METHODS AND APPLICATIONS

The use of spin desalting columns in DMSO-quenched H/D-exchange NMR experiments

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Abstract: Dimethylsulfoxide (DMSO)-quenched hydrogen/deuterium (H/D)-exchange is a powerful method to characterize the H/D-exchange behaviors of proteins and protein assemblies, and it is potentially useful for investigating non-protected fast-exchanging amide protons in the unfolded state. However, the method has not been used for studies on fully unfolded proteins in a concentrated denaturant or protein solutions at high salt concentrations. In all of the current DMSO-quenched H/D-exchange studies of proteins so far reported, lyophilization was used to remove D₂O from the protein solution, and the lyophilized protein was dissolved in the DMSO solution to quench the H/D exchange reactions and to measure the amide proton signals by two-dimensional nuclear magnetic resonance (2D NMR) spectra. The denaturants or salts remaining after lyophilization thus prevent the measurement of good NMR spectra. In this article, we report that the use of spin desalting columns is a very effective alternative to lyophilization for the medium exchange from the D₂O buffer to the DMSO solution. We show that the medium exchange by a spin desalting column takes only about 10 min in contrast to an overnight length of time required for lyophilization, and that the use of spin desalting columns has made it possible to monitor the H/D-exchange behavior of a fully unfolded protein in a concentrated denaturant. We report the results of unfolded ubiquitin in 6.0M guanidinium chloride.

Keywords: H/D exchange; DMSO; 2D NMR; spin desalting column

Abbreviations: 2D, two-dimensional; DCA, dichloroacetic acid; DMSO, dimethylsulfoxide; GdmCl, guanidinium chloride; H/D, hydrogen/deuterium; HSQC, hetero-nuclear single quantum coherence; NMR, nuclear magnetic resonance.

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Introduction

The dimethylsulfoxide (DMSO)-quenched hydrogen/deuterium (H/D)-exchange method, first introduced by Zhang *et al.*,¹ is a very versatile method to characterize the H/D-exchange behaviors of proteins and protein assemblies, and the advantage of this method is twofold. First, DMSO is a strong protein denaturant that can be used as a solubilizer of water-insoluble protein aggregates. Second, the chemical exchange rates of peptide amide protons are substantially reduced in a DMSO solution (most typically 95% DMSO-*d*₆/5% D₂O, pH* 5.0), making it possible in principle to observe the H/D exchange of non-protected fast-exchanging amide protons by two-dimensional nuclear magnetic resonance (2DNMR) spectroscopy; pH* indicates the pH-meter reading. The DMSO-quenched method has thus been used widely for studying the H/D-exchange behaviors of various amyloid fibrils^{2–9} and other protein supermolecular assemblies.^{10,11} The method has also been used for studying the H/D exchange of non-protected fast-exchanging amide protons in the intermediate and the unfolded states of proteins.^{1,12–14}

In the DMSO-quenched H/D-exchange experiments, the H/D-exchange reactions are first carried out in a D₂O buffer solution under a condition used to investigate the exchange behavior of a protein. After a pre-determined exchange time, the exchange reaction is quenched in liquid nitrogen, and the medium is changed from D₂O to the DMSO solution, in which amyloid fibrils or other insoluble protein aggregates are dissolved into monomers. When protein is ¹⁵N-labeled, we can use 2D ¹H–¹⁵N heteronuclear single quantum coherence (HSQC) spectra to monitor the individual amide-proton signals of the protein with different exchange times, because further exchange is effectively quenched in the DMSO solution.

However, in all of the previous DMSO-quenched H/D-exchange studies of proteins so far reported, lyophilization was used to remove D₂O from the protein solution, and the lyophilized protein was dissolved in the DMSO solution.^{1–14} Therefore, the current DMSO-quenched H/D-exchange method has not been used for studies on fully unfolded proteins in a concentrated denaturant (6*M* guanidinium chloride (GdmCl) or 8*M* urea) or protein solutions at high salt concentrations because the denaturants or salts remain after lyophilization, although the DMSO-quenched method is potentially useful for investigating non-protected fast-exchanging amide protons in the unfolded state.

