Chapter 2

Protein Sample Characterization

Tina Daviter and Rémi Fronzes

Abstract

Most biophysical experiments require protein samples of high quality and accurately determined concentration. This chapter attempts to compile basic information on the most common methods to assess the purity, dispersity, and stability of protein samples. It also reminds of methods to measure protein concentration and of their limits. The idea is to make aware and remind of the range of methods available and of commonly overlooked pitfalls. The aim is to enable experimenters to fully characterize their preparations of soluble or membrane proteins and gain reliable and reproducible results from their experimental work.

Key words Purity, Concentration, Solubility, Homogeneity, Electrophoresis, Absorbance

1 Introduction

The main characteristics anyone must know about their sample are its purity and concentration, stability, and solubility. Most biophysical techniques require certain buffers, a certain sample concentration and further require very pure protein. Hence, a lot of problems can be prevented and time saved by checking basic sample properties in the relevant buffers in advance. The methods and assays covered in this chapter are well known, maybe too well: knowledge about them is taken for granted, but rarely comprehensively communicated. Protocols of them are ubiquitous so we will concentrate on reminding of their principles, capabilities, limitations, and pitfalls.

The methods covered in this chapter are various types of gel electrophoresis, spectroscopic methods of concentration determination using dyes or intrinsic absorbance, we refer to dynamic light scattering, mass spectrometry, and analytical ultracentrifugation to determine the heterogeneity of protein samples and we refer to fluorescence spectroscopy, circular dichroism, and differential scanning fluorimetry to determine the foldedness of proteins.

Membrane proteins have moved more and more out of a niche and have become mainstream objects of science and research.

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However, their handling remains more difficult than that of soluble proteins. We try to point out the differences and address some of the issues related to using the techniques described here, particularly in the Subheading 2 of this chapter.

1.1 Assessing Protein Purity, Dispersity, and Oligomeric State

To assess the purity of a protein sample means to detect the impurities in it. The main problem in all methods described here is the detection limit. To detect a contaminant present at a low concentration, a much higher concentration of your sample will have to be analyzed than to detect the protein itself. Many biophysical techniques described in this book require at least 95 % purity.

1.1.1 Gel Electrophoresis

Proteins are commonly run through gels of polymerized and cross-linked polyacrylamide, a method called gel electrophoresis. Proteins will migrate when an electric field is applied and depending on the method used, migration depends on parameters like charge, size, and shape. Subsequent staining of the proteins within the gel will reveal their location.

Most of the protocols can be adapted for membrane proteins; some have been specifically designed for them. We will point this out in Subheading 2.

The most commonly used method is denaturing *SDS-polyacrylamide gel electrophoresis* (SDS-PAGE) first described by Laemmli (1). Its advantages are simplicity, speed, and sample economy. It is used to monitor a protein sample throughout purification and experiments and to detect contaminating proteins.

In principle, proteins are incubated in a reducing and denaturing sample buffer and are heated for a few minutes at >95 °C, so they are unfolded monomers and bind negatively charged SDS (sodium dodecyl sulfate) homogenously along the amino-acid chain. The intrinsic charge of the proteins is abolished and all proteins carry a negative charge proportional to their length, their migration in a gel therefore mostly depends on their size.

Staining of the gel afterwards makes the protein species visible, which have migrated as separate bands. Their size (molecular weight, not dimensions) can be compared to standard proteins applied on the same gel. There are different ways to stain a gel. Silver staining is more sensitive than Coomassie blue staining, and fluorescent stains even more so (*see* Subheading 2).

In *native PAGE* proteins are not denatured and protein–protein interactions are not disrupted if they are of high enough affinity. This allows to monitor purity and homogeneity and to estimate the size of protein complexes. As no SDS is present all buffers must relate to the isoelectric point (p*I*) of the protein/protein complex to ensure that it is charged and will migrate. Usually, the pH and acrylamide concentration for a native PAGE protocol have to be optimized specifically for the sample under investigation.

Blue-native gel electrophoresis is more robust than native PAGE in that it uses a negatively charged blue dye that binds to the protein samples but does not denature them. This means that migration in the gel is independent of their p*I* but dependent only on their molecular weight (size) and shape. For these gels, standards are available.

Furthermore, *isoelectric focussing* is a way to run gels that separates proteins based on their isoelectric point. This can resolve proteins of very similar size, for example a phosphorylated and a nonphosphorylated form of the same protein. It is often used as the first step of 2D gel electrophoresis.

In *2D gel electrophoresis* two gel separation methods are combined. For example, a strip of an isoelectric focusing gel or a lane from a native gel is cut, placed horizontally on a denaturing gel (SDS-PAGE) and run.

Many more variations of gel electrophoresis exist and as they are inexpensive, quick, and do not require much specialist equipment, they are well worth exploiting. Good descriptions can be found in manuals that come with commercial gel apparatuses or with precast gels and on suppliers' Websites.

1.1.2 Gel Filtration

Gel filtration (GF), also called size exclusion chromatography (SEC) is often the first characterization step as well as being a purification step. The column matrix (gel) is a porous resin. It separates the proteins purely on the basis of their molecular mass and shape. Small proteins are able to travel into the porous matrix of the gel. As bigger proteins will be excluded, they will pass through the gel much more quickly, so their elution volume will be smaller. It is important to keep in mind that the elution volume of any protein or protein complex also depends on its shape. Elongated molecules will run faster than expected suggesting a larger than real molecular weight.

One symmetrical peak in a gel filtration chromatogram indicates either the presence of one pure protein or protein complex (dimer, trimer, etc) or it may also be a mix of several species in equilibrium, e.g. monomer and dimer, if association and dissociation are faster than separation of the species in the matrix.

Gel filtration columns can be calibrated with molecular weight standards, and comparison of their elution volumes with that of a given protein sample will give an idea of the size of the protein sample and therefore also indicate if it forms complexes, as well as indicate significant impurities.

The separation efficiency of a column is not buffer-dependent, so the stability of protein complexes in different buffers can be tested with this method.

If detergent is present in the gel filtration buffer, this method can be applied to membrane proteins. However, it is important to keep in mind that the nature of the detergent could modify the

oligomeric state of a protein or protein complex. From one detergent to another, the results obtained may differ. As membrane proteins are embedded in the detergent micelle it will travel through the gel filtration column at a smaller elution volume, i.e. seem of higher MW.

A few rules have to be obeyed for successful gel filtration runs, which are summarized in Subheading 2 as well as basic instructions on molecular weight estimation, column calibration, and the assessment of association equilibria resulting in polydispersity.

1.1.3 Dynamic Light Scattering

Light passing through a protein solution will be scattered. The scattering intensity is proportional to the molecular weight and the weight concentration of the scattering particle; so light scattering (LS) methods are good at detecting large contaminating species like aggregates (2). In static LS, the signal is averaged over time, whereas in dynamic LS (DLS, also called quasi-elastic LS or photon correlation spectroscopy) fluctuations of light-scattering intensity are measured over ns to ms time scales. These fluctuations are due to Brownian motion, so these are measurements of the diffusion coefficient D of the scattering species. The Stokes-Einstein equation (Eq. 1) offers a way to derive the hydrodynamic radius R_h from measurement of the diffusion coefficients:

$$R_h = \frac{k_B T}{6\pi\eta D}, \tag{1}$$

where k_B is the Boltzmann constant, T the absolute temperature in Kelvin and η the solvent viscosity. The hydrodynamic radius is very useful to interpret the peak positions in gel filtration, particularly of large protein complexes and associating systems (3), so the two methods are perfectly complementary and light scattering detectors are often put in line with gel filtration chromatography (size exclusion chromatography-multi angle light scattering or SEC-MALS).

