Protein folding and Chevron plots, How to get the best from your SFM stopped-flow

I. Introduction

Protein folding is the physical process by which a protein chain acquires its native 3-dimensional structure in the cell, a conformation that is usually biologically functional. For some reasons, proteins do not always fold as they should and misfold. This dysfunction is the source of several degenerative diseases such as Creutzfeldt-Jakob and Alzheimer. So, it is essential to truly understand the folding process in the quest of future drugs. The folding process is associated with protein stability. Protein stability is defined as the difference in free energy between the native and the unfolded/denatured state. The folding process is different for all proteins: it can be a direct transition from native to denatured state, but it often goes through folding intermediates or a globen module.

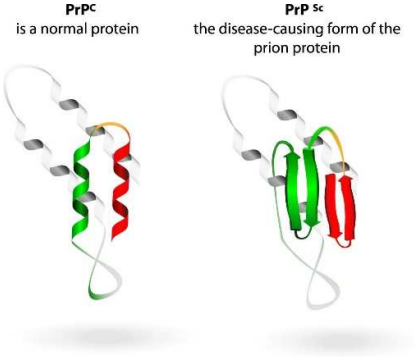

Figure 1 example of misfolding with loss of secondary structure with prion protein

Stopped-flow instruments have been major instruments for protein folding studies for decades[1,2,3] as they offer direct access to free energy of folding and folding rates. But a full series of experiments is highly time-consuming and researchers do not always realize that stepping motor drive stopped-flow can save them both valuable time and samples. This application note concentrates on chemical denaturation and demonstrates the huge advantages that researchers folding proteins can can experience using a BioLogic SFM stopped-flow.

At a glance
In this document you will learn how to:

- Quickly identify the protein denaturation midpoint using the titration accessory.
- Rapidly build Chevron plots.
- Change mixing ratios quickly and easily via Biokine the Stopped Flow interface.
- Execute automated concentration dependence studies (thereby reducing the risk of human error and saving valuable laboratory time)

In this application note a two-state transition model has been selected to demonstrate how the stopped-flow can be used to access stability parameters, while the same method can also be transposed to other folding models.

II. Denaturation midpoint

The denaturation midpoint is usually determined from equilibrium experiments. For a two states model, the denaturation midpoint is obtained when you get the same amount of native and unfolded protein in solution. An efficient way to perform these equilibrium measurements is to use the SFM titration accessory (Figure 2). A standard steady state quartz cell with a micro-stirrer is fitted into the observation head of the stopped-flow in place of the flow cell used for fast kinetics experiments. One SFM syringe is used to deliver denaturant into the cell with as sub-µl

precision and a second syringe is used to compensate the volume before the measurement so the volume is constant in the cell.

Figure 2 SFM with titration accessory

The independent stepping motor technology is ideal for such measurements, as a full titration can be programmed in seconds. Also, the software interface is used for titration and kinetics which makes the system ideal for research laboratories or teaching environments.

In fact, the user only needs to indicate the volume to titrate the initial concentrations, the number of steps and the size of the concentration steps (Figure 3). The software automatically calculates the volume to inject and plots the measured optical signal versus the denaturant concentration.

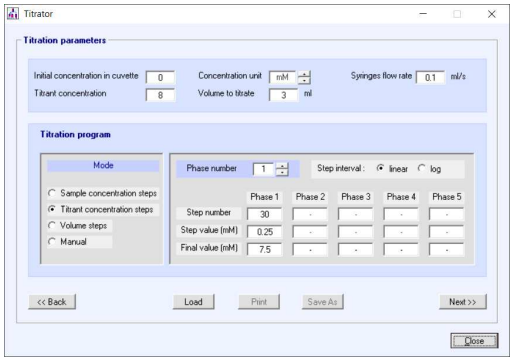

Figure 3 Example of protein titration: concentration of denaturant is increased by 0.25M steps at each measurement

To obtain increased precision around the midpoint it is also possible to change the increment step value so smaller concentration changes are executed in the equilibrium area. The unfolded fraction versus concentration is plotted (Figure 4), the denaturation midpoint is easily determined when the unfolded fraction reaches 0.5 and by fitting.