In this article, we report that the use of spin desalting columns is a very effective alternative to lyophilization for the medium exchange from the D₂O buffer to the DMSO solution in the DMSO-quenched H/D exchange. We show that the medium

exchange by a spin desalting column takes only about 10 min in contrast to an overnight length of time required for lyophilization, and that the use of spin desalting columns has made it possible to monitor the H/D-exchange behavior of a fully unfolded protein in a concentrated denaturant. We report the results of unfolded ubiquitin in 6.0*M* GdmCl.

Results and Discussion

We used Zeba™ Spin Desalting Columns (Thermo Scientific, Rockford, IL) for the medium exchange and 95% DMSO-*d*₆/5% D₂O (pH* 5.0) as the DMSO solution to quench the H/D exchange. When we used the spin desalting column for the medium exchange, the column was first filled with the DMSO solution, and an appropriate volume of a sample protein solution in the D₂O buffer was applied to the column. To investigate the most appropriate volume of the sample, we applied different volumes of 50 mM phosphate buffer in H₂O (pH 7.0) to a column filled with the DMSO solution, and measured 1D ¹H-NMR spectra of the eluates. For a 5-mL spin desalting column, the manufacturer's recommended applied volume is 0.5–2.0 mL. As shown in Figure 1, however, we observed a significant water contamination when

Figure 1. The 1D ¹H-NMR spectra of the eluates from the DMSO solution-filled spin columns when different volumes of 50 mM phosphate buffer in H₂O (pH 7.0) were applied to the column. The applied volumes were (a) 1.5 mL, (b) 1.2 mL, and (c) 1.0 mL. The 1D ¹H-NMR spectrum of the DMSO solution (95% DMSO-*d*₆/5% D₂O, pH 5.0) is also shown in (d). NMR peaks for H₂O, DCA, and DMSO are shown in the figure.

we applied more than 1.0 mL, and hence we determined the best application volume to be 1.0 mL. For a 2-mL spin desalting column, the best application volume was found to be 0.35 mL.

We used ^{15}N -labeled ubiquitin as a model protein to examine the application of spin desalting columns in the DMSO-quenched H/D-exchange 2D NMR (^1H - ^{15}N HSQC) studies; the ubiquitin used in the present study contained an extra 34 residues at the N-terminus (see Materials and Methods) as compared to wild type ubiquitin. The H/D-exchange reaction of unfolded ubiquitin was started by 10-fold dilution of 3 mM ^{15}N -labeled ubiquitin unfolded in 6.0M GdmCl (H_2O) at pH 2.6 into 6.0M deuterated GdmCl in D_2O at pH* 2.6 and 20.0°C. At each pre-determined exchange time, 1.0 mL of the reaction mixture pre-dispensed in a microtube was taken, the reaction was quenched in liquid nitrogen, and the frozen mixture was kept in a freezer at -85°C until the medium exchange and the subsequent NMR measurement. For the NMR measurement, the frozen sample was thawed at room temperature, the medium containing 6.0M GdmCl was exchanged for the DMSO solution by using a spin desalting column, and the ^1H - ^{15}N HSQC spectrum of the protein was measured. The medium exchange by the spin

desalting column took only about 10 min, which is thus a big advantage over the overnight lyophilization that has been used in the conventional DMSO-quenched H/D-exchange method.

Figure 2 shows the HSQC spectra of ubiquitin obtained using different exchange times of 0, 10, and 60 min (panels (a), (b), and (c)), and the spectrum after complete H/D exchange by heating at 55°C for 30 min ((d)); the exchange times shown are the exchange times under the H/D-exchange condition, not including the time required for the medium exchange, and the heating was done in 6.0M GdmCl at pH* 2.6 (90% D_2O /10% H_2O). The amide proton signals were well resolved, and the quality of the spectra was identical to that of the spectrum of the sample solution obtained by direct dissolution of lyophilized ubiquitin in the DMSO solution. In recent DMSO-quenched H/D-exchange NMR studies, pure DMSO- d_6 (or 99% DMSO- d_6 /1% trifluoroacetic acid) was used as a quenching medium instead of the DMSO solution (95% DMSO- d_6 /5% D_2O , pH* 5.0).^{4,10,11} The spin desalting column could also be used for the medium exchange for pure DMSO, and hence we prepared the ubiquitin sample in pure DMSO- d_6 and measured its HSQC spectrum (data not shown). However, the

Figure 2. ^1H - ^{15}N HSQC spectra of ^{15}N -labeled ubiquitin in the DMSO solution with different exchange times under the H/D-exchange conditions (90% D_2O /10% H_2O , 6.0M GdmCl, pH* 2.6, and 20.0°C): (a) The unexchanged sample, (b) 10 min, (c) 60 min, and (d) after complete exchange by heat treatment at 55°C for 30 min.

