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A practical approach to handling protein samples under degradation

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Abstract Protein structure determination using NMR spectroscopy requires a suite of heteronuclear 3-D NMR experiments that can take a couple of weeks for completion. During the experiments, protein samples may suffer from slow degradation due to co-purifying proteases, which complicates and slows down the assignment procedure. Here we describe a practical protocol to avoid unwanted proteolysis during the experiment.

Keywords protease inhibitor, protein degradation, protein stability

Introduction

Protein samples for NMR spectroscopy are frequently labeled with stable isotopes, *e.g.*, ^{15}N, ^{13}C, and ^{2}H to obtain better sensitivity and resolution for resonance assignment and structure calculation.[1,2] Backbone and side chain assignment requires a set of three-dimensional triple resonance NMR experiments that often take more than a week for completion. In addition, collecting structural restraints, such as nuclear Overhauser enhancement and residual dipolar coupling, further takes several days of experiments.[3] When a small compact protein (<15 kDa) is prepared as $^{13}C/^{15}N$-labeled and at a reasonably high concentration (>0.5 mM), it is common to expect two weeks of data collection on a high-field NMR spectrometer. This spectrometer time would extend if the sample is prepared at a lower concentration, or if the spectra suffer from line broadening due to chemical or conformational exchanges.

Given the amount of measurement time, it is important to maintain the sample stability during the data collection. The sample deterioration would result in chemical shift drift and unpredictable spectral changes in the course of the measurement, complicating the interpretation of experimental data. In addition, unstable proteins may lose their structural integrity, yielding inconsistent structural information in the NOESY experiment. Thus, it is highly desirable to keep the protein sample intact for the prolonged period of experiments.

Protein stability in NMR tubes is most likely hampered by aggregation and degradation. Protein aggregation arises from a variety of sources, such as misfolding, lack of partner proteins, and a suboptimal buffer composition.[4] On the other hand, protein degradation mainly results from co-eluting proteases of host bacteria. Here we describe a practical approach to monitoring and handling protein degradations during NMR experiments.

Monitoring protein degradation

Peptide bonds in proteins are very stable at neutral pH and ambient temperature, so that proteolysis observed in the sample suggests that the sample is contaminated by unwanted proteases. Isotope-labeled proteins are usually produced from the bacterial

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culture system using *E. coli* as a host.[5] The bacterial cells contain a number of proteases that are removed during the protein purification steps using chromatography. Affinity chromatography combined with ion exchange and/or size exclusion chromatography commonly purifies the protein of interest enough to pursue the NMR structure determination.

We usually spend a week or two to monitor the 2-D HSQC spectra of the fresh-prepared sample at room temperature. The sample may be kept in the incubator to maintain a specific temperature, when it is required. Since contaminating proteases are in trace amounts after the final purification step, the sample proteolysis is likely slow and may not be apparent at first sight (Fig. 1A). We examine the NMR spectra at the lowest contour threshold to detect potential signs of slow proteolysis, using the NMRView program.[6] Problematic proteolysis is diagnosed by new signals at 7.8–8.5 ppm of ^{1}H chemical shift, and 120–135 ppm of ^{15}N chemical shifts, which signifies the unstructured polypeptide resonances (Fig. 1B).[7] The spurious signals from degradation tend to increase by time, as the proteolysis continues. The degraded protein sample may be investigated using SDS-PAGE, where the sizes of polypeptide fragments might locate the scissile peptide bonds in the original protein. Proteins with modest degradation may be purified by chromatography, if they are difficult to prepare.

If the 2-D NMR spectra of the labeled protein sample remain clean and unchanged, the sample is sent to the spectrometer for a series of long experiments. It is still safe to obtain 2-D spectra for sample check-up every two or three days between 3-D experiments. When all experiments are finished, one can simply compare the initial and final 2-D experiments, and carefully examine the spectra for any discrepancy. When there are noticeable differences between the initial and final spectra, we go through the 2-D spectra between measurements to determine when the spectral changes first appeared. It is worth to mention that a clean sample results in a clean spectrum. When the spectra seem to get messy by time, it is always good to go back and optimize the sample condition.