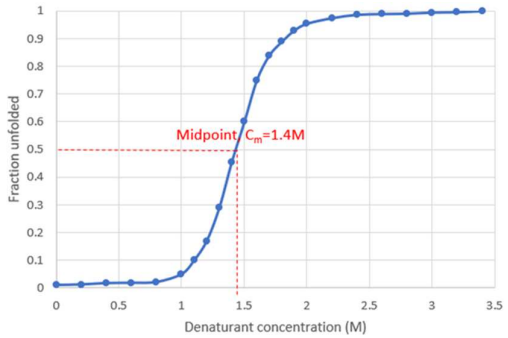

Figure 4 Example of determination of denaturation midpoint

To know the denaturation midpoint is essential to plan correct refolding or unfolding stopped-flow experiments and to obtain access to the stability parameters of the protein (free energy and relaxation time).

III. What is a Chevron plot?

A chevron plot is a representation of the protein folding kinetics data in the presence of varying concentrations of chemical denaturant. The most common chemical denaturants used in the litterature for stability studies are urea and guanidinium hycrochloride (GndHCl). These chemical denaturants need to be handled with care as they cristalize easily at high concentrations. At the end of a series of experiments, a rigorous cleaning procedure needs to be applied to prevent the formation of crystals.

The purpose of the plot is to determine the unfolding stability (the free energy of unfolding) of the protein in the absence of denaturant, but it can also give the observed relaxation rate of the protein.

The stopped-flow is used to mix the protein with different concentrations of denaturant. It generates a change in the fluorophore environment or a change of the secondary structure of the protein which can be tracked by the photomultiplier detector of the stopped-flow. The most popular detection technique is fluorescence because it is highly sensitive and because most proteins contain intrinsic fluorescent amino acids like triptophan.

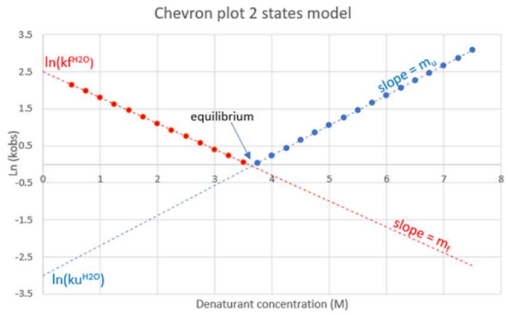

Figure 5 Chevron plot for a two state model (rounds correspond to experimental data points)

A Chevron plots is built in two phases:

• Folding experiments (in red, in figure 4): the protein is kept unfolded in a high concentration of urea or Guanidine hydrochoride (typically 6 or 8M so above denaturation midpoint concentration). The protein is then refolded by mixing with buffer in the stopped-flow. The mixing ratio is chosen so the final concentration of denaturant is below the denaturation midpoint concentration. For example starting from 6M Urea, in a first experiment, a 1:9 mixing ratio would give a final concentration of 0.6M; in a second experiment a mixing ratio of 1:4 would give a final concentration of 1.2M. The user can set the mixing ratio he wants (eg 1:2, 1:3, 1:7.6…) quickly and easily, via the instrument software. There is no need to modify the mechanics of the instrument as with rival Stopped Flow products. This function, which is unique to BioLogic stopped flows, is made possible because of the instrument's independent stepping motor drive and can open up scientific opportunities as well as help researchers save valuable lead time.

For each final concentration of denaturant, the observed rate constant is measured and its logarithm plotted versus the denaturant concentration.

• Unfolding experiments, (in blue in figure 4). The protein is native in buffer. The protein is then unfolded by mixing with a high concentration of denaturant buffer in the stopped-flow. The mixing ratio is chosen so that the final concentration of denaturant is above the denaturation midpoint concentration. For example, by mixing protein with 6M Urea, using a 1:5 mixing ratio final concentration of denaturant, gives 5M. For each final concentration of denaturant the observed rate constant is measured and its logarithm plotted versus the denaturant concentration.

The Chevron plot is the logarithm plot of the observed rate constant versus the concentration of denaturant. The plot is known as "chevron" plot because of the typical chevron shape observed when using a two state model.

In a two-state model, the logarithm for folding and unfolding rates is assumed to depend linearly on the denaturant concentration, thus resulting in the m-values (slopes) mf and mu as shown in figure 4. The sum of these two m-values is the observed relaxation rate.

By extrapolating the folding and unfolding rates to 0 it is possible to get the folding and unfolding rates in water, $\ln(k_f^{H2O})$ and $\ln(k_u^{H2O})$ as shown in Figure 5. With these two rates it is then possible to calculate the equilibrium constant k_f^{H2O}/k_u^{H2O} and the free energy of unfolding using the following formula:

$$\Delta G = RT\ln(k_f^{H2O}/k_u^{H2O})$$

Where T is the temparature in Kelvin, R is the gas constant with a value of 8.314 $J.K^{-1}mol^{-1}$.