Detergent micelles scatter light in the same way as any macromolecule. Therefore, LS cannot easily be applied to membrane proteins, as there will always be a mix of scattering species in the solution.

1.1.4 Mass Spectrometry

The strengths of mass spectrometry are supreme sensitivity as well as accuracy of molecular weight determination. In all mass spectrometry techniques, the sample is vaporized, ionized, and accelerated through an electric field. The trajectory that molecules fly on depends on their molecular weight and their charge. The accuracy of this is close to the Dalton, so that even impurities arising from digestion of a few amino acids or posttranslational modifications become apparent (4, 5).

Specialized methods of mass spectrometry exist to analyze protein complexes (6). Performed at milder conditions, the proteins in a complex stay together and the sample flies as intact

assembly. This is a great method to determine the purity and molecular weight of hetero- or homo-oligomers as it will detect the heterogeneity arising from having one or more of the components in excess, and whether the complex is dissociating. However, some complexes will not survive the conditions of the experiment and fall apart. This may be due to a low affinity, but also depends on the type of interactions holding them together. When there is heterogeneity detected in the mass spectrometry experiment controls are necessary to see if a protein complex was forced apart during the experiment or whether this is a true representation of the complex in solution. In some cases, mass spectrometry will have to be complemented with other methods for clarification.

Because detergent has to be removed, it is commonly admitted that mass spectrometry is incompatible with membrane proteins. However, some techniques have been developed to overcome this problem (7, 8). Denaturing as well as native mass spectrometry can now be applied to membrane proteins.

1.1.5 Analytical Ultracentrifugation

In both modes of analytical ultracentrifugation, sedimentation velocity (SV) and sedimentation equilibrium (SE), samples will stay at equilibrium in their native solution conditions. Unlike in mass spectrometry, there are no dissociating forces acting on the sample. However, it is more time-consuming and will even more likely require specialist help.

An analytical ultracentrifuge allows monitoring the sedimentation process of a sample while moving in the centrifugal field. Absorbance or interference measurements are most common, and fluorescence detection is becoming more available. Sedimentation velocity experiments are done at high speed to analyze the movement of the sedimenting boundaries until all sample collects at the bottom of the cell. In sedimentation equilibrium experiments only the final distribution is of interest. To obtain a nice distribution throughout the centrifugal cell, sedimentation equilibrium experiments are done at a set of lower speeds (9).

SE experiments are more accurate for MW determination; however, to analyze the heterogeneity of a sample, velocity experiments are more suitable. In SV, contaminants and aggregates become visible as separate species. To exclude concentration-dependent association or dissociation, at least three different dilutions of sample are run. Similar to gel filtration a fast equilibrium between species will result in one peak, the sedimentation coefficient of which will change with concentration, i.e. as the equilibrium shifts. This one peak represents the weight-average sedimentation coefficient of the interchanging species (10).

Both SV and SE are in principle able to analyze detergent-bound membrane proteins. Several protocols exist to measure the molecular weight of the protein (rather than protein–detergent micelle) more accurately (11, 12).

1.2 Concentration Measurements

Knowing the concentration of your protein is absolutely critical. Most biophysical techniques described in this book rely on an accurate concentration value when it comes to the stage of data analysis. As the simplest example, any titration in dependence on the protein concentration will give you results that have at least the same error as that of the concentration measurement itself. Lack of reproducibility, e.g. when measuring a dissociation constant, are often problems in determining the protein concentration reproducibly. It is therefore very important to be aware of and estimate the error associated with each method to determine the sample concentration.

If determining the concentration of a homo-oligomer, it will be important to keep track of whether you determine the concentration of monomer or oligomer. Of course, deducing an oligomer concentration makes sense only if it is known that the complex is fully formed and stable at the particular concentration and if the stoichiometry is known.

On a very practical note, many protein preparations have nucleic acid contamination (*see* **Note 1**). In this case, simple absorbance measurements at 280 nm will lead to overestimation of the protein concentration, whereas dye-based methods will not. It may be useful to compare results from different techniques, also as most of them vary in their accuracy from protein to protein.

Dye-based assays are available as kits with extensive documentation on conditions and accuracy, and reading their handbooks is highly recommended.

1.2.1 Absorbance at 280 nm

To measure the absorbance of a protein solution is quick and easy, and it is the only method in which the solution is not consumed, but recoverable for later use. Absorbance (or absorption) is most correctly measured in a peak or trough of a spectrum, i.e. where the slope is (close to) zero. The aromatic amino acids Phe, Tyr, and Trp as well as oxidized Cys have absorption peaks around 280 nm and this wavelength is normally used to measure protein concentrations. The extinction coefficients of these amino acids do not simply add up in the protein environment, so algorithms have been developed to estimate the extinction coefficient of proteins. The ProtParam Webtool on the Expasy servers (13) estimates the extinction coefficient, the pI, and other basic parameters from the sequence of a protein.

To estimate the extinction coefficient of hetero-oligomers by summation, the exact stoichiometry of the different components of the complex has to be known. Detergent micelles often interfere with absorbance measurements through light scattering, so this method is usually incompatible with membrane proteins.

Calculation of the concentration from absorbance (A) uses the Beer-Lambert law (Eq. 2):

$$A = \varepsilon \; c \; d. \tag{2}$$

Hence

$$c = \frac{A}{\varepsilon\, d},$$

where ε is the extinction coefficient (in $M^{-1}\,cm^{-1}$), c is the concentration (in M) and d the pathlength (in cm). A is dimensionless, also referred to as optical density (OD), and is actually the decadic logarithm of the fraction of light passing through a sample:

$$A = \log_{10}\frac{I_0}{I} = \log_{10}\frac{1}{T},$$

where I and I_0 are the intensities after and before the sample, respectively, and T stands for transmission.

1.2.2 Dye-Based Absorbance Assays

There are absorbance assays that use dyes that change their spectral properties upon binding to protein, like the Bradford (14), Lowry (15), or BCA assays (16). These are particularly useful when the buffer absorbs or scatters light (e.g. in the presence of detergent), when the protein does not contain aromatic residues or when the extinction coefficient of a protein is unknown. For example, the Bradford assay uses Coomassie Brilliant Blue G-250 under acidic conditions. The absorption maximum of the dye will shift from 465 to 595 nm upon binding to protein.

Particularly the Bradford and Lowry assays are vastly dependent on the nature of the protein with some proteins being stained better than others. Sample preparation has to follow a strict protocol and there is only a limited time and concentration window in which the measurement will be accurate. It may be advisable to use one of the commercially available kits to increase reproducibility.

An advantage of these assays is that contaminating nucleic acids or nucleotides do not influence the result as they do in normal absorbance measurements at 280 nm.

1.2.3 Dye-Based Fluorescence Assays

Several fluorescent dyes have been found to have a shifted emission peak or changed intensity after binding to protein. Therefore, fluorescence intensity can be used to quantitate proteins. Fluorescamine, NanoOrange, naphthalene-2,3-dicarboxaldehyde (NDA), and *o*-Phthalaldehyde (OPA) are some of them. They detect much smaller quantities of protein than any of the above methods, some down to a concentration of 10 ng/ml. As with dye-based absorbance assays, care must be taken in the preparation of protein samples and standard curves must be obtained in the same experiment for best results, but again, available kits usually make these assays relatively quick and easy. If the instructions are followed closely, they can be very reproducible.

