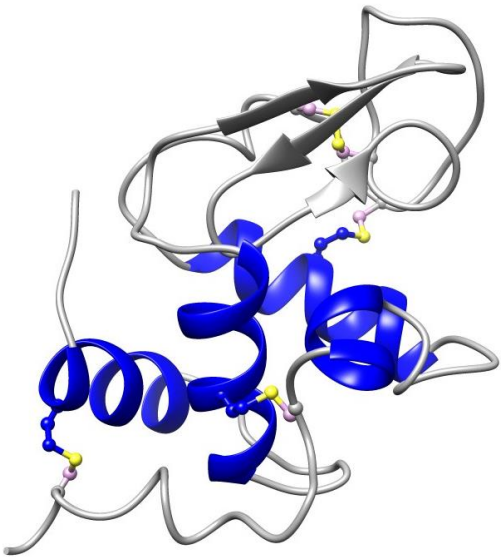
Biophysics Lab Course

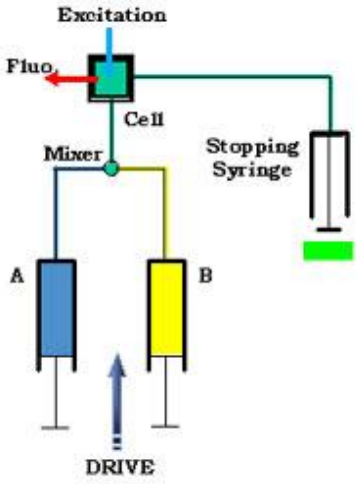
ulm university universität ulm

Stopped-flow kinetics of protein folding

Unfolding and refolding kinetics of lysozyme investigated by relaxation spectroscopy using tryptophan as a fluorescent reporter

Subjects: Protein folding kinetics and thermodynamics, Chemical denaturation, Folding pathways, Two-state model of folding, Tryptophan fluorescence, Relaxation spectroscopy





Experiment:

The unfolding and refolding kinetics of lysozyme is studied by rapid mixing of a protein solution with a buffer solution of different denaturant concentration. Changes of the folding state are followed by the relaxation of the fluorescence signal using tryptophan as an intrinsic fluorescent reporter. Rate coefficients for unfolding and refolding are determined from a 'chevron plot' of the apparent rate coefficients, assuming a two-state model for protein folding.

Please bring your lab coat and safety glasses with you for this experiment

1 Introduction

BSE (*Bovine Spongiforme Encephalopathy*) and some other neurodegenerative diseases cause a spongy degeneration in the brain in form of tangles and plaque. BSE is infectious, which due to the dogma of medicine means, that the trigger for this disease has to be capable of reproduction. However, instead of microorganisms a very resistant agent was found, resistant to attacks of the immune system, high temperatures or disinfectants.

Finally, the American neurologist Stanley Prusiner discovered that proteins are responsible for the disease and called them Prion (*proteinaceous infectious organism*, PrP^{c}). He also found that BSE, like Creutzfeld-Jakob disease, is caused by misfolding of a body's own protein to a pathogen isoform, PrP^{Sc} (nobel prize in physiology and medicine, 1997). Compared to a normal form of the protein, PrP^{Sc} has a higher content of β-sheet structures and less α-helical structures. PrP^{Sc} is able to infect "healthy" proteins by inducing conformational changes leading to the abnormal isoform. Furthermore, misfolded prion protein cannot be digested by proteases and aggregates to highly structured amyloid fibres, which accumulate to form plaques in the brain.

BSE is just one example for a whole set of diseases, which are triggered by misfolded proteins. Proteins perform a wide variety of tasks in every living organism like tissue stability (collagene,..), transport (hemoglobin, ion channels,..), protection (immune globulins,..), regulation of metabolism (enzymes, hormones). Physical and chemical properties of proteins depend on the specific three-dimensional folding of the primary structure. The protein folding process leads to the functional active form, the native structure. This process of arranging a simple amino acid sequence into the native structure of the protein consists of a complex interplay of interactions and is still subject of intense research.

1.1 The protein folding problem

FAKE!

