kJ mol^{-1}.

INTRODUCTION

The quantification of protein stability is of high relevance in both academic research and the pharmaceutical industry. Protein stability is a sensitive quantitative measure for analysing how a protein reacts to alterations in amino acid sequence or environmental parameters such as pH, ionic strength, ligands, excipients, etc. Moreover, protein stability correlates with protein shelf-life, which is of high interest in pharmaceutical formulation processes of, for instance, monoclonal antibodies[1]. During stability measurements, a protein is gradually destabilised by changes in solution conditions, most commonly by elevation of temperature or addition of chemical denaturants. For thermal protein unfolding, various medium-throughput methods like thermal shift assay (TSA), differential scanning calorimetry (DSC), or light scattering are available[1]. However, a major disadvantage of thermal unfolding is that it is often difficult to obtain true equilibrium data because proteins tend to aggregate at high temperatures, thus impeding thermodynamic analysis.

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KEYWORDS

▸ Automated Circular Dichroism	▸ Protein Stability
▸ Chirascan-plus	▸ Excipient
▸ Chemical Denaturation	▸ Titration
▸ Gibbs Free Energy of Unfolding	▸ Automation

For many proteins, equilibrium unfolding induced by chemical denaturants such as urea or guanidinium chloride (GdnCl) is much better suited for quantification of protein stability[2]. In the simplest case, unfolding is described by a unimolecular equilibrium in which only the folded and the unfolded protein species are populated. In the range where both species are populated to a measurable extent (i.e., within the transition range), equilibrium constants, *K*, can be determined. Then, *K* values are used to calculate Gibbs free energies of unfolding, $\Delta G° = -RT \ln K$, which in turn are extrapolated to the Gibbs free energy of unfolding in the absence of denaturant, $\Delta G°(H_2O)$[2]. The long extrapolation from the transition range, which typically occurs at concentrations of several molar urea or GdnCl, to 0 M denaturant results in rather large error margins for $\Delta G°(H_2O)$, which interferes with a reliable quantification of small differences in protein stability.

We have recently demonstrated that automated CD spectroscopy (ACD) yields precise and reproducible spectroscopic data and exemplified how it can be used for chemical equilibrium unfolding experiments[3]. Here, we exemplify how ACD can be applied to quantify subtle changes in protein stability by measuring protein unfolding transitions with very high resolution (i.e., with 48 different urea concentrations), high signal-to-noise ratio (S/N), and excellent reproducibility. To this end, we performed equilibrium unfolding experiments on the same protein (henceforth referred to as protein A) by titrating it with urea in the presence of four different excipients that stabilise the protein to different extents.