1.2.4 Quantitative Amino Acid Analysis

An in-house or commercial service usually carries out quantitative amino acid analysis (QAA), which involves the complete hydrolysis

of a protein into amino acids followed by their separation, detection, and quantitation. QAA is the only truly accurate way to measure the concentration of a sample, so in critical cases this should be done. QAA can also be used to determine the correction factor of any other method for concentration measurement, if they have a systematic error but are otherwise reproducible.

1.3 Protein Stability: Solubility, Degradation, and Structural Integrity

There are at least three aspects of protein stability, the solubility, the stability of its fold (three-dimensional structure) and the stability against degradation. They can be related to each other, i.e. partial unfolding can expose surfaces for aggregation or precipitation.

Complexes that are composed of more than one protein can simply fall apart into their components. If this is irreversible, the relevant sample is essentially lost.

1.3.1 Protein Solubility

Protein solubility often dramatically depends on solution conditions, particularly on the pH and on the salt concentration. This relates to the protein surface, the pK_a and charge of exposed side chains or the presence of hydrophobic patches. Most notably, membrane proteins, which have large hydrophobic areas to interact with membrane in the cellular context, require detergents to keep them in solution when they are purified, and often only certain detergents will work.

Small amounts of precipitate or some aggregates might not be visible by eye, their formation might be reversible or not. In all cases precipitation or aggregation reduce the actual concentration of protein in solution. It is a different case if protein specifically or functionally "aggregates" or assembles to form dimers, oligomers, or polymers.

Many experiments require specific buffers and salt concentrations and a certain concentration of protein, so therefore it is important to check whether a protein is soluble at the required conditions. As a basic test, protein solubility should be tested at the required temperature in dependence of protein concentration, pH, and salt. Varying two parameters at a time and plotting the occurrence of precipitate in dependence of these parameters gives a basic, two-dimensional phase diagram that will suggest suitable buffer conditions for the protein to survive the measurements.

Often, partial unfolding will expose hydrophobic side chains that act as aggregation surfaces or target sites for proteases. Assessing the structural stability more specifically might give crucial information on how to keep a protein in solution (see below).

1.3.2 Protein Degradation

Protein degradation is normally due to proteases. They are either copurified, and even the smallest invisible quantities are sufficient to degrade a sample, or arise from microbial growth within a sample. To slow down any degradation, samples should be kept on ice all the time as it slows both enzymes and growth. Many proteins can

even be frozen for storage and thawed before use without losing their activity. As long as this is done properly, this may be the simplest way to avoid degradation.

Protease inhibitors can prevent digestion and are often added to the very early stages of protein purification, i.e. cell lysate. Sodium azide is typically used to stop growth in the purified sample, and some protein preparations might require protease inhibitors until the end of the procedure. This is particularly true with membrane proteins that are highly sensitive to proteases.

Both protease inhibitors and sodium azide have disadvantages and may be incompatible with the methods described in this book. It is preferable to improve the purification protocol with the aim of reducing the presence of proteases. Sometimes purifying more stable constructs of the protein is the way forward.

1.3.3 Assessing Structural Stability

Various methods exist to establish protein folding and the stability of its fold or structural integrity. Almost certainly, the structural integrity—like the solubility—depends on solution conditions (buffer, pH, salt, reducing agents, detergents) and also on the temperature. Various methods exist to determine structural changes, e.g. the CD spectrum, and the NMR spectrum undergo characteristic changes upon unfolding and the fluorescence spectrum is likely to change, too (*see* **Note 2**). Protein denaturation (unfolding, melting) experiments are usually performed as temperature scans (thermal denaturation), or as titrations with chaotropic agents like urea or guanidine hydrochloride (*see* Subheading 2.3.1).

Circular dichroism is the property to absorb left- and right-handed circularly polarized light to a different extent. All chiral molecules have this property and proteins are chiral. CD spectroscopy measures this property. Certain secondary structural elements have certain CD signals, so the method can be used to assess the fold of protein samples. CD instruments are capable of temperature scanning and can be used to measure protein melting more accurately, but one condition at a time (*see* Chapter 8 for details on the method).

A very simple and convenient way to measure thermal denaturation is *differential scanning fluorimetry*. It is often used for screening of many conditions simultaneously and very quickly. The assay uses a fluorescent dye, the emission peak of which shifts and increases massively upon binding to hydrophobic patches on proteins when they get exposed through protein unfolding (17). Detergents interfere somewhat with the assay, but membrane proteins have nonetheless been tested successfully by this method (18).

Differential scanning calorimetry (DSC) provides a thorough thermodynamic characterization of protein fold stability (19), but requires a lot of sample, is quite slow and might be more

sophisticated than required if the aim is just to obtain soluble protein sample.

1.3.4 Stability of Multimeric Protein Complexes

Finally, multimeric protein complexes can dissociate over time. This can happen to a purified complex, if dissociation is very slow, but thermodynamically favorable. It might be worth trying different solution conditions, as association could be favored in other conditions.

Dissociation can also be caused by degradation or unfolding in any of the single components. However, the dissociation might be the only or first observable change.

The methods to detect dissociation are the same as discussed for assessment of purity and monodispersity, e.g. various types of gel electrophoresis, gel filtration, DLS, AUC, and mass spectrometry.

1.3.5 Membrane Proteins and the Role of Detergents

Membrane proteins display large patches of hydrophobic residues required for their insertion into or interaction with membranes. In solution, these patches promote protein aggregation through strong hydrophobic interactions. Detergents are required to prevent aggregation and to keep membrane proteins soluble in aqueous solutions. Detergents are amphiphilic molecules that usually contain both hydrophobic and hydrophilic parts. Their hydrophobic portion binds the protein and their hydrophilic portion interacts with the aqueous solution. Therefore, they make hydrophobic patches at the surface of proteins inaccessible to the solution, preventing protein aggregation through these patches.

Detergents are classified with regards to the nature of their hydrophilic or hydrophobic moieties. The hydrophilic moieties can be nonionic, anionic (negative charge), cationic (positive charge), or zwitterionic (both charges). Nonionic or zwitterionic detergents are the most commonly used detergents for membrane protein purification. The hydrophobic moiety of detergents can be classified by size (i.e. length of the alkyl chain) and flexibility (i.e. linear alkyl chain, branched aromatic, or aliphatic ring moieties).

The physicochemical properties of detergents in solution are described by several characteristics such as the critical micellar concentration (CMC), the aggregation number, or the size of the micelles:

- The *CMC* is the concentration below which monomers of detergent are soluble. Above this concentration, micelles form spontaneously. In these aggregates, the hydrophilic part of the detergent monomers are in contact with surrounding solution while the hydrophobic part is sequestered in the micelle center. The CMC depends on the physicochemical properties of each detergent, but also on the temperature, ionic strength, or pH of the solution. Some methods exist to determine precisely the CMC of a given detergent in a particular buffer condition (20). Usually suppliers indicate the CMC

in water of commercially available detergents. It is in most cases safe to work with at least twofold this value.

- The *aggregation number* gives the average number of detergent monomers within the micelle. The micelle size is the average size of the detergent micelle.
- Some suppliers (Novagen) provide tables where the micelle sizes are expressed in kDa. This corresponds to the molecular weight of a protein with the hydrodynamic radius equal to that of the detergent micelle. This value is particularly useful to implement the techniques described below, many of which are size dependent.

It is very often necessary to adjust buffer conditions or concentrate the sample during protein purification or for a given assay. When detergents are present in the sample buffer, it complicates the basic procedures that are usually used for this. How this can be resolved, or how the detergent can be exchanged, is summarized in Subheading 2.3.5.

2 Materials and Methods

2.1 Methods to Determine Purity, Dispersity, and Oligomeric State

Many manufacturers produce gel-running equipment. Protein gels are usually run vertically between glass plates with the top immersed in anode buffer and the bottom dipping into the cathode buffer. Some manufacturers offer precast gels, and they will very often fit only into their own running equipment.