Figure 3. The kinetic progress curves of H/D-exchanged ^{15}N -labeled ubiquitin in 6.0M GdmCl at pH* 2.6 and 20.0°C (open circles). Panels (A), (B), and (C) represent the kinetic progress curves for H/D exchange of amide protons corresponding to signals A, B and C, respectively, shown in Figure 2. The filled triangle in each panel represents the sample after complete exchange by heating at 55°C for 30 min.

spectrum was collapsed, and its quality was worse than the spectral quality in the DMSO solution in the case of ubiquitin.

Figure 3 shows typical H/D-exchange curves of three amide proton resonances labeled “A,” “B,” and “C” in Figure 2. We calculated the predicted half times of the H/D exchange for the non-protected amide protons in the whole sequence of the protein under the present exchange conditions (pH* 2.6 and 20°C),^{15,16} and they ranged from 1.5 to 19 min except for the amide proton of the second amino acid residue in the sequence, which had a predicted half time of 0.7 min. Loftus *et al.*¹⁷ previously reported that the presence of 6.0M GdmCl resulted in a two-fold deceleration of the H/D exchange rate of the peptide amide groups, and hence the predicted half

times ranged from 3.0 to 38 min. Amide proton A may belong to a glycine residue according to its chemical shift values. Because the H/D-exchange rate of the glycine residue was relatively fast in the DMSO solution, a significant portion of the amide proton signal was lost during the NMR measurement, but nevertheless, we observed a single-exponential decay of the signal with a half time of 18 min [Fig. 3(a)], which was within the range of the predicted exchange half times. Amide proton B showed a stronger signal with a half time of 16 min [Fig. 3(b)], which was also within the range of the predicted exchange half times for the non-protected amide protons. Amide proton C, which showed an even stronger signal, however, was exchanged much more slowly, with a half time of 117 min, which was at least three times longer than the predicted half times for the non-protected protons. Among the 12 additional amide protons for which we analyzed the H/D-exchange kinetics, three showed a half time longer than 90 min. Ubiquitin was shown to be fully unfolded at 6.0M GdmCl at pH 2–3.¹⁸ It thus remains to be determined whether the amide protons that showed an exchange half time longer than 90 min arose from a weakly protected portion of a locally structured region in the unfolded protein or rather from a slight inaccuracy in the predicted exchange rates.

Finally, we also investigated whether or not, the present method is equally applicable to a protein dissolved in DMSO, because DMSO is often used as a solubilizer of insoluble protein aggregates such as amyloid fibrils.⁹ For this purpose, we dissolved lyophilized powder of ^{15}N -labeled ubiquitin in 100% DMSO, exchanged the medium (100% DMSO) for the DMSO solution by a spin desalting column, and measured the ^1H – ^{15}N HSQC spectrum of the eluate. The spectrum thus obtained was identical to that shown in Figure 2(a), indicating that the present method is not only useful for water-soluble proteins but also for insoluble protein aggregates that can be dissolved in DMSO.

In conclusion, the use of spin desalting columns in the DMSO-quenched H/D-exchange studies of proteins was very effective, and allowed us to successfully obtain the H/D-exchange kinetics of the individual amide protons, H/D-exchange-quenched by DMSO and detected by ^1H – ^{15}N HSQC spectroscopy, of unfolded ubiquitin in 6.0M GdmCl, which was previously impossible by using the conventional DMSO-quenched H/D-exchange technique with lyophilization for the medium exchange. Because the medium exchange by a spin desalting column is relatively easy to handle and takes a much shorter time than lyophilization, the use of spin desalting columns is superior to lyophilization, and will be more widely employed in future DMSO-quenched H/D exchange studies.

Materials and Methods

Chemicals

DMSO- d_6 and D_2O were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). Dichloroacetic acid (DCA) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). GdmCl was purchased from Nakalai Tesque Inc. (Kyoto, Japan). Deuterated GdmCl was produced by repeated cycles of dissolution of GdmCl in D_2O followed by lyophilization.