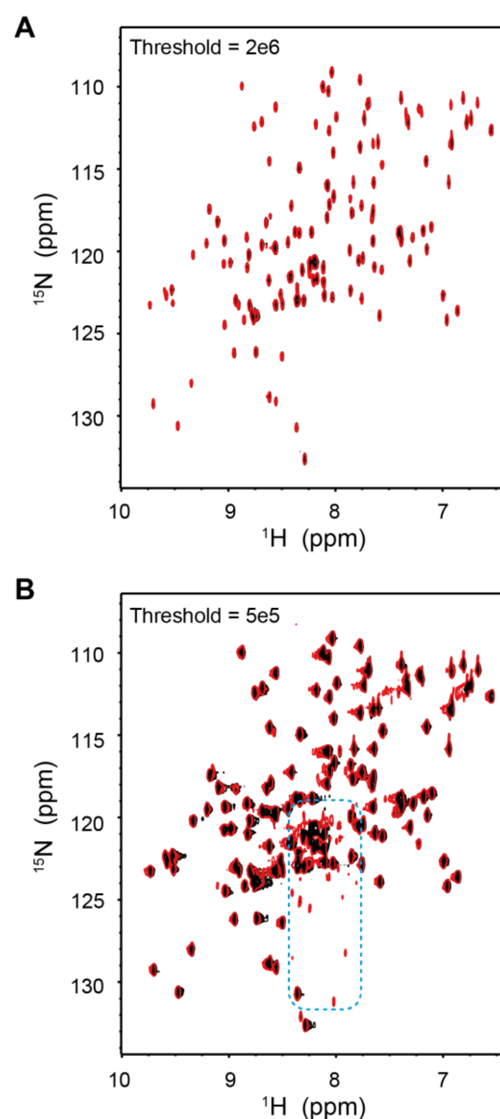

Figure 1. ^{1}H–^{15}N HSQC spectra of ^{15}N-labeled AcrIE4-F7 with different contour threshold of (A) 2e6, and (B) 5e5. The spectra were obtained from the same sample fresh-prepared (*black*) and after 2-week (*red*). The *cyan* box in part B highlights the degradation signals.

Dealing with protein degradation

If the protein sample appears to degrade, what can we do? The proteolysis may be very slow without affecting main signals, and then the degradation can be safely ignored. Little degradation should not affect the interpretation of major sample signals between

3-D spectra. Otherwise, extra signals from degradation increase with time, and it would be necessary to slow down or stop the degradation. Protease inhibitors can be used to prevent the sample proteolysis. From our experience, serine proteases are often responsible for the sample degradation. The contaminating serine protease can be of host origin, or may be added during purification to remove tags from the protein sample (e.g., thrombin and enterokinase). If this is the case, benzamidine can be used to potently inhibit the serine protease. Benzamidine sepharose is commercially available to remove serine proteases using a small column, but it costs less to purchase the chemical from companies. In practice, we first try 1 mM of benzamidine to check whether serine proteases cause the problem. We also try an inhibitor cocktail to collectively quench different types of proteases, in case other proteases are involved.

Inhibitor cocktails are available from several vendors, and the SIGMAFAST protease inhibitor tablet (cat. no. S8820) worked well for us. The inhibitor tablet contains six different protease inhibitors that include small molecules such as sulfonyl fluorides and also an endogenous inhibitor protein such as aprotinin. The cocktail conveniently quenches serine protease, cysteine proteases, metalloproteases, amino-peptidases, etc. The single or multiple inhibitors should be used with caution, if the protein sample itself belongs to a protease family, since the inhibitors may remain bound to the sample or cause covalent modifications at the active site. We show an example of the AcrIE4-F7 protein that does not belong to a protease family, but interacts with the inhibitor cocktail. AcrIE4-F7 showed a problematic degradation over a weak, as we mentioned earlier (Fig. 1). When we tried 1 mM benzamidine to prevent the proteolysis, AcrIE4-F7 remained stable for more than two weeks (Fig. 2A). AcrIE4-F7 was also stable in the presence of SIGMAFAST, but the inhibitor cocktail caused noticeable chemical shift perturbations in the 2-D spectra (Fig. 2B). Thus, any inhibitor cocktail should be used with caution, and less is often better in the sample tube.