2.1.1 Gel Electrophoresis

We have chosen the most common protein gel methods to present here. There are many more and if the standard protocols do not give good separation or clear bands it is well worth investigating the literature and optimizing protocols for your own application.

Modes of Gel Electrophoresis

The most common gels run are *denaturing polyacrylamide gels (SDS-PAGE)*, where SDS is kept in sample and running buffer and is included in the gel to keep the protein entirely denatured.

The classic protocol (1) involves a stacking (focusing) and a resolving gel as two layers in the gel, which differ in acrylamide concentration as well as pH (discontinuous gel electrophoresis). The acrylamide concentration, ratio of mono- and bis-acrylamide and concentration of the crosslinker TEMED determine the pore size of polymerized acrylamide and is chosen depending on the sizes of proteins that need to be resolved. Gradient gels with the acrylamide concentration rising towards the bottom make separation and visualization of small (10 kDa) and large (200 kDa) proteins on the same gel possible. They retain fast moving small proteins in the bottom of the gel while large proteins can still enter at the top. Commercially available precast gels are expensive, but extremely reproducible and give nice bands.

Importantly, some general rules need to be followed when preparing and running gels:

- Some of the components need to be prepared fresh (*see* **Note 3**).
- Gels can be poured in bulk and stored, if only for a limited time (*see* **Note 4**).
- The lower detection limit is around 100 ng (Coomassie) or 5–10 ng (silver stains/fluorescent stains) of protein. To detect small amounts of contaminants, the gel should be overloaded and/or sensitive staining methods used.
- Rinse out the wells after removing the comb, so samples can sink in more easily. Typical minigels of 1 mm thickness and 15 wells take about 15 µl sample.

It is worth trying the following simple variations, certainly if results are unexpected:

- Try some samples twice so you can compare them boiled and not boiled (*see* **Note 5**).
- Try leaving out reducing agent from the sample buffer and also adding more/fresh reducing agent.

Boiled and reduced proteins should run at monomer molecular weight. Bands at multiples of the molecular weight indicate Cys-bridge formation, which might be specific and present in the original sample (*see* **Note 6**) or be formed unspecifically during sample denaturation or electrophoresis (*see* Fig. 1).

Running buffer can often be reused; however, if the current or voltage drops when running the gel this indicates the running buffer has gone off and needs replacing. With old buffer run times are much longer and the bands are blurry.

SDS-PAGE can be used for most membrane proteins. However, they are more likely to be resistant to SDS-denaturation or more heat sensitive than soluble proteins. Therefore, the results of SDS-PAGE have to be analyzed with caution with this type of protein. For example, the β-barrel fold of outer membrane proteins is SDS-resistant and if samples are not boiled the protein appears to have smaller molecular weight on SDS-PAGE. This phenomenon is well described for OmpA (21, 22).

Even slightly modifying a gel protocol may make a big difference to the outcome with a particular sample. This can be a problem if inaccurate work leads to lack of reproducibility, but it can also be a blessing and consciously be used to characterize the sample (*see* Fig. 1).

In *native PAGE* the denaturant SDS is omitted, so proteins retain their fold and migrate according to shape as well as size. Also, gels are continuous and run at just one constant pH with identical gel and running buffer. Standards exist, but will only be approximate due to inevitable differences in shape.

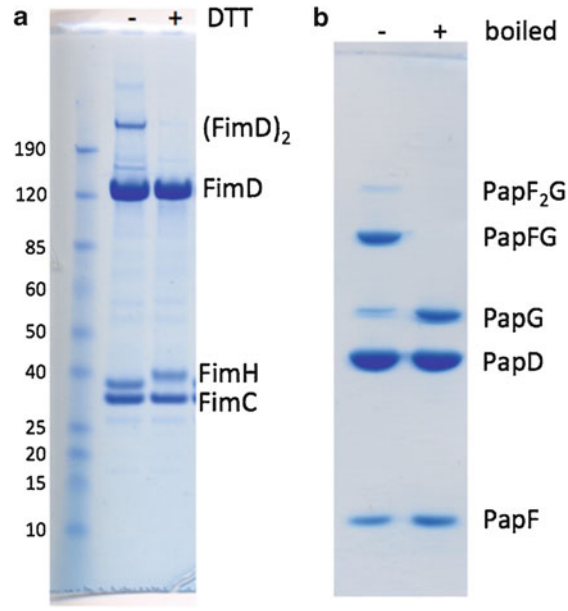

Fig. 1 Differences due to treatment of samples for SDS-PAGE. (**a**) FimD (92 kDa) membrane protein complex with FimC-FimH (23 and 29 kDa, respectively) run with and without 10 mM DTT on SDS-PAGE (NuPAGE run in supplied MES buffer, Invitrogen). There are four Cys-residues per monomer capable of forming two intramolecular disulfide bridges in the oxidizing environment of the outer membrane. Possibly because of the over-expression condition, there is a small fraction of reduced Cys-residues available to form dimers that are most likely nonfunctional (40). The standard is known to be inaccurate. NB FimH also shows changed behavior when DTT is present. (**b**) PapD-PapF ("PapDF") and PapD-PapG ("PapDG") preincubated and run on a standard, 12 % SDS-PAGE with and without prior boiling of samples. In boiled sample all proteins, PapD, PapF, and PapG run as separate bands. However, in sample that has not been boiled, a heavier band appears while the PapG band is nearly depleted, indicating formation of a PapDFG complex with an SDS-resistant PapF-PapG interaction. PapDF was used in excess, and some $PapDF_2G$ seems to have formed as indicated by another, faint heavier band that likely consists of two PapF and one PapG. (*Panel (a) courtesy of Gilles Phan, panels (a, b) with the kind agreement of Gabriel Waksman, Institute of Structural and Molecular Biology, London, UK*)

The critical issue is to find a buffer that suits a particular protein, but buffers of a wide range of pH values suitable for proteins of a wide range of p*I* values are known (23). Native gels are run for a long time, at high voltage, and on ice/in the coldroom to keep them reasonably cool.

This method can be applied to detergent-solubilized membrane proteins if the specific detergent required by the protein is present in the gel and running buffer.

The blue-native PAGE method (24) is designed to keep proteins in their native state, but to ensure that migration is predominantly by

size, not by shape. This is achieved with a charged, blue dye (Coomassie Blue G-250) kept in the running buffer, which does not denature proteins, but uniformly binds to their surface. Due to their charge, proteins will migrate in the gel once an electric field is applied. These gels are also run for a long time and on ice.

This method is very commonly used for the analysis of membrane proteins and membrane protein complexes. The blue dye replaces the detergent bound to the protein and keeps the protein soluble.

For a review on the significance and methods of native gel protocols for protein complexes and membrane protein complexes, *see* ref 25.

Isoelectric focussing (IF) is mainly used as one step in 2D gel electrophoresis, often combined with SDS-PAGE for analysis of protein mixtures or even cell lysates. There are many reviews on these techniques (26, 27), and on a newer development of immobilized pH gradients (28) and sample preparation for isoelectric focusing gels is discussed in (29) including preparation of membrane protein samples. As the name suggests, in IF gels a protein will stop migrating in the pH region of the gel that is identical to their own p*I* as it is then uncharged and unresponsive to the electric field. This is what constitutes the separation power between proteins with a charge difference, e.g. phosphorylated and nonphosphorylated isoforms.

Gel Staining Methods

Visualization of proteins in gels can be achieved using organic dyes, silver stains, or fluorescent dyes and the choice will depend on the required sensitivity, reproducibility, and downstream applications like Western blotting (30, 31).