As mentioned before, only a correctly folded protein is a functional protein. A linear polypeptide chain, which is released by the ribosome, is folded almost instantly (milliseconds to seconds) to its native conformation. The information needed for this process is completely encoded in the primary structure of the protein – at least for small proteins. Both thermodynamic and kinetic requirements have to be fulfilled for producing a functional protein: the native structure has to be stable under physiological conditions and it has to be reached very fast, at least very much faster than in a random search of conformations.

In order to exemplify the problem of protein folding, imagine a protein consisting of 101 amino acids, where two neighboring amino acids may adopt three different conformations. Even if we assume that the protein tests 10^{13} new conformations per second from a total of 3^{100} possible conformations,

a random search of all conformations would take 10^{27} years, much longer than the age of the universe (about $1{,}37 \cdot 10^{10}$ years)! This Gedankenexperiment was done by Levinthal in the sixties of the last century and is called Levinthal's paradox. Consequently, he suggested that protein folding occurs along defined pathways towards the native structure of the protein. In contrast to this, the now widely accepted model describes the folding process as a random search in a rugged potential energy landscape, consisting of the different conformations of the polypeptide chain. The energy landscape of folding is similar to a funnel (Fig. 1). The native structure, which is assumed to be the global minimum of free enthalpy, can be reached via a multitude of pathways and possibly local minima (intermediate states). Hence, protein folding can be described as a stochastic, but highly directed probing of different conformations down to the final native structure. In detail, the polypeptide starts with many possible open conformations, represented by the broad margin of the funnel. Due to thermal fluctuations, distant parts of the amino acid chain can make 'native' contacts and thus limit the number of possible conformations on the way to the native state. Due to the limited conformational space, the random search for further native contacts is much faster.

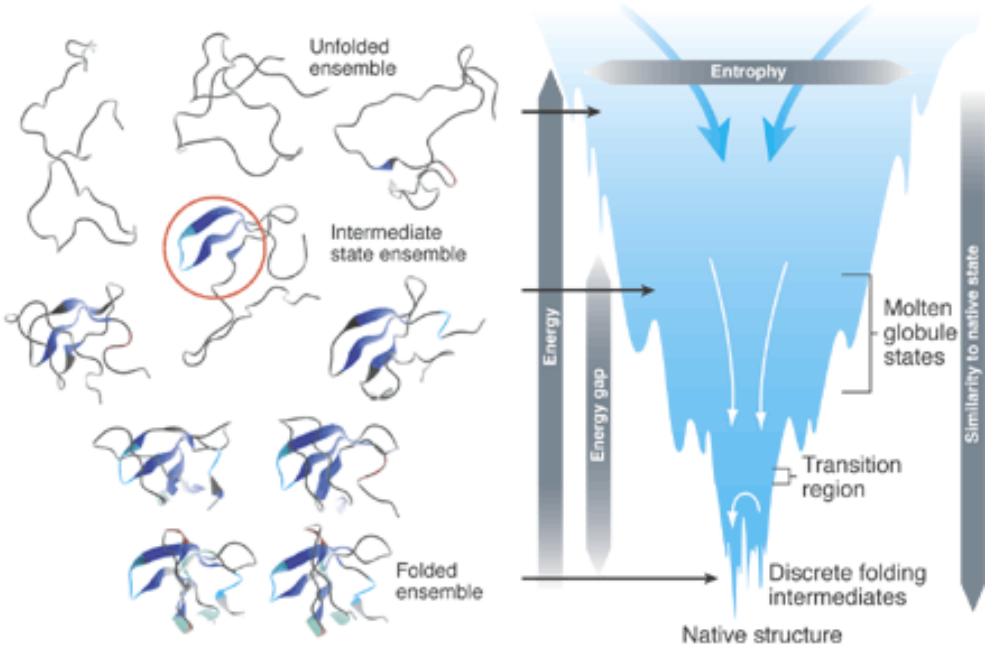


Fig. 1: The free energy landscape of folding ('folding funnel'). Conformational entropy loss during folding is compensated by the free energy gained as more native interactions are formed. (from Brooks et al. Science 293 (2001) 612-613)

STILL FALSE!

