Detection of early unfolding events in a dimeric protein by amide proton exchange and native electrospray mass spectrometry

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Abstract: Oligomeric proteins generally undergo unfolding through a dissociation/denaturation mechanism wherein the subunits first dissociate and then unfold. This mechanism can be detected by the fact that the proteins exhibit a concentration dependence of the denaturation curve. However, the concentration dependence does not answer the question of whether there are thermally induced conformational changes that facilitate subunit dissociation. To fully probe these mechanisms it is desirable to have an analytical approach that is capable of measuring both subunit dissociation and protein denaturation in a highly sensitive manner. In this article, we demonstrate that the combined use of native mass spectrometry to detect subunit mixing, and amide hydrogen/deuterium exchange to detect transient unfolding events can provide a very unique insight into the pre-melting transitions in a protein oligomer. Both methods keep an isotopic record of each transformation event, without the dependence on equilibrium of the unfolding reaction. Here, we use a combined form of H/D exchange/mass spectrometry and isotopic labeling/native electrospray mass spectrometry to study the pre-unfolding events of *Bacillus subtilis* NAD⁺ synthetase, a symmetrical dimer protein, which plays a vital role in the lifecycle of the bacteria. In the experimental outcome provided, we were able to clearly illustrate that at elevated temperatures, the NAD synthetase dimer undergoes reversible dissociation without monomer unfolding, while at temperatures where monomer unfolding is observed to take place, the rate of dimer dissociation still yet exceeds the rate of unfolding. Information provided by combining these two mass spectrometric methods was found to be very robust, and allowed us to establish an NAD synthetase unfolding model, where primary dissociation occurs prior to the complete unfolding of the NAD⁺ synthetase.

Keywords: *B. subtilis*; NAD⁺ synthetase; unfolding; mass spectrometry; H/D exchange; amide proton exchange; isotopic labeling; native mass spectrometry

Introduction

Bacillus subtilis NAD⁺ synthetase is an amidotransferase whose biological role is to catalyze the final step of conversion of nicotinic acid adenine dinucleotide to

NAD⁺, which requires ATP and Mg²⁺. This specific species of the enzyme has become a promising drug target against anthrax¹ as it plays a vital role in the lifecycle of this highly visible organism, especially since NAD⁺ is an essential cellular cofactor of many biochemical pathways. To date, *B. subtilis* NAD⁺ synthetase has been thoroughly characterized, with structures available in the presence and absence of cofactors, and in the substrate bound and non-bound forms with resolutions as detailed as 1 Å.^{2–5} It is a tightly

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formed homodimer with an α/β topology. The dimer interface is extensive, covering $\sim 20\%$ of each monomer surface.

Protein unfolding is typically characterized using calorimetry and/or spectroscopy-based approaches.^{6–8} Although these very important technologies provide important information regarding the kinetics and thermodynamics of unfolding transitions, to completely understand the unfolding mechanisms of multimeric proteins, it is equally important to determine the association state of the protein throughout the unfolding reaction. However, obtaining this kind of information is highly challenging with conventional methods such as size exclusion chromatography and ultracentrifugation, as the monomeric and oligomeric forms are in constant equilibrium. In addition, the more common analytical methods have the potential to perturb the final equilibrium of multimer forms. Additionally, it is of considerable interest to study the transformations of proteins such as *B. subtilis* NAD⁺ synthetase at the sub-unfolding levels effected as a direct result of increased heat, and/or differing concentrations of denaturant, which also provides information regarding the initial unfolding reaction. At the very beginning of unfolding, the population of protein molecules undergoing transition is very low, and for that reason undetectable by “bulk” methods like DSC or spectroscopy.