The standard gel staining procedure uses solutions containing Coomassie Brilliant Blue G-250 or R-250, but other organic dyes are suitable as well. A standard protocol would use 0.1 % Coomassie brilliant blue R-250 in 40 % ethanol and 10 % acetic acid as a staining solution, destain with a solution of 40 % ethanol and 10 % acetic acid, and use a solution of 20 % ethanol and 10 % glycerol for storage. Variations of this try to dispense of the destaining step or increase sensitivity. Typically, 15 μg of protein can be quantitatively stained in a band, and down to 1 μg detected, however as the dye interacts with certain amino acids and not others, staining is sequence and hence protein dependent.

Silver staining is of roughly 100-fold higher sensitivity than Coomassie staining. However, it is more protein dependent and less reproducible due to varying amounts of background staining. Many different protocols exist.

Finally, fluorescence staining can reach the high sensitivities of silver staining at higher reproducibility, but requires a fluorescence imaging system for visualization. Different specialist fluorescent dyes exist to specifically stain posttranslational modifications.

The efficiency of all these stains is protein-dependent (*see* **Note 7**) (30, 31), so quantitation by densitometry requires comparison with samples of the same protein at known concentrations, as well as on even staining throughout the gel.

2.1.2 Gel Filtration Chromatography

Gel filtration (size exclusion) chromatography separates macromolecules purely based on their size and shape (*see* **Note 8**). The molecular weight of the species to be separated will have to be considered when choosing the right column matrix. A variety of matrices is commercially available. Depending on their pore size, the separation range will vary. This range is usually expressed in terms of molecular weight (32).

Molecules too large to diffuse into the pores will elute with the void volume V_0, the volume equivalent to the mobile phase, these molecules do not interact at all with the matrix. The smallest molecules will elute at a volume close to the total column (bed) volume V_t, i.e. mobile (V_0) plus stationary phase volumes (V_s) together (with $V_s = V_t - V_0$) as they can occupy the pores in addition to the mobile phase. Intermediate size molecules will have part of the stationary phase available to diffuse into and therefore elute at volumes V_e that are larger than V_0 and smaller than V_t. An elution volume smaller than the void volume indicates channeling through the matrix and means the column needs to be repacked. An elution volume larger than the total column volume indicates unspecific interaction with the matrix and a different resin should be chosen.

Not only the matrix but also the column geometry and the sample size will determine how well it separates different species. Generally, longer columns better separate species of similar molecular weight and are also better with larger sample volumes. Columns for analytical use will be a smaller diameter and shorter length than those for preparative use. They run faster, but have a much smaller sample capacity. The sample volume may have to be as small as 0.5 % of the column bed volume in any gel filtration application to achieve good separation.

The sample concentration can be very high, but above protein concentrations of 70 mg/ml the sample viscosity will impede separation (32). There is an upper limit of the speed (usually given in ml/min) specific for each column matrix. Above this speed, separation will suffer as sample diffusion between the stationary phase (buffer inside the pores) and mobile phase (moving buffer outside the pores) is not given enough time. Furthermore, excess speed leads to column pressure that will collapse the pores, irreversibly damaging the separation power of the matrix (32).

Using Gel Filtration to Determine the Molecular Weight

A standard curve is required to determine the molecular weight of a protein. This is done using a set of standard proteins of known molecular weight (available as calibration mixes). The distribution coefficients K_{av} of these standard proteins are calculated from Eq. 3

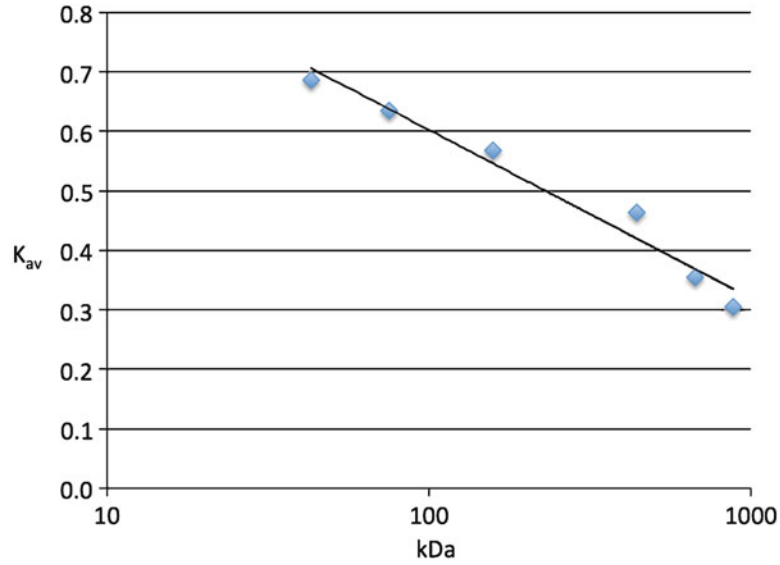

Fig. 2 Gel filtration calibration (Superose 6). Ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) are injected on the gel filtration column. Their elution volume is recorded (V_e). The void volume (V_0) of the column is measured by injecting Dextran 2000 onto the column. To obtain the standard curve, K_{av} is calculated (*see* Eq. 3) and plotted against the molecular mass of the standard proteins

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}, \tag{3}$$

where V_e is the elution volume for each of them, V_0 the void volume and V_t to total column volume. These distribution coefficients are plotted against the logarithm of their molecular weights and should lie on a straight line (*see* Fig. 2). V_0 can be determined by running 1 mg/ml of blue Dextran 2000 over the column. V_e of the sample in question will then give a molecular weight estimate by reading off the calibration plot or by calculating from the equation for the fitted regression line.

Any deviation from the true molecular weight is likely due to either a difference in shape of the protein species in the sample and those in the calibration mix or it might be due to an association reaction resulting in a peak representing one or even more different species.

Using Gel Filtration to Assess Protein Dispersity

Polydispersity is immediately obvious if there is more than one peak in a GF chromatogram of a sample that looked clean on SDS-PAGE. Any peaks in the void volume suggest vary large species. Depending on the separation range this is most likely unspecific aggregate. Any peaks at multiples of the monomer MW are likely homo-oligomers. If two or more peaks exist, this indicates that the species are not in a fast equilibrium with each other, i.e. they are kinetically stable. At such a slow equilibrium of associating

species there will be a peak per species. To investigate a shift in the equilibrium, samples have to be pre-incubated at their different concentrations to equilibrate entirely before they are run. A lower concentration sample should give higher peaks for the species of lesser MW (higher elution volume), and vice versa, than for samples preincubated at a higher concentration. If peak heights do not change at all, the protein species could either be extremely stable or they might have lost their ability to interchange.

But also a single peak can suggest polydispersity. If the MW is unexpectedly high for a monomeric species this could not only be due to an elongated shape but alternatively (or additionally!) to a fast associative equilibrium with dimers, trimers, or higher order oligomers. In this case, lowering the concentration of a sample would shift the equilibrium towards the smallest stable species, and therefore the peak in a gel filtration chromatogram towards higher elution volumes, i.e. lower MW, and vice versa. The peak represents all species present in the sample. As they are in fast interchange the different species will never separate.

In any of these cases it will be informative to run peak fractions on SDS-PAGE and native PAGE concurrently to confirm the identities of these complexes.

