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Characterization of the solution properties of *Pichia farinosa* killer toxin using PGSE NMR diffusion measurements

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Abstract

The solution behaviour with respect to pH and NaCl concentration of the tertiary structure and propensity for aggregation of salt-mediated killer toxin (SMKT) from *Pichia farinosa* was examined using pulsed-gradient spin-echo NMR diffusion measurements. It was found that in 0.15 m NaCl the tertiary structure of SMKT was constant below pH 5.0, with the native SMKT existing as an unaggregated heterodimer containing the β-subunit in a compactly folded form. However, above pH 5.0 the β-subunit dissociated and lost its compact structure, becoming a random coil with an ∼37% increase in effective hydrodynamic radius. To determine the effects of NaCl concentration on the tertiary structure of SMKT, diffusion measurements were performed at pH 3.5 and NaCl concentrations up to 2 M. Both the tertiary structure and aggregation state of SMKT were found to be insensitive to the salt concentration which indicates that the activity of the toxin is not a direct result of salt–protein interactions.

Introduction

Killer toxins are secreted by certain strains of yeasts and fungi and kill the competitive strains. One of these killer toxins is produced by the halotolerant yeast, *Pichia farinosa* KK1 strain. The toxin exhibits maximum activity under acidic conditions (pH 2.5–4.0) in the presence of 2 M NaCl (Suzuki and Nikkuni, 1989) and is therefore termed salt-mediated killer toxin (SMKT). Under acidic conditions, SMKT is in the form of a tightly bound heterodimer. The two polypeptide subunits are not linked by any disulphide bridges (Suzuki and Nikkuni, 1994). The α-subunit has a long hydrophobic section (35 residues) which infers a high potential for forming a membrane spanning region. Under neutral or base conditions, the heterodimer easily dissociates into its subunits: α (MW 6344) and β (MW 7874), with the α-subunit precipitating and the β-subunit remaining in solution. From X-ray crystallographic analysis of the native heterodimeric form (Kashiwagi et al., 1997), it has been determined that in the native form the α-subunit has one α-helix and two β-strands whilst the β-subunit has one α-helix and three β-strands (see Figure 1). The five β-strands form one β-sheet. It has been shown from circular dichroism (CD) measurements that the secondary structure of SMKT changes drastically in the pH range 5.1–5.5, and that after dissociation of the subunits the β-subunit loses its well-defined secondary structure (Suzuki et al., 1997). This unfolding appears to be irreversible. From the CD measurements, it was found that the salt concentration had no noticeable effect on the SMKT secondary structure.

The crystal structure indicates that the intact heterodimer has an ellipsoidal structure (41 Å × 29 Å × 34 Å) with most of the hydrophobic residues of the α-subunit being buried in the core of the β-subunit (Kashiwagi et al., 1997). Further, about 60% of the surface of the β-subunit comprises hydrophobic residues and the majority of these are involved with interactions with the α-subunit. Four pairs of acidic

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Abbreviations: PGSE, pulsed-gradient spin-echo; SMKT, salt-mediated killer toxin.

residues exist and presumably form hydrogen bonds that stabilize the heterodimer. As the pH is increased, these interactions are likely to become repulsive and destabilize the structure.

Since rather drastic changes in the protein structure are expected as the heterodimer dissociates, the translational diffusion of the heterodimer and the β-subunit should provide information on the changes in tertiary structure that occur, without requiring the spectral assignment of the peptide subunits. The Stokes–Einstein relation relates the diffusion coefficient, *D*, of a particle (rigorously at infinite dilution) to its molecular shape via a friction coefficient, *f*,

$$D = \frac{kT}{f} \tag{1}$$

where T is the temperature and k is the Boltzmann constant. In subsequent analysis we assume that the shapes of the heterodimer and β-subunit are spherical. In the case of a sphere,

$$f = 6\pi R_{\mathrm{D}} \tag{2}$$

where R_{D} is the effective hydrodynamic (or Stokes) radius. The diffusion coefficient is a much more reliable guide to the apparent molecular weight and tertiary structure of a molecule than NMR relaxation time or line width measurements, especially in the absence of resonance assignments (Price et al., 1997).