The molecular details of protein folding are mostly unknown. From a present-day perspective, the process of protein folding is determined by two kinds of driving forces: (1) the hydrophobic effect, which leads to an accumulation of hydrophobic residues in a hydrophobic core shielded from the usually polar environment, (2) early formation of some secondary and tertiary structure. There is

evidence, that partially folded proteins or stable intermediates are the reason for multiphasic folding kinetics. However, it is still under discussion if the intermediates are kinetically trapped transition states which slow down the folding process, or if these states are even necessary for an efficient folding reaction.

The change in free energy during the folding process is quite small, due to the fact, that the stabilizing interactions of the secondary and tertiary structure are weak. For example, the change in free enthalpy for a protein consisting of 100 to 200 amino acids is about 45 ± 15 kJ·mol^{-1}. This weak stabilization is important as it enables structural flexibility which is essential for protein function.

High temperatures, chemical agents or extreme pH-values can destabilize the native structure and thus denature the protein. Chaotropic agents, like GdmCl or urea, reduce the order in the hydrogen bonding network of water. Thus, the entropy of the solvent surrounding the protein is increased, which reduces the hydrophobic effect and destabilizes the native structure of the protein.

1.2 Investigation of protein folding using tryptophane fluorescence

Tryptophan is an essential amino acid and usually active as a *L*-stereoisomer. Like tyrosine and phenylalanine it belongs to the group of aromatic amino acids (Fig. 2), which are responsible for the intrinsic fluorescence of proteins. Changes in this intrinsic fluorescence can be used to monitor conformational changes of proteins.

Fig. 2: Structure of aromatic amino acids in stick model. ***A****: phenylalanine,* ***B****: tyrosine,* ***C****: tryptophan.*

UV B

Proteins can be excited by light with a wavelength of 190 – 295 nm. Tryptophan makes the largest contribution to the intrinsic fluorescence of proteins for several reasons: The three aromatic amino acids differ in their absorption and emission maxima as well as in their extinction coefficients and quantum yields (QY). Table 1 shows the very strong absorption and a considerably higher quantum yield for tryptophan.

Table 1: Spectral characteristics of aromatic amino acids within proteins

Amino acid	Abs_{max} /nm	ε_{max} /$M^{-1}\cdot cm^{-1}$	Em_{max} /nm	QY
tryptophane	280	5600	348	0.20
tyrosine	274	1400	303	0.14
phenylalanine	257	200	282	0.04

The fluorescence emission of tryptophan depends on the surrounding solvent. An increase in the polarity of the surroundings results in a shift of the fluorescence emission maximum to higher wavelengths. If the tryptophan residue is hidden in the hydrophobic core of a protein and therefore shielded from the polar solvent, the emission maximum is around 335 nm. If the protein is unfolded, the emission maximum is redshifted (Fig. 3). This phenomenon is due to the effect of solvent relaxation: during absorption of a photon, the electronic transition induces a larger dipole moment in the excited state of the fluorophore. The surrounding polar solvent molecules rearrange in order to reduce the energy of the excited state dipole and therefore the fluorescence emission from the solvent-relaxed state is shifted towards longer wavelengths (corresponding to lower energy).