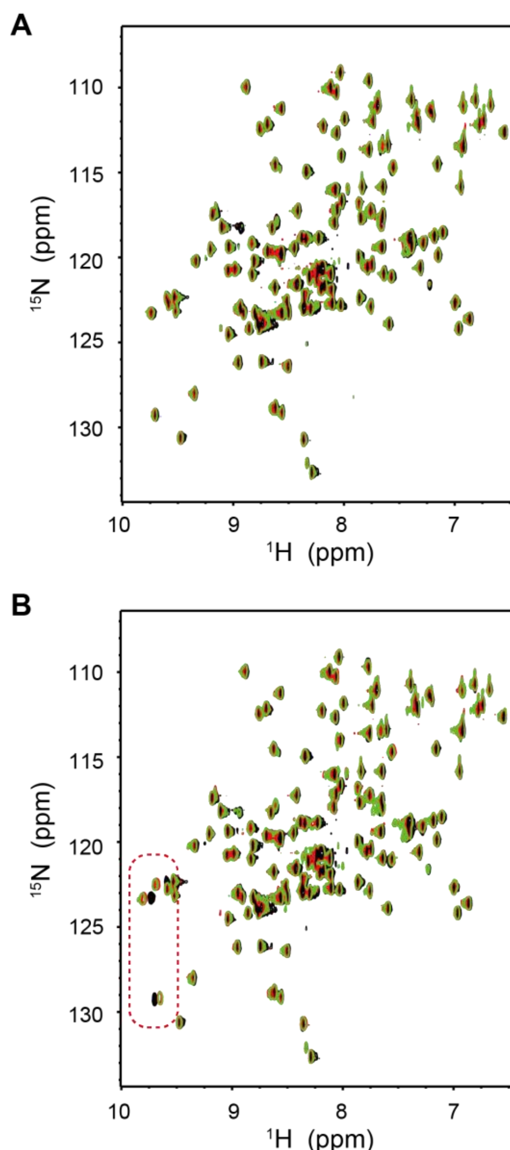

Figure 2. ^{1}H–^{15}N HSQC spectra of ^{15}N-labeled AcrIE4-F7 in the presence of (A) 1 mM benzamine and (B) a tablet of SIGMAFAST inhibitor cocktail (S8820). HSQC spectra of fresh AcrIE4-F7 alone (*black*), fresh AcrIE4-F7 with each inhibitor (*green*), and 1-week old AcrIE4-F7 with the inhibitor (*red*) are superimposed. The dashed box in *red* highlights chemical shift perturbations caused by the inhibitor cocktail.

In the second case, we introduce another protein AcrIE5. This protein also suffered from slow proteolysis over a week (Fig. 3A). We employed the

same approach to this protein, and examined the effect of benzamidine and the inhibitor cocktail. This time, benzamidine failed to keep the protein intact, and only the inhibitor cocktail maintained the integrity of the protein sample (Fig. 3B and C). The contaminating protease in this case was apparently other than one of the serine protease family proteins. Further, the inhibitor cocktail did not cause unwanted chemical shift perturbation in the 2-D spectra of AcrIE5 (Fig. 3C). Taken together, we decided to use the inhibitor cocktail in the final buffer for a suite of high-field NMR experiments.

When protease inhibitors do not work

We experienced that some protein samples are highly sensitive to proteases, especially when the protein contains a long loop or flexible linker region. Proteins with intrinsically disordered regions are potentially vulnerable to contaminating proteases. A single inhibitor or a mixture may be good to slow down the sample proteolysis by contaminating proteases. We experienced a few cases, however, that the inhibitors were ineffective to stop the sample degradation. When the inhibitors are not good enough, we can always return to the protein purification step, searching for a different strategy to separate the contaminant. A new purification strategy can be employed, such as the hydrophobic interaction chromatography. Changes in the existing protocols, such as different buffer compositions or gradient elution strategy may also be useful. If the sample protein can refold easily, chemical-induced or temperature-induced refolding may inactivate the contaminating protease.