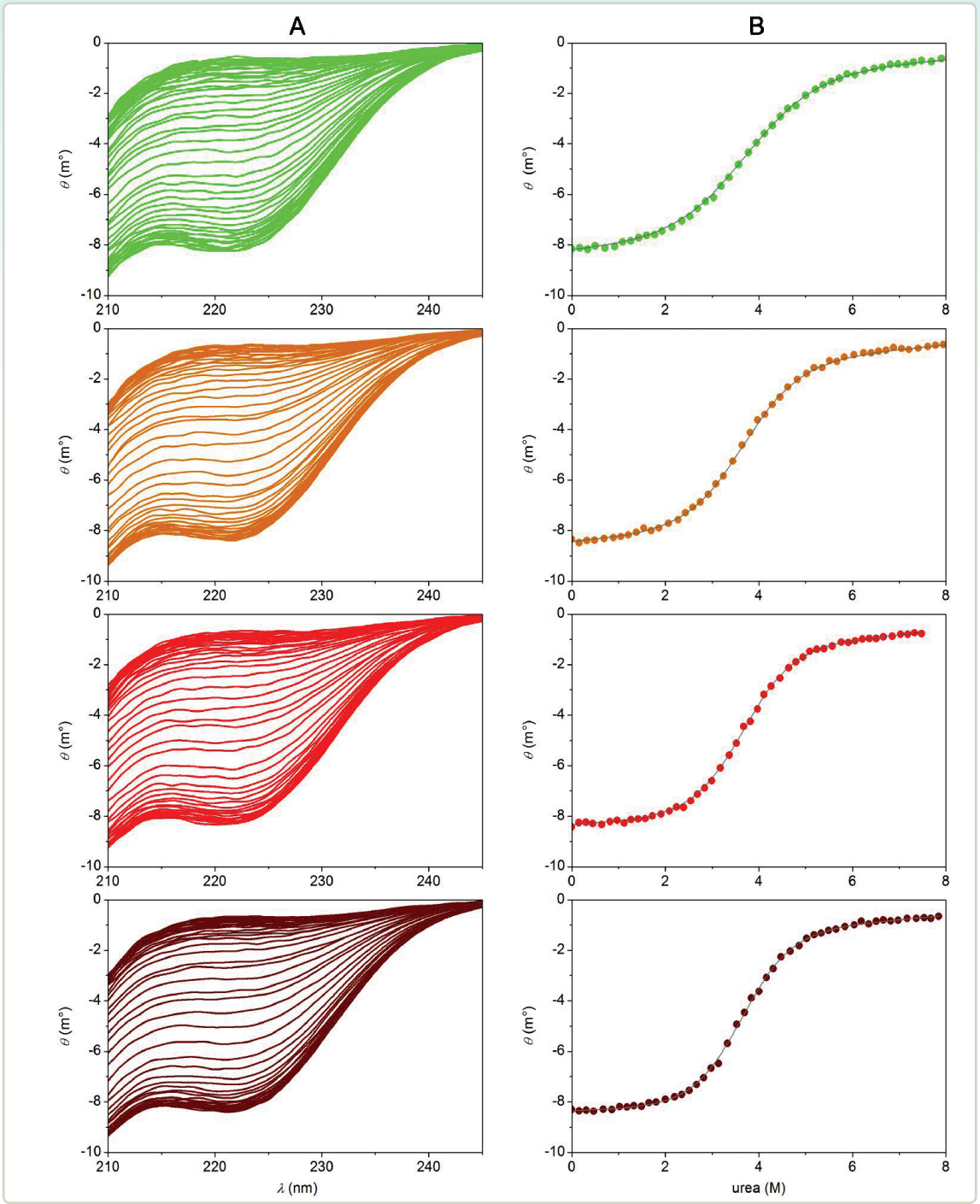

Figure 1. **A**) CD spectra acquired upon urea titration (0–7.5 M or higher) of protein A in the presence of excipients 1, 2, 3, and 4. **B**) Unfolding isotherms at λ = 222 nm for protein A in the presence of excipients 1, 2, 3, and 4. Lines represent nonlinear least-squares fits according to equation 1.

For each of the four excipients, we monitored the transition from the folded state at 0 M urea to the unfolded state at urea concentrations >7 M using a total of 48 urea concentrations per titration (**Figure 1A**). CD spectra were recorded at a protein concentration of 0.25 mg/mL and represent averages of 10 repeated scans, each of which was acquired with a digital integration time of 1 s, yielding excellent S/N ratios over the entire wavelength range investigated. The corresponding unfolding isotherms also exhibit very low noise levels (**Figure 1B**). All experiments were performed in triplicate and were highly reproducible.

The steepness of the unfolding transitions increased from excipient 1 to excipient 4, whereas the transition midpoint, c_M, was rather unaffected, always falling in the range of 3.5–4 M urea. To elucidate how $\Delta G°(H_2O)$ of protein A depends on the presence of the four different excipients, we analysed the unfolding isotherms according to the linear-extrapolation model[4] (LEM) using nonlinear least-squares fitting (NLLSF)[5]. The LEM is described by the following equation:

$$Y_{obs} = \frac{Y_U - Y_F + c_D(\beta_U - \beta_F)}{1 + e^{\frac{\Delta G°(H_2O) - m\, c_D}{R\, T}}} + Y_F + \beta_F\ c_D \qquad (1)$$

With c_D being the concentration of denaturant, R the universal gas constant, and T the absolute temperature. The six fitting parameters include the two thermodynamic quantities $\Delta G°(H_2O)$ as the sought measure of protein stability and m, which reflects the sensitivity of protein stability against the denaturant, as well as four parameters describing the pre- and post-transition baselines (i.e., the y-axis intercepts Y_f and Y_u and the slopes β_f and β_u). As **Figure 2A** reveals, protein A is least stable in excipient 1, with $\Delta G°(H_2O)$ = 12 kJ/mol. The stability increases to $\Delta G°(H_2O)$ = 15 kJ/mol for excipient 2 and reaches a maximum of $\Delta G°(H_2O)$ = 17 kJ/mol for excipients 3 and 4.