2.1.3 Notes on Dynamic Light Scattering Experiments

Samples for DLS can be prepared in nearly any buffer that does not itself contain large scattering particles like detergent micelles, colloids, or crowding agents. A relatively large concentration of about 1–10 mg/ml is required to obtain a good signal for most proteins of 50–100 kDa. The volume required varies massively from instrument to instrument, from only a few µl to a couple of ml. It is hugely important that the sample does not contain dust, so only filtered buffers should be used, or the sample itself be filtered before the experiment.

In theory, the concentration and diffusion coefficient can be obtained for each of a mix of species. But in practice distinct species are visible only if they are at least factor eight apart in molecular weight (*see* **Note 9**), which would be the case for aggregates, but not for small oligomers.

If investigating an associating system, samples of different concentration should be tested to check whether the suggested molecular size moves. In that case the size given by any one of these experiments is an average size of the mix of species present. If the species are sufficiently different in size, like a monomer and a hexamer, then even a fast equilibrium between these species will give a peak for each species as the measurement is done on such small time scales (different from gel filtration where these would be averaged).

2.1.4 Sample Preparation for Mass Spectrometry

It is absolutely critical that MS samples do not contain any salt or detergent. These would bind to the macromolecules to be analyzed in different ratios and unspecifically, so that peaks

are enormously broadened and resolution is lost. For some techniques, the sample is transferred into a volatile, organic solvent, for others, ammonium acetate can be used as a buffer as it evaporates in the experiment.

Standard protocols for buffer exchange can be used like several volume exchanges in a spin concentrator, dialysis, or gel filtration. Most convenient might be the use of a small desalting column. Most importantly, this has to be done thoroughly. In particular, also detergents have to be removed entirely e.g. when running membrane proteins. Newer, special mass spectrometry methods allow keeping detergents in the protein sample. The removal of the detergent from the protein is performed within the mass spectrometer itself (7, 8).

2.1.5 Notes on Analytical Ultracentrifugation Experiments

In principle, AUC experiments can be performed in any buffer compatible with absorption or interference spectroscopy. Total absorbance of buffer plus protein should not exceed about 1.2 OD, and if wavelengths shorter than 280 nm are to be used reducing agents have to be carefully chosen or avoided not to absorb at the required wavelength. Interference optics is even more versatile, however even the sedimentation of salts will be detected if sample and reference buffer do not match perfectly. Extensive dialysis is recommended. Buffers containing glycerol or a high concentration of salt may form concentration gradients, so that the sedimenting protein will encounter different solution properties at different positions in the cell. This will require special treatment during data analysis (33), so should be avoided if possible.

To discover large aggregates or precipitate the first scans are of tremendous importance and scans may have to be taken particularly quickly as heavy aggregates will sediment very quickly.

After fitting the traces for several concentrations, the sedimentation coefficient should be plotted over the concentration: if it goes up with concentration components in the sample associate (*see* **Note 10**). If the sedimentation coefficient goes down with rising concentration this indicates nonideal behavior and more dilute samples should be run (34, 35).

2.2 Concentration Determination

Any of the following methods can only be as accurate as the pipetting, e.g. when making dilutions (*see* **Note 11**).

All of the following methods have systematic errors that are protein dependent and entirely reproducible. If accurate concentrations are needed, QAA should be used to determine a correction factor for the day-to-day method of concentration measurements for each protein.

2.2.1 Absorbance Measurements

There are two common mistakes made during concentration measurements using absorbance at 280 nm. The first is to ignore the limits of the UV/Vis spectrometer (*see* **Note 12**) in relation to the

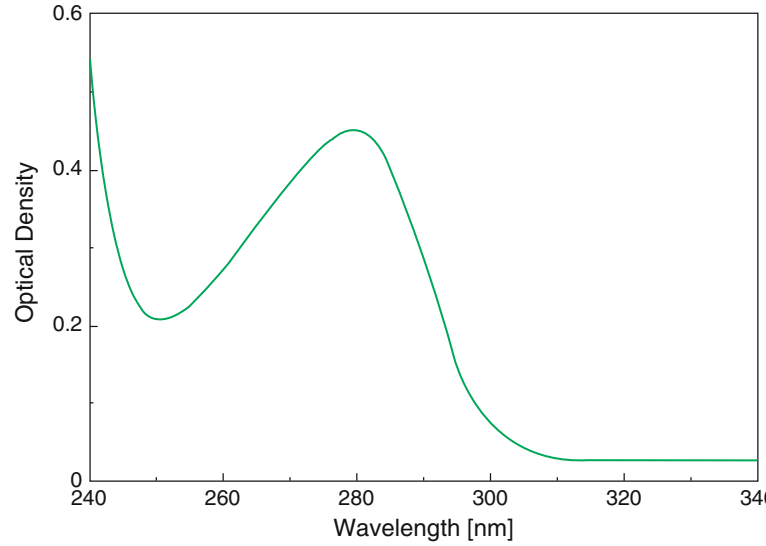

Fig. 3 UV/Vis-absorbance (OD) spectrum of a membrane protein. Recorded in 50 mM Tris pH 8, 200 mM NaCl and 5 mM LDAO taken on Jasco V-650 spectrometer in a 0.05 mm capillary. A largely flat baseline between 340 and 320 nm indicates little contribution from scattering to the peak at 280 nm. However, the baseline is offset and also, there is a tiny bump at 260 nm suggesting a small contribution of nucleotides/nucleic acids to the main peak (*Courtesy of Natalia De Val, Institut Pasteur, Paris, France*)

total absorbance of sample, buffer, and cuvette taken together. The sensitivity of the instrument should be checked in the manual, and the absorbance of empty cuvette and buffer should all be measured separately as well as that of the sample.

The second common mistake is to overlook the presence of contaminants. While the contribution from contaminating proteins will have to be estimated from other methods, other contaminants would often be apparent if a spectrum was run and inspected (*see* Fig. 3). Usual protein spectra have a fairly symmetrical peak at 280 nm resulting in a trough at 250 nm before absorbance becomes very high below 240 nm. Contaminating nucleic acids (for their removal *see* **Note 1**) would show extra absorbance around 260 nm and reducing agents would also show extra absorbance within a spectrum and quite possibly at and below 280 nm. Certainly many detergents absorb at relevant wavelengths, so in fact the method is often inappropriate for membrane proteins. If the baseline rises from 400 nm towards the peak at 280 nm this indicates light scattering by large particles like aggregate or micelles. The contribution of scattering can be subtracted from the absorbance due to soluble protein (*see* **Note 13**).

This is a thorough, recommended procedure to measure a protein spectrum for concentration determination:

1. Switch on the instrument and allow it to warm up for 20 min.
2. Run a baseline with nothing in it from 340 nm down to 190 nm (at least 240 nm).
3. Insert cuvette and take a spectrum of the empty cuvette to identify contamination and to make sure it is transparent at the required wavelengths (*see* **Note 14**).
4. Add buffer and run a spectrum to measure the absorbance of cuvette plus buffer. The absorbance will go up at low wavelengths as carbonyl and amide bonds as well as salts absorb here, DTT and β-mercaptoethanol absorb at even higher wavelengths.
5. Run a baseline with buffer.
6. Remeasure buffer to make sure it is consistently at 0 Abs, this also checks whether the lamp is warm and excludes lamp intensity fluctuations.
7. Run a spectrum with the protein solution and inspect the spectrum shape: peak at 280 nm, trough at 250 nm, then absorbance going up steeply towards 200 nm. Any absorbance decrease below 250 nm is likely an artifact due to very low and therefore noisy light levels (poor signal to noise ratio).
8. Calculate from the two spectra whether $Abs_{280,\ prot} + Abs_{280,\ buffer\ +\ cuvette}$ is below the specified dynamic range of the instrument (*see* **Note 12**). If not, dilute protein to repeat or preferably use a less absorbent buffer.