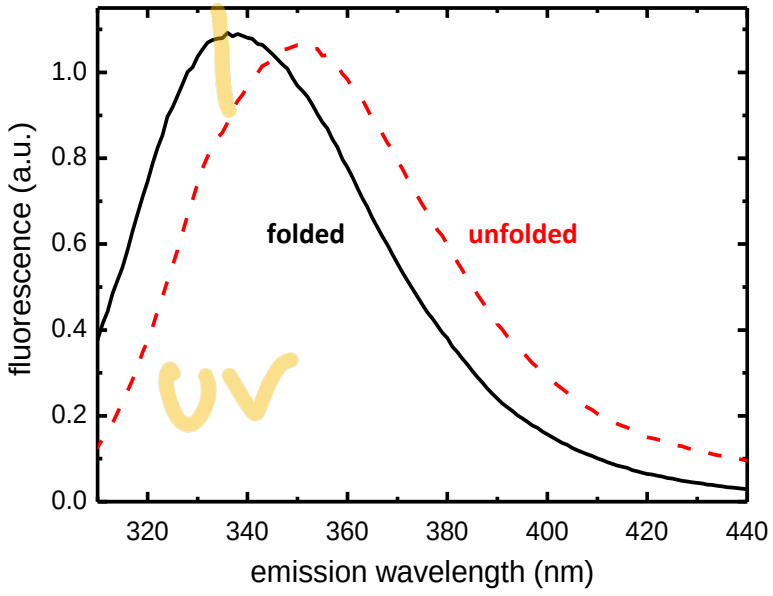


Fig. 3: Example for fluorescence emission of tryptophane in folded and unfolded protein

Denaturation of a protein with urea or guanidinium chloride (GdmCl) causes unfolding of the protein and a 'bathochromic' shift in the fluorescence emission maximum due to the contact of tryptophan with the polar solvent which causes a much larger Stokes shift than the unpolar protein core.

If we observe the variation in tryptophan fluorescence in response to a rapid change of the denaturant concentration, we are able to gain information about the folding/unfolding kinetics of proteins from chemical denaturation studies.

2 Kinetics of protein folding

2.1 Two state model of protein folding

Disregarding the burst phase (hydrophobic collapse) and the slow phase (proline isomerization), we will try to approximate the main part of the folding process by a two-state model. Many small proteins fold according to this simple model without going through any intermediate states.

$$U \underset{k_u}{\overset{k_f}{\rightleftharpoons}} F$$

For this case, the equilibrium constant between folded and unfolded state is given by

$$K_{eq} = \frac{[F]_{eq}}{[U]_{eq}} = \frac{k_f}{k_u} \qquad (1)$$

The consistency of experimentally determined thermodynamic (K_{eq}) and kinetic parameters (k_f, k_u) are a good test for the validity of the two-state model. According to transition state theory, the rate coefficients for folding and unfolding are governed by the corresponding activation free energies:

$$k_f = a \cdot e^{-\frac{\Delta G_f^{\ddagger}}{RT}}, \qquad k_u = a \cdot e^{-\frac{\Delta G_u^{\ddagger}}{RT}}, \qquad (2)$$

where the preexponential factor represents the limiting rate coefficient in absence of any free energy barrier and $\Delta G_f^{\ddagger} = \Delta G_{U \to TS}$, $\Delta G_u^{\ddagger} = \Delta G_{F \to TS}$, as shown in Fig. 4.

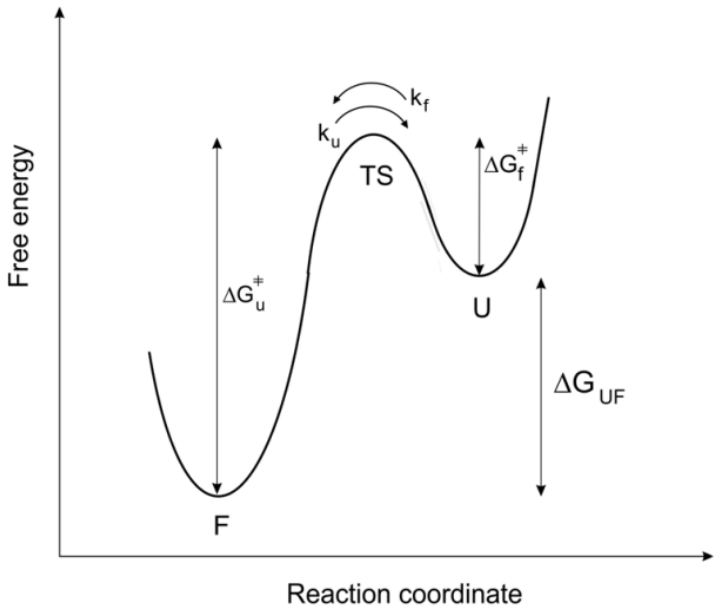


Fig. 4: Free energy landscape for the two-state model of protein folding

In general, reaction kinetics are investigated by relaxation techniques, where an experimental parameter (temperature, pressure, pH, concentration...) is rapidly changed and the formation of a new equilibrium state is tracked spectroscopically. The apparent rate coefficient for the relaxation process is given by the sum of the two first order rate coefficients for the forward and backward reaction, here:

$$k_{app} = k_f + k_u. \tag{3}$$

For protein folding/unfolding experiments, a rapid change in denaturant concentration can be used to initiate the relaxation into a new equilibrium in presence of a denaturant concentration [D]. Denaturants are chaotropic agents (urea, guanidinium chloride) which disrupt the hydrogen bonding network between water molecules and thus destabilize the folded state of proteins by weakening the hydrophobic effect.