Here, we have characterized the unfolding pathway of *B. subtilis* NAD⁺-synthetase by applying a combination of H/D exchange mass spectrometry, and native electrospray mass spectrometry with an ¹⁵N-isotopically enriched protein. Although amide protons intrinsically exchange rapidly, they cannot exchange while hydrogen bonded, and in a folded protein they are protected from exchanging by their involvement in hydrogen bonds. The degree of protection reflects the stability of the hydrogen bonds. During low energy local “breathing” motions or higher energy unfolding transitions, the amide hydrogen bonds are broken and the amide protons are free to exchange. Kinetically, there are two H/D exchange regimes EX1 and EX2. In the EX2 regime, the rate of refolding or closing and H-bond reformation is fast relative to the chemical exchange. Under these conditions, many cycles of opening and closing are required for full exchange, and therefore, the mass of a protein increases monotonically with time.^{9,10} For EX1 type exchange, the open time is long relative to the chemical exchange step, and when a region opens, it fully exchanges before closing. Under these circumstances, the exchange presents as a bimodal pattern, whose peaks at any given time, represents the fraction of molecules which have opened, and which have not. Under these circumstances, the opening rate is reflected in the rate at which the unexchanged peak translocates into the exchanged peak.

What H/D exchange experiments cannot tell is the oligomerization state of the protein during unfold-

ing. Here, we decided to use the high resolving power of mass spectrometry and observe the dissociation of NAD⁺-synthetase dimers using normoisotopic and ¹⁵N-labeled forms of protein. During protein unfolding, dimers dissociate and re-associate randomly with formation of normoisotopic and ¹⁵N-labeled heterodimers. Native electrospray mass spectrometry was capable of measuring the accurate masses and relative intensities of all three oligomeric species (normoisotopic and ¹⁵N-labeled homodimers and heterodimers), and provide kinetic information on the association state of the protein. Comparison of H/D exchange and dimer interchange kinetics allowed us to show that dimer dissociation precedes complete unfolding of the NAD⁺-synthetase, and that dissociation also takes place at temperatures where no monomer unfolding takes place, indicating the existence of a compact monomer intermediate in the unfolding pathway of *B. subtilis*.

Results

Labeling of NAD⁺ synthetase with ¹⁵N

Electrospray mass spectrometry produces a series of multiply charged ionic species of the form (z , $z+1$, $z+2$...), and mass analyzers determine the m/z ratio. The mass of a protein can be accurately determined from this series of simultaneous equations. For normoisotopic NAD⁺ synthetase, the mass was measured on our instrument at 30262.54 ± 0.34 Da, which is within 3 Da of the theoretical mass of 30264.00 Da [Fig. 1(A)]. NAD⁺ synthetase contains 364 nitrogens; therefore, the completely ¹⁵N-labeled protein will be ~ 362.9 Da heavier than the normoisotopic protein. The mass of our ¹⁵N-labeled NAD⁺ synthetase was measured at 30620.2 ± 0.45 Da, which is 358 Da heavier than the normoisotopic protein [Fig. 1(B)]. This result suggests that 99% of all nitrogens in the labeled protein are ¹⁵N. Correspondingly, the ¹⁵N labeled homodimer and ¹⁴N/¹⁵N heterodimer will also be 716 Da and 358 Da heavier than normoisotopic protein, respectively.

NAD⁺ synthetase displays an EX2 exchange pattern at room temperature

We studied the hydrogen/deuterium exchange of normoisotopic NAD⁺ synthetase over a temperature range from room temperature to 45°C. In all cases, the protein was diluted into a D₂O-based buffer, and at the various time points indicated in the methods section, the exchange reaction was quenched. The subunit mass measurements were subsequently determined by liquid chromatography ESI–ToF mass spectrometry. Figure 2 illustrates the 33+ charge state of the protein. At room temperature [Fig. 2(A,C), gray squares], the observed m/z values (and therefore the mass of the protein) increases monotonically with deuterium incorporation over time in a pattern typical of EX2

Figure 1. Determination of masses of normoisotopic and ^{15}N -labeled NAD^+ synthetase. (A) Normoisotopic protein, measured mass is 30262.54 ± 0.34 Da, theoretical mass is 30264.0 Da. (B) ^{15}N -labeled protein, measured mass is 30620.20 ± 0.45 Da, theoretical mass assuming 100% labeling efficiency is 30630 Da, which suggests that the actual labeling efficiency is 98%.

exchange. The endpoint of the H/D exchange reaction was determined by 10 min of exchange at 100°C , conditions under which the protein is denatured. The fully exchanged NAD synthetase incorporated 183 deuterons out of a possible 247, thereby suggesting that $\sim 25\%$ were back-exchanged. However, this protein is not fully exchanged even after 960 min at room temperature suggesting the existence of a stable folded core.