IV. Stopped-flow to build a Chevron plot

One of the major advantages of BioLogic stopped-flow technology is the use of independent stepping motors to drive the syringes. Thanks to this driving method the user can easily select the mixing ratio (or volume) applied between syringes. This means the concentration in the observation cuvette can be changed from one shot to the other just by using the software and without changing the content of the syringe. This change of mixing ratio can be carried out manually at each shot or can be fully automated. The two modes will be described so a Chevron plot can be drawn in few minutes .

1. For folding experiments

The protein is kept unfolded in a high concentration of denaturant eg GndHCl 6M. The protein is then refolded by mixing with the buffer in the stopped-flow, or to be more precise, with a pre-mix GndHCl versus buffer

achieved in mixer 1, so the final concentration of denaturant is below the denaturation midpoint concentration. At the same time, the protein concentration is kept constant by keeping the mixing ratio (S1+S2):S3 for all concentrations.

An example of mixing sequence is given in Figure 6. A 1:9 mixing ratio is selected for the folding. For each concentration the user only needs to adjust the ratio between S1 and S2 keeping the sum equal to 9. The final concentration of GndHCL is the sum of the two concentrations given in the 'Final concentrations' area in the configuration window (see Figure 6).

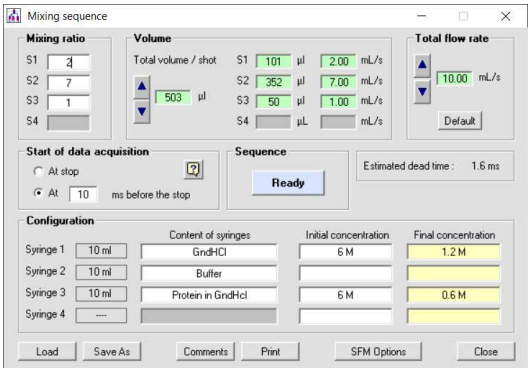

Figure 6 Example of a driving sequence to obtain GndHCl 1.8M

In the example shown in Figure 6, a 2:7 ratio between syringe S1 and S2 is shown, so 9:1 between (S1+S2): S3, would give a final concentration of 1.8M.

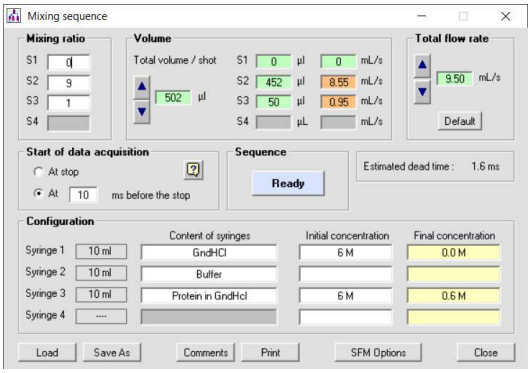

Figure 7 Example of a driving sequence to obtain GndHCl 0.6M

If S1 is not used then the unfolded protein is only folded by buffer and 0.6M is the minimum concentration of denaturant you can reach.

A series of concentrations can be carried out very quickly as the user does not need to do manual dilutions or reload the instrument. All traces are fitted independently using a single exponential model to obtain k_f and $\ln(k_f)$ is used to build the low concentration part of the chevron plot.

2. For unfolding experiments

The independent motor drive also opens unique automation possibilities so the user saves precious laboratory time. Biokine software includes a 'concentration dependence studies' mode (Figure 8) so the change of mixing ratio can be fully automated .

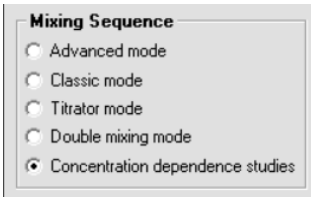

Figure 8 Automation mode in Biokine software.

The automation mode is based on the advanced mode used previously and includes the same safety limits for mixing parameters. This automation mode requires two mixers.

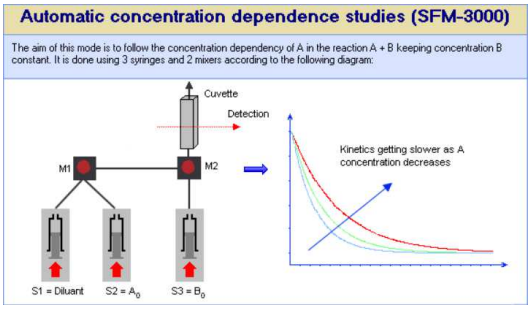

Figure 9 Principle of concentration dependence studies mode.