The activation energies typically change linearly with the denaturant concentration:

$$\Delta G_{\mathrm{f}}^{\ddagger}([D]) = \Delta G_{\mathrm{f}}^{\ddagger}(0) + \mathrm{m_f} \cdot [\mathrm{D}]\,, \qquad \Delta G_{\mathrm{u}}^{\ddagger}([D]) = \Delta G_{\mathrm{u}}^{\ddagger}(0) + \mathrm{m_u} \cdot [\mathrm{D}] \tag{4}$$

Thus, the expected apparent rate coefficient for a relaxation experiments is

$$k_{app} = k_f^0 \cdot e^{-\frac{\mathrm{m_f}\cdot[\mathrm{D}]}{RT}} + k_u^0 \cdot e^{-\frac{\mathrm{m_u}\cdot[\mathrm{D}]}{RT}} \tag{5}$$

where k_f^0 and k_u^0 denote the rate coefficients in absence of denaturants. The apparent rate coefficient is dominated by the folding rate at low denaturant concentration and by the unfolding rate at high denaturant concentration (note that $\mathrm{m_u}$ must be negative, as the activation energy for unfolding is lowered by the denaturant). This feature can be visualized in a semi logarithmic 'chevron plot' (Fig 5).

Data analysis yields the rate coefficients (k_f^0, k_u^0) and the denaturant dependence of the activation free energies ($\mathrm{m_f}$, $\mathrm{m_u}$). From the extrapolated rate coefficients the stability (the free energy of unfolding) of the protein in the absence of denaturant $\Delta G_{UF}^0 = RT \cdot \ln(\frac{k_f^0}{k_u^0})$ can be determined. Thus, consistency with experimental results for the thermodynamics of folding can be inspected in order to judge validity of the two-state model.

Chevron

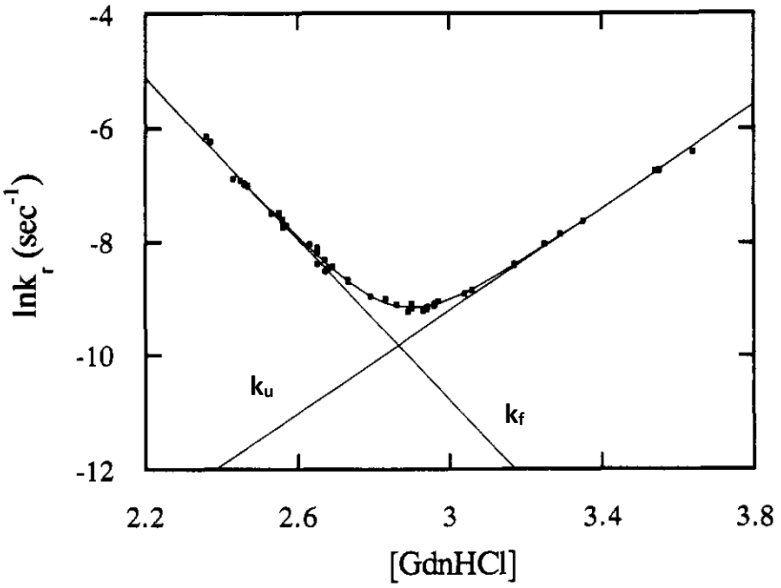


Fig. 5: Example for a plot of the apparent rate coefficient vs. concentration of guanidinium chloride. Due to the logarithmic scaling of k_{app}, the characteristic chevron shape appears. The points are experimental data for T4 lysozyme, the curve shows a fit to the data according to eq. (5) , the straight lines are extrapolations for the individual rates k_f and k_u (taken from: Chen, B.L. et al., Biochemistry (1992), 31, 1464).