In the current study, translational self-diffusion measurements were performed using the pulsed-gradient spin-echo (PGSE) NMR method. The theoretical and experimental aspects of the PGSE NMR method have recently been reviewed (Price, 1997, 1998). PGSE NMR measurements have previously been used to study the helix to coil transition in poly-L-glutamic acid (Moll, 1968). The helix to coil transition resulted in a nearly threefold decrease in the diffusion coefficient, although the experimental diffusion data contained large error margins. In a later study of the urea-induced unfolding of lysozyme (Jones et al., 1997), PGSE NMR measurements revealed that the effective hydrodynamic radius increased by 38% (i.e. the diffusion coefficient decreased by ∼28%).

In the current study, diffusion measurements were performed on SMKT at various pH values and in the presence of salt concentrations up to 2 M to probe for changes in tertiary structure and to determine if the SMKT had any propensity for aggregation.

Materials and methods

SMKT was prepared as described previously (Suzuki and Nikkuni, 1994). A solution containing ∼30 mg of SMKT in 15 ml of 10 mM phosphate buffer was concentrated using a centriprep (cutoff MW 3000; Amicon) until the solution volume was reduced to about 1 ml. Then 14 ml of 10 mM phosphate buffer containing 0.15 M NaCl at the appropriate pH was added to the concentrated SMKT solution. This was again concentrated to about 1 ml using a centriprep. This dilution/concentration step was repeated for each sample with buffer of the appropriate pH until the target pH was achieved. Next, the dilution/concentration step was repeated twice with 2 mM phosphate buffer and 0.15 M NaCl and the same pH value. Finally, the protein concentrations of the samples were adjusted to 1 mM and the concentration checked using UV spectroscopy. SMKT solutions at various salt concentrations were prepared in the same manner with appropriate changes in the buffer NaCl concentration.

To separate the β-subunit from the heterodimer, NaOH solution (1 M) was added to a portion of the SMKT solution as prepared above until the pH was greater than 8. The solution became cloudy as the subunits separated due to precipitation of the α-subunit. The α-subunit was removed by centrifugation (10 000 × g, 3 min) leaving the β-subunit in solution. The pH of the β-only solution was adjusted using dilute HCl solution. We have previously found, using tricine SDS-PAGE, that this method allows the β-subunit to be separated with no α-subunit detectable (Suzuki and Nikkuni, 1994).

All ^{1}H NMR experiments were performed at 500 MHz using a Bruker DMX 500 equipped with a 5 mm triple-resonance probe with three-axis shielded magnetic field gradients. In all cases, a spectral width of 7.5 kHz was digitized into 32 K data points and presaturation was used to suppress the water resonance. A recycle delay sufficient to allow for full relaxation (i.e. $>5 \times T_1$) was allowed between each transient. For the PGSE experiments only the *z*-gradient was employed and its strength was first calibrated using the known diffusion coefficient of water (Mills, 1973). All measurements were performed at 298 K. The temperature in the NMR probe was calibrated using methanol. Samples (∼ 0.1 ml) for the PGSE experiments were placed in susceptibility-matched microtubes (BMS-005; Shigemi, Tokyo). The stimulated echo pulse sequence was used to measure the translational diffusion coefficients. For a single diffusing species, the