Use the A_{280} and the extinction coefficient to calculate the concentration of the protein dilution in the cuvette. Bear in mind that both the measurement and the extinction coefficient contain errors, e.g. preparing dilutions inevitably introduces significant pipetting errors. Also, the extinction coefficient varies with solution conditions, so use a buffer close to that suggested on the ProtParam site on the ExPasy server (13).

If the extinction coefficient and the protein sequence are unknown, the protein concentration might be estimated using Eq. 4 (36):

$$[\mathrm{P}] = 1.55A_{280} - 0.76A_{260}, \tag{4}$$

where [P] is the protein concentration in mg/ml, and A_{280} and A_{260} the absorbance at 280 and 260 nm, respectively. This relies on the sample being free of nucleic acids or nucleotides which absorb at 260 nm. Similarly inaccurate is to assume what is the "average" protein extinction coefficient of 1 OD per 1 mg/ml sample. Both these methods do not take into account that different proteins contain a different number of aromatic side chains and can therefore be grossly wrong.

2.2.2 Using the Bradford Assay

The Bradford assay, and all kits based on it, requires that your protein is soluble at acidic conditions, as only soluble protein binds dye and is measureable. The blank measurement (dye plus buffer, no protein) usually has an absorbance of 0.4–0.5 already, so also with these methods care has to be taken not to overstretch the dynamic range of the photo spectrometer (*see* **Note 12**). The assay itself also has a limited linear range.

Preparation of a standard curve is usually done with BSA, but this may not be a good standard for your protein as they might not stain to the same extent. The best standard is a preparation of your purified protein of known concentration. Quantitative amino acid analysis (see below) could be used to establish this standard.

2.2.3 Using Fluorescent Dyes for Protein Concentration Determination

Dyes are available from companies such as Molecular Probes (Invitrogen) and Promega. It is essential to follow the instructions closely as some have maximum tolerance of salts or other buffer components and as some are dependent on temperature and other conditions. Only small volumes of protein solution are usually required and the suitable concentration range varies from kit to kit.

As with dye-based absorbance assays, the accuracy of these fluorescence assays for the case of your protein is worth checking once by comparison with quantitative amino acid analysis. The reproducibility of results is usually high.

Of course measurement requires a fluorescence spectrometer. If none is available, some manufacturers of these assays sell a relatively cheap mini-fluorometer designed for assay use (Quant-iT assays, Molecular Probes). These are set up to generate standard curves and calculate results from sample measurements.

2.3 Protein Stability

2.3.1 Assessing Protein Solubility

A systematic check of protein solubility would at least involve varying the pH from about 4 to about 9, trying 0–1,000 mM NaCl and varying the temperature from 4 to 37 °C or higher if the protein is from a thermophilic organism. Not only the concentration but also the nature of the salt may be important for protein solubility. While many ions might be able to shield surface charges, a specific ion might be required for correct protein folding and therefore its stability. A few different monovalent and divalent salts should be tried. Bear in mind also, that some buffers require pH adjustment with bases, which will contain a metal ion that might itself influence solubility.

The solubility of some proteins depends on glycerol, but its presence generally is a disadvantage as it makes concentrating difficult, is hard to remove and interferes with many experiments.

Often, solution conditions are changed systematically during protein purification, i.e. a salt gradient is applied for elution from an ion exchange column. Careful observation at this stage gives an initial idea of protein solubility. A more systematic variation of the

pH, salt concentrations, and protein concentration with small aliquots of the purified protein in a dedicated experiment will give a wider picture of solubility. Plotting the protein state in dependence of two (or more) conditions gives a two (or more)-dimensional phase diagram which is of prime interest to protein crystallographers, but very informative to establish safe conditions to keep a protein soluble (37, 38).

Precipitation is often visible by eye as white fluff or streaks. But some aggregates are soluble or small amounts of precipitate might not be visible by eye. Spinning samples in a tabletop centrifuge (25,000–30,000 RCF) might visualize a small pellet, and supernatant can be removed carefully (*see* **Note 15**). Importantly, the concentration of the cleared protein solution will have to be measured again as it will be lower and to determine protein loss.

Other methods are intrinsically more quantitative: Gel filtration will show larger amounts of aggregate which would not enter the column matrix and go straight through with the void volume and appear as a peak in the beginning of the chromatogram. Dynamic light scattering is far more sensitive, detecting the smallest quantities of precipitates or unspecific aggregates. The early scans in a sedimentation velocity AUC experiment would also indicate heavy species in protein samples. All these methods are discussed in the paragraphs on "monodispersity".

2.3.2 Detecting Degradation

Most methods to detect protein degradation have already been introduced previously in this chapter. Gels or gel filtration will often give the first indication, particularly when multiple bands or peaks appear or the molecular weight appears to be smaller than expected. But these methods will not detect small amounts of breakdown products or those only different in a few amino acids' length.

Mass spectrometry will give unequivocal information on the molecular weight and is therefore the method of choice in case of suspicion. *N-terminal sequencing* will identify the N-terminal amino acids of each breakdown product, i.e. identify the cleavage site. N-terminal sequencing is done by Edman degradation and often provided as in-house service or available as commercial service.

2.3.3 Preventing Degradation

Protease inhibitors and mixes of inhibitors are available from many of the standard companies. Many, but not all contain EDTA, which might not be wanted (*see* **Note 16**).

Azide will prevent microbial growth in protein samples which eliminates a source of proteases.

Snap freezing is done in small protein aliquots (20–50 µl) in thin-walled PCR tubes in liquid nitrogen to prevent ice crystal formation that can damage proteins. For slow freezing, glycerol can be added as cryoprotectant. However, glycerol is not easy to remove and might not be suitable in future experiments. Whether freezing is appropriate needs to be tested for each protein by

checking for precipitation and aggregation and investigating whether the protein has retained its fold after thawing. Ideally, a functional test should be used to assess damage through freeze–thawing or at least one of above methods to establish structural integrity. Often, a protein aliquot should not be frozen a second time.

If the cleavage site of a protein is known this information can sometimes be used to express just that fragment of the protein to ensure that the sample is homogeneous. Of course this only makes sense if this fragment retains the overall fold and function of the full-length protein. Sometimes, adding a protein tag (or leaving it on after purification) can hinder degradation from the N- or C-termini.

2.3.4 Assessing Fold Stability

To assess the stability of a protein's fold with respect to chaotropic agents, a titration should be performed, e.g. from 0 to 6 M urea in 0.5 M steps. CD or fluorescence readings could be taken if the chaotropic agent is pure and does not interfere with these methods. The protein concentration should be identical in all these samples and generally needs to suit the measurement method chosen, e.g. to get enough, but not an oversaturated signal in a fluorimeter.

Differential scanning fluorimetry to asses thermal fold stability is often set up in a real-time PCR instrument programmed to ramp up the temperature slowly while taking frequent fluorescence measurements. 25–50 μl protein solution at 1–10 mg/ml in different conditions are tested usually in 96 (or 384)-well plate format. For screening, conditions to be tested may be pH and salt concentration initially as described for solubility studies. A dilution of SyproOrange (*see* **Note 17**) is added which becomes fluorescent upon binding to hydrophobic patches exposed in denaturation. In a melting event the fluorescence rises sharply, and the melting temperature can be determined. If this varies for different solution conditions it suggests that they affect protein stability.