Frequently, folding kinetics cannot be fully described by a simple two-state model. Depending on the experimental design, more complex kinetic traces are observed, indicating transiently populated intermediate states and branched folding pathways. Furthermore, additional slow processes like proline isomerization and 'reshuffling' of disulfide bonds may contribute to the folding process *in vitro*. *In vivo,* these processes are often catalyzed by specialized enzymes.

2.2 Subject of study: Lysozyme

Lysozyme from hen egg white is a small globular protein consisting of 129 amino acids. Due to its ability of hydrolysing glycosidic bonds in the bacterial cell wall and thus destroy their structural integrity, it shows antibiotic activity. Structurally, lysozyme consists of two domains which are separated by the deep substrate binding cleft of the enzyme: an alpha-helical domain, which contains both the amino- and the carboxy- terminus and a smaller beta-sheet domain (Fig. 6). This kind of combined α- and β- structure is also found in other proteins and is generally called a lysozyme-like fold.

Lysozyme from hen egg white belongs the 'c-type' lysozymes, which are closely related to alpha-lactalbumin and contain eight highly conserved cysteins (forming four disulfide bonds).

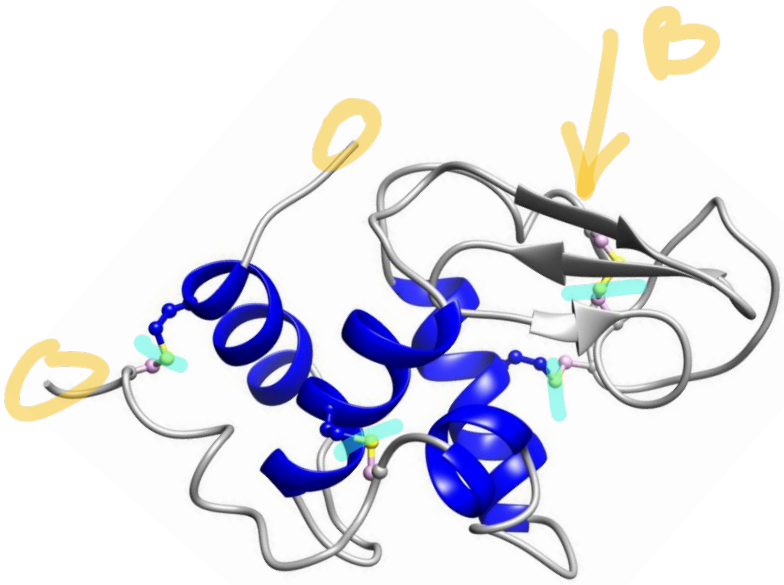


Fig. 6: Structure of hen egg lysozyme (PDB entry 132L)

2.3 Stopped-flow technique

In relaxation techniques, a (mostly small) perturbation of an equilibrium by a rapid change in an external parameter like temperature, electric field, pressure, pH or denaturant concentration is applied. Typically, the process of reaching a new equilibrium state ('relaxation') is followed by observing changes in a spectroscopic parameter like absorbance or fluorescence. This allows to determine rate coefficients for the processes involved. Here, we use a rapid mixing technique to rapidly change the denaturant concentration surrounding our protein and follow the kinetics of folding/unfolding using the intrinsic tryptophan fluorescence as a reporter.

In the stopped-flow technique, two syringes containing the initial solutions are pushed simultaneously and the solutions are combined in a turbulent mixing process. High flow rates on the order of ml/s are necessary for channel diameters on the order of 1 mm to achieve turbulent flow conditions. When the observation cell with a volume of typically several hundreds of microliters is purged and filled, the flow is stopped abruptly by a third syringe which hits a stopping block after being filled by the waste from the observation cell (Fig. 7a). The dead time of an instrument, which is a limiting factor for kinetic studies, can be quantified by observing extremely fast test reactions. Commercial stopped-flow instruments reach dead times in the millisecond range. The upper time limit for reliable stopped-flow measurements is given by the stability of the mixture in the observation cell, which is limited by movement of the reagents.