NAD synthetase displays a bimodal HDX pattern at elevated temperatures

To characterize the pre-unfolding dynamics, we performed H/D exchange at 40 and 45°C , which was based on previously published differential scanning calorimetry (DSC) experiments.¹¹ At 40°C , no changes in the heat capacity were seen while 45°C represents the beginning of the heat capacity change peak.

The H/D exchange pattern at 40°C is similar in appearance to that seen at room temperature, [Fig. 2(A,B)] although the overall rate of exchange, and the extent reached, was found to be somewhat greater [Fig. 2(C)]. However, upon close inspection, it is evident that the 240 min peak is broadened compared to the peak at other time points [indicated by a gray arrow in Fig. 2(B)]. This peak broadening is suggestive of a bimodal H/D pattern (EX1 exchange) in which the two peaks are not well resolved.¹² Under these conditions, the time of broadest peak width (240 min) corresponds to the half time for the EX1 opening transition.

When H/D exchange was performed at 45°C , the pattern of exchange was clearly bimodal in the 3–10

min time frame [Fig. 2(D)] suggesting an opening motion and EX1 type exchange. As the fraction of protein molecules that have undergone the opening transition increases over time, the intensity of the higher mass peak increases at the expense of the lower mass peak. The rate of this redistribution directly reflects the opening rate of the transition. Although theoretically it should be possible to quantify the opening rate by fitting the time dependent redistribution, this proved difficult given the limited peak resolution. However, the peaks were well resolved to estimate their intensity relative to each other, and estimate the half time of the reaction (where the heights of the peaks are the same) as ~ 6 min. The higher mass peak [Fig. 2(D)] is at lower mass than the fully deuterated protein, which means that the open state retains elements of compact structure.

The NAD^+ -synthetase dimer dissociates at 40°C and higher

The exchange experiments suggested that at increased temperature, a long lived open form of the protein existed. To determine whether the opening represented dimer dissociation a subunit mixing experiment was performed using ^{15}N labeled protein as depicted in Figure 3(A). An equimolar mixture of normoisotopic and ^{15}N -labeled protein was incubated at different temperatures for different amount of time. Subunit interchange results in the formation of hetero-dimers whose presence can be detected by native state mass spectrometry. Native electrospray mass spectrometry utilizes direct injection of the analyte into the mass spectrometer in a non-denaturing aqueous buffer,

Figure 2. H/D exchange on NAD⁺ synthetase at different temperatures. (A) Exchange at room temperature. Monotonic increase in the mass is observed, this is EX2 type of exchange. Small peak at $\sim +180$ Da from the main peak is a covalent adduct of an electrophilic protease inhibitor used to prevent protein degradation. (B) Exchange at 40°C. Largely monotonic increase, however, peak broadens at around 240 min (indicated by gray arrow), which suggests that the elements of EX1 exchange might be present. However, the peaks are very close to each other and are not resolved, which suggests small scale of the change that leads to the increase in the mass. (C) Plot of the mass increase of NAD synthetase at room temperature (gray squares) and 45°C (black squares). (D) Exchange at 45°C. Step-wise increase of the mass is obvious (EX1 type of exchange), as second peak with mass 40 Da heavier appears in a time-dependent manner. The half-time of transition of first peak into a second one is about 6 min (spectra in the middle).

typically ammonium acetate. Electrospray mass spectrometry under native conditions revealed that homotypic ^{14}N and ^{15}N dimers have masses 60,524 Da and 61,241 Da, respectively [Fig. 3(B) lower spectrum; only charge states +15 and +14 are shown]. The mass difference between the dimers is 717 Da, which is almost exactly double the value of 358 Da measured for the mass increment due to ^{15}N incorporation. It is worth noting that both proteins were found to remain in a dimeric state even at the highest attainable cone voltage (200 V), with only traces of monomeric and tetrameric species (not shown).