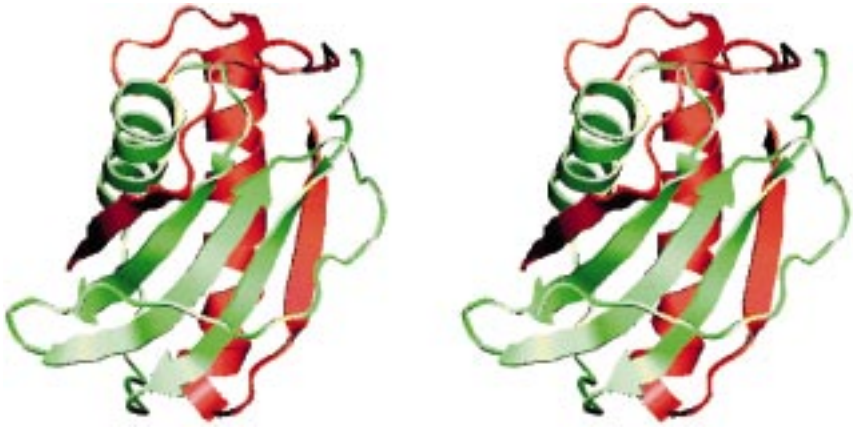

Figure 1. Stereoview of the X-ray crystallographic structure of the intact heterodimer (Kashiwagi et al., 1997). The α- and β-subunits are shown as red and green, respectively. The α-helix and β-strands are clearly visible. The illustration was drawn with the program QUANTA (Molecular Simulations Inc.).

echo signal attenuation, E, is related to the experimental parameters and the diffusion coefficient, D, by (Stejskal, 1965; Price, 1997)

$$E = \exp(-\gamma^2 g^2 D \delta^2(\Delta - \delta/3)) \quad (3)$$

where γ is the gyromagnetic ratio, g is the magnitude, δ is the duration of the gradient pulses and Δ is the separation between the leading edges of the gradient pulses. Further experimental details can be found in the figure captions.

Results and discussion

One-dimensional ^{1}H NMR spectra of the intact SMKT and of the β-subunit are shown in Figure 2. A typical set of experimental PGSE spectra of SMKT is shown in Figure 3, and the results of the diffusion measurements for the intact SMKT and β-subunit are summarized in Figure 4.

The diffusion coefficient of the intact heterodimer was nearly a constant value of 1.15×10^{-10} m^2 s^{-1} up to pH 5, above which the heterodimer dissociated. The slight decrease in the diffusion coefficient near pH 5 probably reflects the onset of this dissociation. Given the similarity of this diffusion coefficient to that of monomeric lysozyme (1 mM, pH 3 under the same experimental conditions), 1.16×10^{-10} m^2 s^{-1} (Price et al., unpublished results), and that lysozyme and SMKT have almost the same MW (i.e. 14.3 kDa and 14.2 kDa, respectively), it is clear that SMKT exists in the heterodimeric state below pH 5. Although SMKT

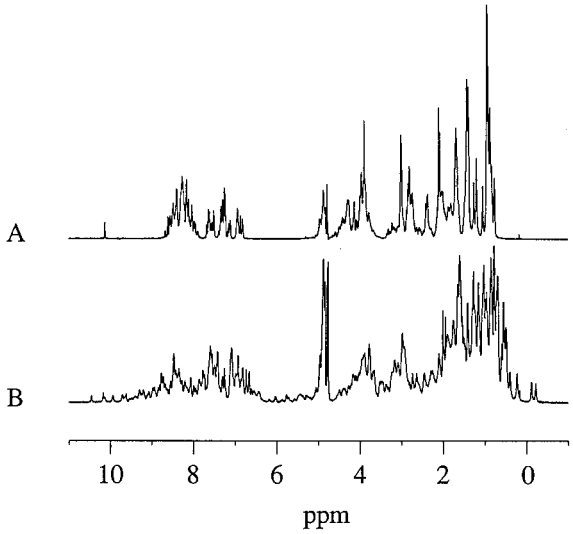

Figure 2. One-dimensional NMR spectra of (A) the β-subunit and (B) the intact heterodimer. Both samples contained 1 mM protein at pH 4 in 0.15 M NaCl. Each spectrum is the average of eight scans. From these spectra alone, it is very difficult to gauge either the aggregation state or the degree of unfolding of either the intact SMKT or the β-subunit.