2.4 Instrumentation and experimental details

The setup consists of a stopped-flow unit for the rapid turbulent mixing of two liquids (Fig. 7a) and a detection unit, a photomultipier. Light from a LED lamp is fed into the sample chamber. Emitted fluorescence light from the sample reaches a photomultiplier detector, after being spectrally

selected by an emission filter. The photocurrent is converted to a voltage, fed into an analog-to-digital converter card and recorded by a PC ProData SX software. The subsequent kinetic analysis of the recorded traces is done with the fitting tool of this software, typically by using a single-exponential model in a limited time range.

The results of the analysis are documented in a table (table 2) during the lab course.

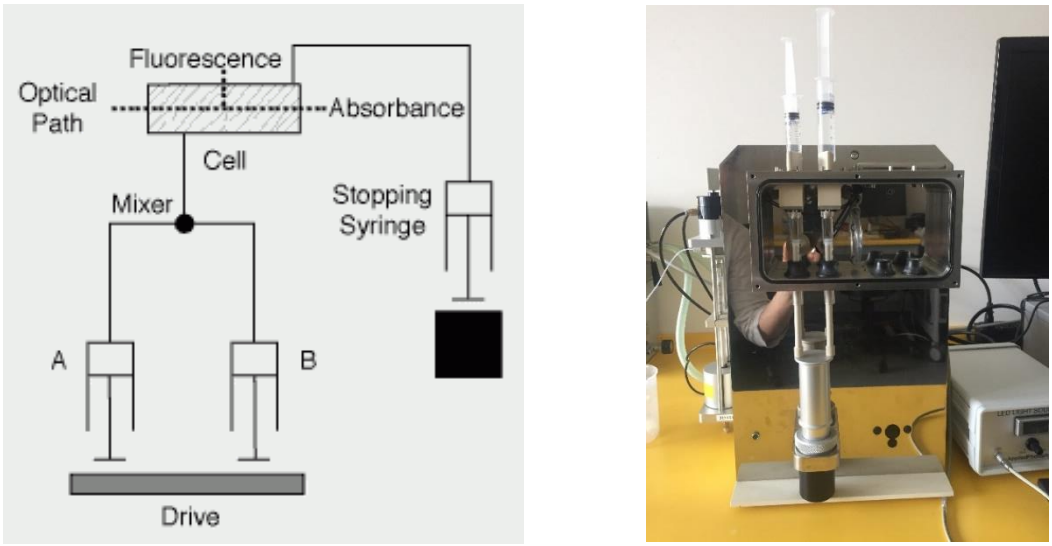


Fig 7: (a) Schematic drawing of a stopped-flow unit for rapid mixing of the liquids A and B for optical detection (source: http://www.hi-techsci.com/techniques/stoppedflow/). (b) Photograph of the SX20 Stopped-flow spectrometer from Applied Photophysics

Under the experimental conditions used here, the spectral shift upon unfolding of lysozyme is accompanied by an increase in quantum yield (Fig. 8). Thus we expect an increasing fluorescence signal upon unfolding and an opposite signal change for the folding reaction.

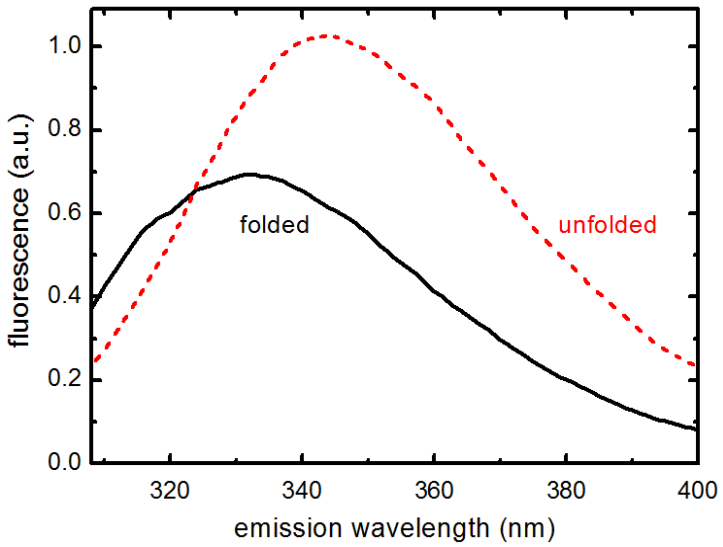


Fig. 8: Fluorescence spectra for for lysozyme in glycin buffer at pH 2.2 and for unfolded lysozyme in the same buffer with additional 6 M GdmCl.