One may go back further to the cloning stage, and try different tags for the purification. The His-tag has been most popular, but MBP- or GST-tags can provide a different purification profile to remove proteases. In addition, the construct of the sample protein may be trimmed, so that flexible N- and C-terminal tails are removed to avoid proteolysis during protein purification and NMR data collection. Lastly, proteases are often introduced to the sample to remove tags, and there are several proteases

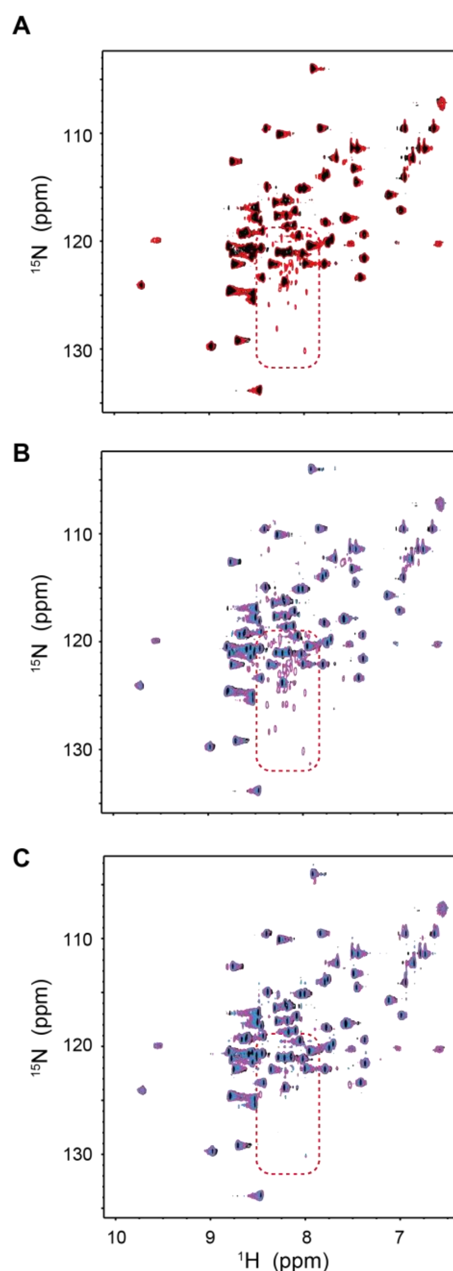

Figure 3. ^{1}H–^{15}N HSQC spectra of AcrIE5. (A) fresh AcrIE5 alone (*black*) and 1-week old AcrIE5 alone (*red*), (B) AcrIE5 and 1 mM benzamidine, and (C) AcrIE5 and an inhibitor cocktail. In parts B and C, HSQC spectra of fresh AcrIE5 alone (*black*), fresh AcrIE5 with each inhibitor (*light blue*) and each 1-week old sample with the inhibitor (*violet*) are superimposed. Dashed boxes in *red* highlight the signature region of sample degradation.

for this purpose. Thrombin and enterokinases are serine proteases. Thrombin is efficient and handy, but may cause secondary cleavage reactions at unwanted sites of your sample. Enterokinase (also known as enteropeptidase) is more specific, and can be used to avoid the side reaction. For the tag removal, we recommend engineered proteases of viral origin, such as a Tobacco Etch Virus (TEV) protease or a Human Rhinovirus (HRV) 3C protease. These viral proteases are cysteine proteases with high specificity, and easy to produce in the laboratory or purchase from vendors.

Conclusion

We described a simple and quick protocol in our lab to cope with potential sample degradation. As long as we produce recombinant proteins with a heterologous expression system, there always exists a threat of sample degradation due to co-eluting proteases. We recommend to observe the 2-D HSQC spectra at least a week to check sample integrity. If the protein seems to degrade by time, two pilot experiments seem to be useful to contain the situation. Benzamidine is useful to destroy serine protease activity, and an inhibitor cocktail will inform us of general possibility to stop the protease activity. When the inhibitor cocktail interacts with the sample protein, its individual components can be separately purchased to test if they stop the proteolysis without interfering with your protein. Protein purification seems complicated and frustrating at times, but as the old saying goes, *Quaerendo invenietis*. As you seek, you shall find.

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