is not perfectly spherical, small deviations from perfect sphericity have little effect on the diffusion coefficient (Price et al., 1997). Using Equation 1 and taking the viscosity of pure water at 298 K (8.904×10^{-4} kg m^{-1} s^{-1}) (Weast, 1984) and the measured diffusion coefficient of SMKT (1.15×10^{-10} m^2 s^{-1}) we can calculate the effective hydrodynamic radius of the SMKT heterodimer to be 213 nm. We have neglected the extremely small effects of the 0.15 M NaCl and 2 mM phosphate buffer on the water viscosity. By

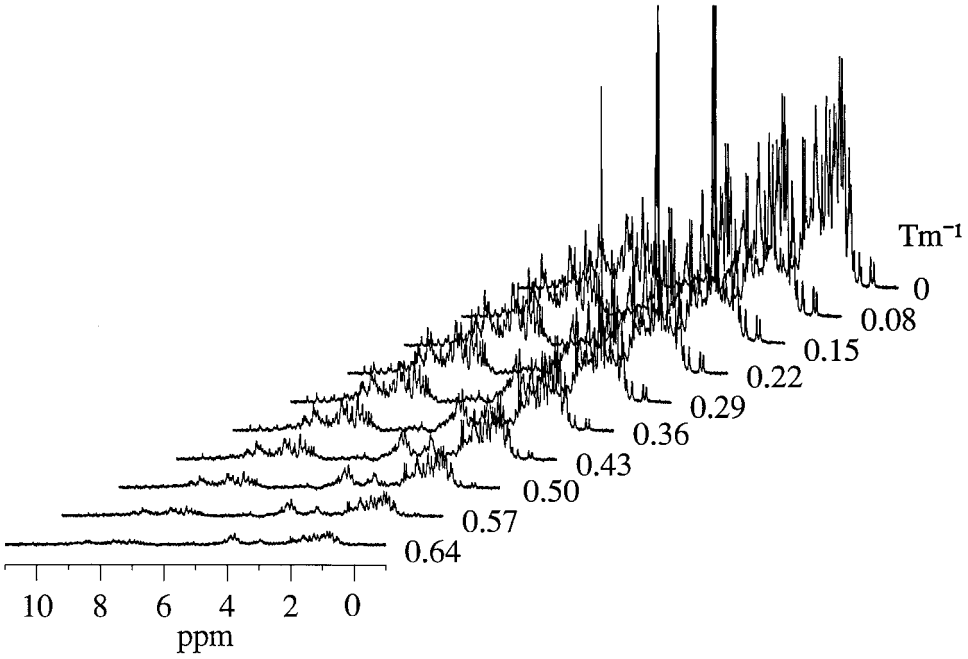

Figure 3. A typical set of ^{1}H PGSE NMR spectra for SMKT (1 mM) in 2 mM phosphate buffer and 0.15 M NaCl at pH 3. The acquisition parameters were $\Delta = 34$ ms and $\delta = 5$ ms. Each spectrum is the average of 64 scans. The gradient strength was varied up to a maximum of 0.64 T m^{-1}. The vast difference in the diffusion coefficient of the solvent water and the protein is reflected by the very different rates of attenuation with the water peak disappearing by $g = 0.22$ T m^{-1}. By regressing Equation 3 onto the spin-echo attenuation data, the SMKT diffusion coefficient was determined to be $1.15 \pm 0.02 \times 10^{-10}$ m^2 s^{-1} (the true accuracy of the measured diffusion coefficient is in reality of the order of 3% due to factors such as limited signal-to-noise and temperature drift).