No heterodimer peak was seen in the mixture of $^{14}\text{N}/^{15}\text{N}$ after incubation at room temperature for as long as 72 h [not shown but similar to Fig. 3(B), lower spectrum], which suggested that no dimer dissociation takes place at these conditions. However, incubation at both 40 and 45°C resulted in time-dependent appearance of heterodimer [Fig. 3(B,C)]. All peaks were resolved adequately to perform quantitative analysis of peak dynamics. Assuming random association of

monomers after dimer dissociation, and that ^{15}N and normoisotopic protein dimers have the same association constant, the distributions of peaks at equilibrium were expected as follows: 25% for both normoisotopic and ^{15}N , and 50% for the heterodimer [Fig. 3(A)]. This ratio was observed at late time points [Fig. 3(B,C), upper spectrum]. At 40°C, peak interchange was shown to take place [Fig. 3(B)], with a half-time measured at ~ 60 min. The 60 min half time suggests that at 40°C, equilibrium still lies strongly towards the dimer form. However, the peak broadening clearly observed in the exchange experiment is not likely a result of interdimer surface exposure due to dissociation, since the half time for subunit mixing (60 min) is much faster than the half-time for EX1 exchange (240 min). Instead, it appears from this data that there is likely a relatively rare, region-constrained cooperative unfolding event taking place in the protein. While in principle the number of amide protons in the opening region is reflected by the size of the mass increment, in this instance it is difficult to

Figure 3. Electrospray mass spectrometry of ^{14}N and ^{15}N NAD $^{+}$ synthetase mixture. Charge states are indicated in the bottom spectra, ^{15}N -labeled protein peaks are marked with asterisk in the bottom spectra. (A) Schematic of the dimer interchange reaction. Dark and light paired ovals are normoisotopic and ^{15}N -labeled NAD $^{+}$ synthetase dimers. A mixture of the dimers (left section) is heated, which results in dimer dissociation (middle section). Upon cooling, the monomers randomly re-associate to form dimers and hetero dimers (right section). (B) Dimer interchange at 40°C. Timedependent appearance of the hetero dimer is obvious (in the middle of the peaks observed in the bottom spectrum). (C) Dimer interchange at 45°C. Hetero dimer appears faster, suggesting higher dissociation rate. (D) Plot of the relative peak intensities after incubation at 45°C [Fig. 4(C)]. Half-time for transition is around 3 min.

estimate due to the concurrence of EX2 exchange. Nevertheless, we believe that a clear disconnect between H/D exchange, and dimer interchange kinetics is illustrated in this data, with repeated cycles of dimer dissociation/association preceding the EX1 H/D exchange event. This strongly suggests the transient existence of a compact monomer at 40°C.

In sharp contrast to the 40°C data, a much higher rate of dimer interchange was observed at 45°C [Fig. 3(C)]. Interestingly, the half time of dimer interchange at this temperature was ~3.5 min [Fig. 3(D)], which is approximately twice as fast as the bimodal H/D exchange at the same temperature [Fig. 2(D)]. If the H/D exchange bimodality were caused by dimer dissociation, we would expect the dimer interchange rate to be twice that of the EX1 rate, since every opening of the dimer should result in mass jump due to higher deuterium incorporation. However, only every second dissociation should result in the formation of heterodimer, as monomer subunits have the same probability of interacting with either ^{15}N -labeled or normoisotopic monomer. Thus, the half-time of 6 min observed

in the H/D exchange experiment and 3.5 min in dimer interchange experiment would indicate that dimer dissociation proceeds about four times faster than the process that results in the step-wise mass increase in H/D exchange experiment. This in turn would further indicate that for every four dimer dissociation events, there is one dimer unfolding (or rather one unfolding of both monomers of a dimer), again confirming the presence of compact monomer intermediate on the way to complete unfolding. This is in good agreement with data obtained at 40°C.