2.3.5 Handling of Detergent-Solubilized Membrane Proteins

For exchange or removal of detergent, many techniques can be used:

- Affinity chromatography is an easy way to exchange or remove detergent. If it is possible to bind the protein on an affinity column (ion exchange, hydroxyapatite, Ni-NTA, streptactin, etc...) removal or exchange of detergent can be achieved by extensive washing by a buffer with no or new detergent. However, it is important to note that this method could lead to the removal of bound lipids from the purified membrane protein. This removal could affect its properties.
- Depending on the size of the protein, gel filtration could be used to exchange or remove detergent. However, if the

detergent micelles have a similar size than the sample, this method will not work.

- Dialysis is very often used to remove and exchange detergents. However, several criteria have to be taken into account: The size of the protein, the size of the detergent micelles and the CMC of the detergent(s). If it is possible to use a dialysis membrane with a cut-off large enough to let the micelles pass through it but not the protein, then dialysis will be very efficient. If only detergent monomers can pass through the dialysis membrane, then this technique will be effective only for detergents with high CMC (>1 mM).
- Finally, detergent can be removed by adsorption to hydrophobic beads. This technique is particularly useful to remove low concentrations of detergents. However, it has to be noted that membrane proteins can also be adsorbed to the same surface.

To concentrate protein–detergent mixes, two techniques can be used:

- Ultrafiltration is a widely used method to concentrate protein samples. Water and small molecules will pass through the filter. The pore size of the filter membrane has to be adjusted in function of the protein size. If the detergent micelles are much smaller (at least 2–3 times smaller) than the pore size, then detergent will also be filtrated and only the protein will be concentrated. However, because the size difference between the protein and the detergent micelles is often not big enough, it is very common that ultrafiltration also leads to concentration of the detergent.
- Affinity chromatography can be used to concentrate a protein sample. Large amounts of protein can be bound on the column and eluted in a small volume. This method will not concentrate the detergent but the protein will be in the elution buffer. A dialysis step may be required to remove some reagents that could interfere with following assays.

3 Notes

1. Nucleic acids can be removed from protein samples in many ways, the safest of which is an anion exchange step (DEAE sepharose) in the purification. Alternatively, use of DNase during cell lysis is recommended, and the small nucleic acid fragments and nucleotides should then be removed in a gel filtration or dialysis step. RNA contamination is less common.
2. Proteins that contain tryptophan or tyrosine, and to a lesser extent phenylalanine, are fluorescent. Fluorescence is usually strongly dependent on the close environment of the

fluorophore (the fluorescent group or molecule). So when a protein unfolds its intrinsic fluorescence may change (*see* Chapter 7 for details on the method).

3. The APS stock needs replacing every few days and if gels fail to set APS is the prime suspect. TEMED should be kept at 4 °C or on ice at all times for safety reasons.
4. Gels can be poured in bulk and stored wrapped in damp paper towel and cling film for a limited amount of time. The acrylamide network will deteriorate, proteins move faster and protein bands loose their sharpness. In discontinuous gels the pH difference between stacking and resolving gel will blur. Many kinds of gels can be purchased as precast gels, these usually have a longer shelf life, but are expensive.
5. For better denaturation, samples should be incubated at 90 °C for a few minutes. However, some proteins may precipitate, particularly membrane proteins or highly concentrated proteins. It needs to be tested which procedure is better. Also, some protein–protein interactions are SDS-resistant and will only be dissociated if the sample is denatured entirely by boiling.
6. Cys-bridges will be native to the protein if it is active in a nonreducing cellular compartment, e.g. the periplasm in bacteria or the Golgi apparatus in eukaryotes. It would have to be assessed whether experiments are better done at oxidizing or reducing conditions.
7. Not all proteins stain equally well. This is true for any known method. In addition, a larger protein will likely take up more dye than a small one. So the darkness of bands cannot be compared in terms of the molar concentrations of different proteins, but only in terms of the weight concentrations (mg/ml).
8. Gel filtration really is a hydrodynamic method, i.e. the hydrodynamic radius of any protein species determines how fast it moves through the resin. The hydrodynamic radius is larger for molecules of a larger molecular weight as well as for more extended molecules of the same molecular weight. In rare cases, proteins may be unspecifically adsorbed by the column material with or without permeating into the pores. This will interfere with the method.
9. For spherical proteins, an eightfold difference in molecular weight only translates into a twofold difference in diameter, which is what limits the separate detection of different species.
10. Sedimentation velocity analysis is an equilibrium method. However, analysis of a complex at a concentration near its K_D will not resolve the components into its various species, unless the rates of association and dissociation are slow compared to

the time scale of the experiment (hours). This is a similar phenomenon as in gel filtration. So here also, the experiment may give a single peak with a particular sedimentation coefficient, but this will be a mix of all species present. Importantly, a control of different concentrations is required to see whether this peak shifts in a concentration dependent manner.

11. Never pipette less than 2 μl, preferably 5 μl and never make dilutions higher than 1:100 in one go, i.e. to reach 1:1,000 do two sequential dilutions. Check the accuracy of pipettes frequently to avoid errors due to lack of calibration. Doing all measurements in triplicate will identify random errors.
12. In any photo spectrometer, a certain amount of light has to get to the detector for the measurement to be accurate. In many instruments the measurement should not be below 0.1 or above 1.0. Importantly, the upper limit of 1.0 is for the total sample, i.e. including buffer absorbance. The dynamic range of an instrument depends on the light intensity, the quality of the optics (stray light etc) and the detector sensitivity. They range from 0.7 absorbance units (Nanodrop-type instruments) to 6 absorbance units (on CD instruments), but it is usually safe to work below 1 absorbance unit of total absorbance, i.e. cuvette, buffer plus sample taken together. Check the instrument manual for the high absorbance limit.
13. It is recommended to take protein scans from at least 340 nm down, so that light scattering can be detected between 320 and 340 nm, in which region the baseline should be flat. The contribution of scattering to the overall absorbance at 280 nm can be calculated from scans starting at 400 nm or higher using the near-linear relationship between log $A_{scatter}$ and log λ for extrapolation (39).
14. The range of cuvettes available is huge. Most plastic cuvettes are not transparent in the UV range. Quartz cuvettes are certainly best. When choosing a small-volume cuvette, bear in mind the beam size and that the center height of the cuvette needs to match the location of the beam. If the window is smaller than the beam, then blacked out cuvettes only give a correct measurement; however, these cuvettes have absorbance on their own as they block part of the beam, so this needs to be added to buffer and protein absorbance to calculate the maximum sample concentration acceptable before the Beer-Lambert law breaks down. Cuvettes need frequent and thorough cleaning. Hellmanex is a specialist product to clean quartz cuvettes. Use a dilution per instructions and do under no circumstance incubate for longer than the advised time, a few minutes are nearly always sufficient, additional sonication will help to remove contaminants.

15. Protein pellet might be light and fluffy and be stirred up easily, so remove samples from the centrifuge carefully. Use a small tip and draw up slowly to remove supernatant to avoid picking up the pellet at the same time. Bear in mind in centrifuges that are not cooled samples heat up during spinning, so there is also a temperature effect.
16. Protease inhibitors are mixes of inhibiting agents and often contain EDTA, which will chelate all bivalent metal ions. This is to inhibit metallo-proteases, but will also inhibit most proteins that bind nucleotides and nucleic acids and others that depend on bivalent ions. EDTA-free protease inhibitors exist and are preferable. Both protease inhibitors and sodium azide are toxic and have spectral properties that prohibit their use for certain methods. Think carefully before adding them to your final purified sample.
17. SyproOrange is usually used at a final dilution of 1:500 to 1:5,000. Make a solution of dye stock of which to add 1 µl to every 25 µl sample. Final protein concentrations of 0.5 to 5 mg/ml usually give a good signal depending on the size of the hydrophobic core. Discard any leftover of the dilution as it will lose activity within hours.

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