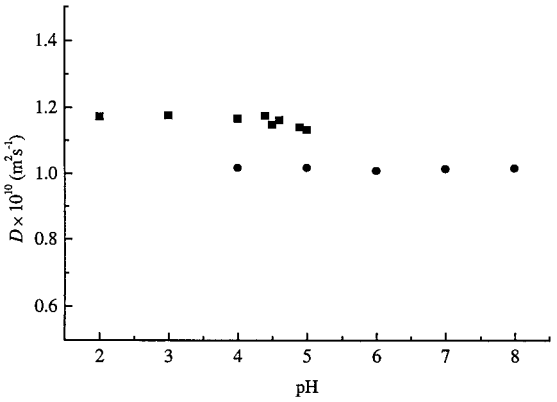

Figure 4. Diffusion coefficient versus pH for the intact SMKT (■) and β-subunit (●). In both cases, the samples were dissolved in 2 mM phosphate buffer to give a final concentration of 1 mM. The diffusion coefficient of the SMKT could not be measured above pH 5 due to dissociation of the heterodimer. There is evidence of a slight decrease in the SMKT diffusion coefficient approaching pH 5. Each data point represents the results of one diffusion measurement. The very small scatter of the data in the pH ranges where there was no structural change shows the high reproducibility of the diffusion measurements.

comparison with some recent work on lysozyme diffusion (Price et al., unpublished results), it is reasonable to expect that at the protein concentration used in the present work the diffusion coefficient determined from the PGSE NMR measurement is very close to the infinite dilution value and is unaffected by crowding (e.g. see Han and Herzfeld (1993)). Further, if we assume that upon dissociation of the heterodimer the subunits themselves are spherical and considering the mass ratio of the two subunits, the hydrodynamic radius of the β-subunit when folded in the compact form as part of the heterodimer should be 175 nm.

The diffusion coefficient of the β-subunit was nearly constant at 1.02×10^{-10} m^2 s^{-1} throughout the pH range 4–8. This value was significantly smaller than that of the heterodimeric SMKT. Using the same procedure as above for the heterodimer we calculate the effective hydrodynamic radius of the β-subunit to be 240 nm. Thus, the effective hydrodynamic radius of the β-subunit increases by 37% upon dissociation. This seemingly counterintuitive finding can be reconciled by noting that previous CD work has shown that the dissociated β-subunit does not have secondary

structure. Thus, when the β-subunit dissociates from its compact state in the heterodimer, it becomes a (much less compact) random coil. In contradistinction to the CD finding which reports on the secondary structure, the diffusion data allow the scale of this decompaction to be estimated since it reports on the tertiary structure. It is interesting that the observed increase (as a percentage) was almost exactly the same as that found for the urea-mediated unfolding of lysozyme (Jones et al., 1997).

To determine the effects of NaCl concentration on the tertiary structure and aggregation state of SMKT, we measured the diffusion coefficient of the heterodimer at pH 3.5 and 298 K in salt concentrations up to 2 M. The diffusion coefficient of water under the same conditions, but in the absence of SMKT, was also measured (data not shown). The diffusion coefficient of the SMKT was observed to decrease with increasing salt concentration. However, after allowing for the change in solution viscosity due to the salt concentration (using the measured water diffusion coefficients), the diffusion of the SMKT was found to be nearly constant at about 1.1×10^{-10} m^2 s^{-1}. This clearly indicates that the SMKT does not aggregate as the salt concentration increases nor does it undergo any significant changes in tertiary structure. Hence, the increase in SMKT activity with salt concentration is not due to a change in the tertiary structure. This result also shows the need for appropriate control experiments in order to make cogent interpretations of diffusion data.

Our diffusion measurements showed that the aggregation state and tertiary structure of native SMKT and the dissociated β-subunit were unaffected by pH. The value of the diffusion coefficient for native SMKT was consistent with it existing in the heterodimeric state. Since the SMKT concentration used in the present study is rather higher than that found in nature, it is most likely that heterodimeric SMKT is the normal form in nature. As the salt concentration did not affect the SMKT structure, we surmise that the importance of the high salt concentration with respect to the activity of the toxicity of SMKT is due to osmotic pressure effects. The high osmotic pressure may make the cells of the sensitive yeast strains more susceptible to the toxic effects of SMKT or, conversely, the SMKT may make the cells more sensitive to osmotic pressure. Our results also showed that upon dissociation from the intact heterodimer structure of SMKT, the β-subunit lost its compact state. Based upon these findings, genetic and biochemical studies are under way to elucidate the killer mechanism of SMKT.

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