The aim of this mode is to change the concentration of one reactant at each shot keeping the concentration of the second reaction constant. To achieve this, the samples need to be loaded carefully, the solution where you want to keep the concentration constant must flow to the second mixer, that is to say syringe 3 (see Figure 9) and the one where you want to vary the concentration goes to syringe 2.

The native protein is loaded in syringe 3. At each shot, the protein can be mixed with an excess of denaturant so the final concentration of denaturant is above the transition unfolding midpoint. Concentrated denaturant is thus loaded in syringe 2 and a first mixing with the buffer in mixer 1 will adjust the concentration of denaturant at each shot. The user needs to

enter the concentrations of the loaded solutions and the mixing ratio he/she wants to apply at each shot between the protein and denaturant, so that the concentration of protein remains constant.

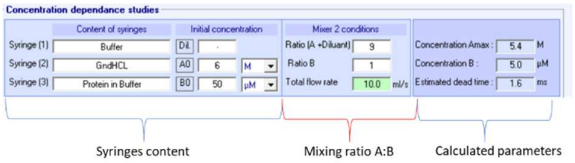

Figure 10 Setting the parameters

The mixing ratio chosen determines the maximum concentration A_{max} of denaturant that can be reached (see Figure 10). Also, all shots are carried out with the same total flow rate, so the dead time is constant, which makes data comparison much easier. The estimated dead time is indicated in the calculated parameters area (see Figure 10).

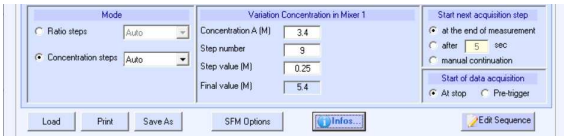

Figure 11 Concentration steps definition

The concentration steps can be achieved with a fixed incremental concentration or can be manually adjusted. The software will run the experiment from the lowest to the highest concentration. The user only needs to indicate the lowest concentration he wants to reach, the step value and the number of concentration steps. For a protein having a denaturation midpoint at 3.5M as shown in Figure 5 , the user can program some denaturations from 3.6M to 5.4M with a 0.25M concentration step (see Figure 11). The Biokine software automatically builds a mixing sequence taking in to account considerations all above parameters (see Figure 12).

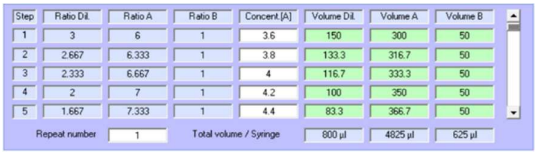

Figure 12 Mixing table generated (only steps 1-5 are displayed), 50µl of protein per shot.

For all concentration steps the calculated mixing ratio is displayed and the user can also see how much solution is required to carry out the full series of experiments. The data collection time must be selected with care as it is the same for all concentrations. The concentration is automatically saved in the title of the data file.

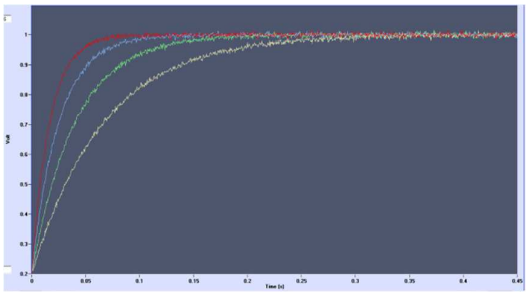

Figure 13 Example of unfolding data at different concentrations

All traces can be fitted independently (Figure 13) using a single exponential model to obtain k_u, and $\ln(k_u)$ is used to build the high concentration part of the chevron plot.

V. Conclusion

This application note demonstrates how to get the very best out of your stopped-flow instrument in order to measure protein stability. Thanks to its independent stepping motor drive with a sub-µl precision, the BioLogic stopped-flow can be converted in seconds, into a powerful titrator, to determine the chemical denaturation midpoint of a protein. Concentration dependence studies can be achieved without manual dilution to reduce human handling errors and experimental time.The user can fully automate the denaturation process and a complete Chevron plots can be built in minutes when it could take a day with other stopped-flow !

REFERENCES

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