^{15}N -Labeled ubiquitin

Human ubiquitin was bacterially expressed as a recombinant protein and purified as described in the literature¹⁹ with slight modifications. The plasmid vector was constructed and cloned using the pET28a(+) vector (Novagene, Madison, WI), then transformed into *Escherichia coli* strain BL21-Codon-Plus (Stratagene, La Jolla, CA). For the production of isotopically labeled ubiquitin, cells were grown in M9 minimal media containing [^{15}N]NH $_4$ Cl (1 g/L). Ubiquitin thus expressed was flanked by an N-terminal hexahistidine-tag moiety, MGSSHHHHHSSGLVPRGSHMASMTGGQMGRGS, and a C-terminal amyloid β segment. After purification by a Ni $^{2+}$ -nitrilotriacetic acid affinity column (GE Healthcare, Buckinghamshire, UK), ubiquitin was enzymatically cleaved from the C-terminal amyloid β segment. Ubiquitin with the N-terminal extension was further purified by reverse-phase chromatography using an octylsilane column (Sunniest C8; ChromaNik, Osaka, Japan) with a linear gradient of acetonitrile. The fraction containing ubiquitin was collected and lyophilized.

DMSO-quenched H/D-exchange experiments

The H/D-exchange reaction of unfolded ubiquitin was started by 10-fold dilution of 3 mM ^{15}N -labeled ubiquitin unfolded in 6.0M GdmCl (H_2O) at pH 2.6 into 6.0M deuterated GdmCl in D_2O at pH* 2.6 and 20.0°C. Immediately after the dilution, 1.0 mL of the reaction mixture was dispensed into each of 10–20 microtubes with a screw cap sealed by an O-ring to prevent water contamination, and the solutions in the tubes were incubated at 20.0°C for H/D exchange. At each pre-determined exchange time between 5 and 240 min, the reaction mixture in a tube was taken, and the reaction was quenched in liquid nitrogen. The frozen mixtures were kept in a freezer at –85°C until the medium exchange and the subsequent NMR measurement.

For the medium exchange for the DMSO solution (95% DMSO- d_6 /5% D_2O , pH* 5.0), we first removed the storage solution from a spin desalting column (ZebaTM Spin Desalting Column 89891, 5 mL; Thermo Scientific) by centrifuging the column at 1000g for 2 min. We then added 2.5 mL of the DMSO solution to the column, and centrifuged the column at 1000g for 2 min to remove the excess DMSO solution.

This process was repeated two or three additional times to fill the column with the DMSO solution. Before sample loading on the spin column, the frozen reaction mixture was thawed at room temperature, and the sample solution thus obtained was slowly applied to the center of the compact resin bed of the column. The sample in the DMSO solution was collected by centrifuging the column at 1000g for 2 min, and immediately subjected to NMR measurement to detect the amide proton signals of the protein.

NMR measurements

All NMR spectra were acquired at 25°C on a Bruker Avance 500 spectrometer. The standard 1H – ^{15}N HSQC experiment was carried out on ^{15}N -labeled ubiquitin in the DMSO solution. The 1H chemical shifts were directly referenced to the resonance of tetramethylsilane, while the ^{15}N chemical shifts were indirectly referenced with the ratio of the ^{15}N and 1H chemical shifts.²⁰ All NMR data were processed using NMRPipe²¹ and NMRView.²²

Data analysis

The NMR signal intensities of the amide protons observed by the 1H – ^{15}N HSQC spectra of the protein showed single-exponential decay curves with respect to the exchange time under the H/D-exchange condition (6.0M GdmCl, 90% D_2O /10% H_2O and pH* 2.6 at 20.0°C) (Fig. 3). The exchange half times $t_{1/2}$ of the amide protons were given by $t_{1/2} = (\ln 2)/k_{app}$, where k_{app} represents the apparent rate constants of the H/D-exchange reactions. The predicted half times of the H/D exchange for the non-protected amide protons were calculated by the methods of Bai *et al.*¹⁵ and Connelly *et al.*¹⁶ We used the program SPHERE for the calculation of the predicted half times; SPHERE is accessible through the internet at the following URL, <http://www.fccc.edu/research/labs/roder/sphere/sphere.html>.

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