Discussion

Both H/D exchange with mass spectrometry^{13–18} and native electrospray mass spectrometry¹⁹ have been successfully applied for the study of protein folding. H/D exchange allows monitoring phase transition (unfolding), and the measuring of the specific kinetics as unfolding processes heralds itself in the appearance of EX1 exchange pattern. However, utility of this method is limited when unfolding of multimeric proteins is followed, as it does not provide information on

Figure 4. Hypothesized unfolding pathways of NAD⁺ synthetase. Top: two state unfolding. Monomers are not stable by themselves and unfold immediately after dissociation. Bottom: three state unfolding. Monomers have higher stability than their dimer interaction. Additional unfolding intermediates are likely present but are beyond the resolving power of the methods in this work.

the association state of the proteins. Yet, dimer interchange assays, such as one we have developed here, do provide this type of information. Although study of non-labeled proteins would not be of help with this type of analysis (as dissociated/re-associated dimers are not discernable from never-dissociated dimers), ¹⁵N-labeled proteins allowed us to monitor the dynamics of dimer dissociation. Upon dissociation, normoisotopic and ¹⁵N-labeled monomers re-associate randomly with the formation of heterodimers, and by measuring the area under each of the mass spectrometric peaks, we were able to quantify the rate of dimer dissociation. Since both of these methods are non-equilibrium approaches, they do not need a large portion of the studied molecules to be unfolded to achieve reliable detection, since every molecule that undergoes transformation (even once) retains a newly acquired isotopic state. Therefore, over time the amount of heavy labeled molecules accumulate and becomes large enough to measure, even if the population at any static moment is below detection.

We considered two models of the protein unfolding: two-state (Fig. 4, top) and three-state (Fig. 4, bottom). In the first model, monomers are not stable by themselves and unfold as soon as dimer falls apart. If that model was correct we expected dimer dissociation (as measured by the interchange assay) to coincide with the protein unfolding (measured by H/D exchange), both incidentally and kinetically. The second model assumes that intramolecular, rather than intermolecular interactions provide most of the stabilization energy. Assuming that model was correct, we expected to observe dimer dissociation without unfolding at temperatures close to the start of the phase transition. Observation of NAD⁺ synthetase dimer dissociation at 40°C (by dimer interchange assay) without unfolding (by H/D exchange) strongly suggested that dimer dissociation precedes unfolding. Furthermore observation of dimer dissociation at 45°C was measured to be ~4-fold more rapid than complete unfolding was clearly compatible only with the second model, where for every dimer dissociation event there

is only one unfolding event (unfolding of both monomers).

The peak transition observed in the H/D exchange experiments was taken as evidence of unfolding, since the mass of the second peak was very close to the final deuteration state (equilibrium). In the folded protein, complete deuteration would have taken a considerable amount of time as the protein core is usually very stable and inaccessible to solvent. After unfolding of the monomer, nearly all of the amide protons come into equilibrium with the solvent composition, and so this serves as a record of the unfolding event. Unexpectedly to us, after the phase transition at 45°C, the second peak was not at the end point, which indicated that some elements of structure remained in the protein during unfolding at this temperature.

Observation of slight peak broadening in the H/D exchange experiment performed at 40°C suggested that some kind of EX1 process takes place and there could be intermediates on the unfolding pathway that are beyond the resolving power of the methods used in this work. It was also reasonable to assume that this increase is due to the exposure of the dimer interface, which makes up to 20% of the overall monomer surface.²⁻⁵ However, the EX1 exchange was only obvious at very late time points, while the dimer interchange assay indicated that the dissociation proceeds at a significantly larger pace. This suggests that some other processes lead to the peak broadening, most likely region-limited and/or partial unfolding. That would suggest presence of other unfolding intermediates that are beyond the resolving power of our methods due to high EX2 component of the H/D exchange and a small scale of the EX1 component. The presence of relatively long-lived folding intermediates was previously observed by H/D exchange pulse-labeling and mass spectrometry.¹⁸