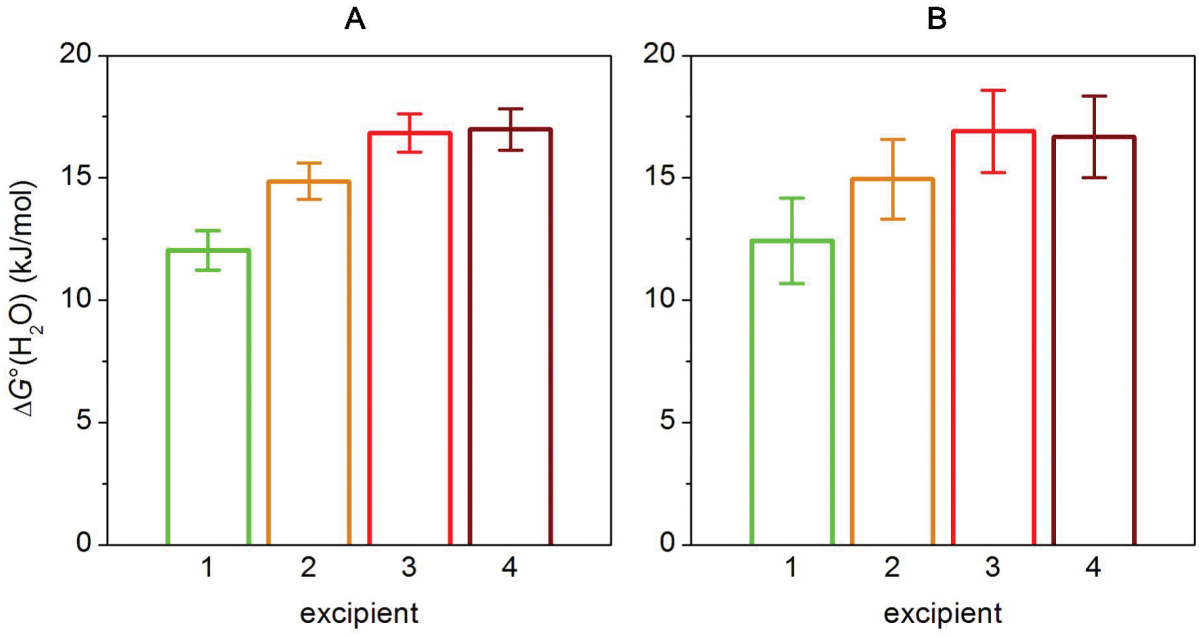

Figure 2. **A)** Stability of protein A in four different excipients as obtained by ACD. **B)** Stability of protein A in the same set of excipients as derived from a simulated urea titration with a reduced number of data points typical of manual experiments. To this end, ACD datasets were reduced to 12 urea concentrations per unfolding isotherm. For both original and reduced datasets, $\Delta G°(H_2O)$ was determined by global analysis of triplicate urea titrations. Error bars represent 68.3% confidence intervals (corresponding to ±1 SD) derived by error surface projection[5].

Rather than fitting each of the three unfolding isotherms for each of the excipients separately, the high S/N and the excellent reproducibility of the data allowed a global analysis of the three isotherms sharing all six adjustable parameters. Thus, $\Delta G°(H_2O)$ could be determined with 68.3% confidence intervals (corresponding to ±1 SD) of ±0.8 kJ/mol in all four experiments. This enabled reliable quantification of $\Delta\Delta G°(H_2O)$ values as small as 2 kJ/mol, as is the case for protein A in excipient 2 compared with excipients 3 and 4. It should be noted that the confidence intervals cited here were determined using error surface projection[5] and that error analyses implemented in standard NLLSF programs would yield even narrower error margins.

Figure 2B shows the results obtained when the same analysis was applied to a simulated manual urea titration with a reduced resolution of 12 urea concentrations in each isotherm. Most importantly, the resulting decrease in resolution adversely affects the precision of the fit by a factor of two and impairs the quantification of small $\Delta\Delta G°(H_2O)$ values. Moreover, assuming an average time of 30 min spent on each sample (including data acquisition as well as cell cleaning and drying), a manual urea titration comprising 12 samples and 12 corresponding buffer blanks would keep a user busy for 12 h. Besides this obvious advantage of saving precious human resources, automated data acquisition and cell cleaning result in better experimental reproducibility and, consequently, higher precision than usually achieved with a manually operated instrument.

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