3 Experiments

3.1 Samples and settings

All solutions contain the buffer 12 mM Glycin*HCl, 0.6 M KCl, pH=2.2
and varying concentrations of GdmCl +/- 40 µM Lysozyme

The solution for the refolding experiments which contains Lysozyme and 6 M GdmCl was prepared at least 2 hours before the measurement to accomplish complete unfolding of the protein.

Experimental settings of the stopped-flow instrument:

Excitation wavelength = 280 nm
Emission wavelength >320 nm

3.2 Refolding experiments with lysozyme

In refolding experiments, denatured lysozyme in a solution containing GdmCl is rapidly mixed with solvent in order to decrease the GdmCl concentration and therefore induce refolding of the protein. A series of experiments with different final GdmCl concentrations allows to draw the left branch of a 'chevron-plot' (Fig. 5).

3.3 Unfolding experiments with lysozyme

In unfolding experiments, native lysozyme is rapidly mixed with a denaturing solvent in order to unfold in presence of a distinct GdmCl concentration. A series of experiments with different final GdmCl concentrations allows to draw the right branch of a 'chevron-plot' (Fig. 5).

Table 2: Table for documentation of results during the lab course

	Results			
	2.5 ml syringe	**0.5 ml syringe**	**GdmCl (M)**	**k_{app} (s^{-1})**
		Refolding	0 + 6 / 2	
rf 1	0 M GdmCl	6 M GdmCl + 40 µM Lysozym		0,158
rf 2	0.5 M GdmCl	6 M GdmCl + 40 µM Lysozym		
rf 3	1 M GdmCl	6 M GdmCl + 40 µM Lysozym		
rf 4	1.5 M GdmCl	6 M GdmCl + 40 µM Lysozym		
		Unfolding		
f 1	3 M GdmCl	40 µM Lysozym		
f 2	3.5 M GdmCl	40 µM Lysozym		
f 3	4 M GdmCl	40 µM Lysozym		
f 4	4.5 M GdmCl	40 µM Lysozym		
f 5	5 M GdmCl	40 µM Lysozym		
f 6	5.5 M GdmCl	40 µM Lysozym		
f 7	6 M GdmCl	40 µM Lysozym		

We are interested in K
so if K has big residuals it is wrong

3.4 Analysis of the kinetic data from the chevron plot

Determine the rate coefficients (k_f^0, k_u^0) and the denaturant dependence of the activation free energies (m_f, m_u) from the intercepts and slopes of the limiting straight lines (Fig. 5).

Determine the equilibrium constant between folded and unfolded state: $K_{eq}^0 = \frac{k_f^0}{k_u^0}$ from the extrapolated rate coefficients in absence of denaturant and also the stability (the free energy of unfolding) of the protein $\Delta G_{UF}^0 = RT \cdot \ln(K_{eq}^0)$ in absence of denaturant.

Preparation before lab course

- Lab coats and safety goggles are needed
- USB stick is needed to save data during the lab course
- Laptop allows to analyze data while measuring

4 Literature

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User Manual SX20 Stopped Flow Spectrometer, Applied Photophysics

- Discuss cherry picking for when selecting traces for chevron plot

- the syringes are 5 to 1 in ration

- you don't want bubbles

- Wash it & consider that
- When the decay function doesn't seem right we exclude that point.

- Reset it at the end only when everything is done

- Buffer G + Buffer A
  6M Guanidine 0 M Guanidine
  Actually super solution
- We prime the Chambers
- All screen shots of Decay Plots go into Appendix

the Solutions could be sistematically shifted for all solutions

2 state - folder but it's not

Temperature not Stabilized.