We have shown that the dimer interchange assay as designed in this work, can be of great utility in studying unfolding of multimeric proteins. Mass spectrometry allows measurement of protein masses with unrivaled accuracy and resolution: with native mass spectrometry it was possible to distinguish 11- and 12-mer non-covalent oligomers of P22 portal protein with mass accuracy less than 1%.²⁰ Charge state and the number of charges of the protein molecules electrosprayed under non-denaturing conditions are usually low relative to the denatured protein,²¹ which also contributes to higher signal intensity and resolution. The prerequisite for the successful implementation of the dimer interchange assay is high stability of the studied oligomer both in solution and in gas phase and its good solubility in a volatile buffer with low ionic strength (ammonium acetate). NAD⁺ synthetase fulfilled all of those conditions. In this case, the gas phase stability was very high, which allowed the use of very high cone voltage, which improved the molecule's desolvation, and the mass spectrometric resolution.

Materials and Methods

Protein production

NAD synthetase was expressed and purified as previously described (1–3). For the ^{15}N -labeled NAD synthetase, a minimal media with ^{15}N -ammonia (Cambridge Isotope Laboratories) was utilized as a sole source of nitrogen.

H/D exchange on NAD synthetase and mass spectrometry

NAD synthetase (5 μL of 20 μM) in 20 mM EPPS buffer pH 8.5 was quickly diluted 10-fold with 20 mM EPPS in D_2O pH 8.5 (no correction for pD). Reaction at 40 and 45°C was performed on an iCycler thermocycler (BioRad, Hercules) using thin-walled 0.2 mL PCR tubes. Before dilution into the D_2O buffer, both protein and the buffer were preheated for 1 min at the specified temperatures. At different intervals, the H/D reaction was stopped by the addition of 50 μL of ice-cold 8M urea, 1% formate, and snap freezing in liquid nitrogen.

The samples were rapidly thawed just before measurement, and 15 μL of the mixture was injected into C4 micro trap (Michrom), submerged into ice bath, and connected to an LCT mass spectrometer (Micromass, UK) equipped with a micro-flow electrospray interface. Spectrums were analyzed using MassLynx software (Micromass, UK). Protein elution was performed using an ultimate LC system (Dionex, Sunnyvale) controlled by MassLynx software (Micromass, UK) with the following gradient: (buffer A: 5% acetonitrile, 94.9% H_2O , 0.1% formate; buffer B: 5% H_2O , 94.9% acetonitrile, 0.1% formate) with a 6 min linear gradient of 100% A to 100% buffer B and a flow rate 64 $\mu\text{L}/\text{min}$. The mass spectrometer was calibrated with a 1 mg/mL poly alanine solution.

Dimer interchange and native mass spectrometry

Normoisotopic NAD synthetase (10 μL of 40 μM) was mixed with 10 μL of 40 μM ^{15}N -labeled NAD synthetase, incubated at each specified temperature after which, the mixture was quickly chilled in an ice bath. To exchange the ion suppressing EPPS buffer to an electrospray compatible buffer, the protein mixture was diluted with 5 mM ammonium acetate pH 7.9, and concentrated using Ultrafree Centrifugal filter with a 5K cutoff at 4°C as per the manufacturer's instructions (Millipore). This procedure was performed four times to ensure that the final EPPS concentration was well below 10 μM and that the final protein concentration was close to 10 μM . The resulting mixture was directly infused into an LCT mass spectrometer and software as mentioned above, but at a flow rate of 1 $\mu\text{L}/\text{min}$. The mass spectrometer parameters were: capillary voltage 2600 V, cone voltage 200 V, source temperature 60°C, pressure in the first stage 5.9 mbar

(adjusted with a speedivalve accessory mounted on the rotary pump), and with the RF lens set to 750 V. The mass spectrometer was calibrated with 10 mg/mL CsI solution in H_2O just before the experiment with.

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