ASSIGNMENTS, STRUCTURE AND DYNAMICS OF STAPHYLOCOCCAL NUCLEASE

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Introduction

Proteins are complex natural polymers that are composed of twenty different amino acid monomer units. In their native states nearly all proteins adopt well-defined but irregular three-dimensional structures. A typical globular protein is composed of alpha-helices, beta-strands and various types of turns arranged to form a complex but compact structure. The intimate relationship between protein function and structure has been recognized for many years, and is the reason that protein structure and dynamics have been intensively studied. Such studies have received recent impetus because of advances in molecular biology which permit proteins to be produced in high yield with an almost limitless variety of variations in the amino acid sequence[1].

For nearly forty years three-dimensional structures of crystalline globular proteins have been determined by X-ray diffraction[2]. More recently two-dimensional NMR techniques have been developed that yield structures of proteins in solution[3]. Because resolution and sensitivity of the NMR experiment decrease as molecular weight increases, structural determination using NMR is limited to unlabeled proteins having molecular weights of less than ca. 10kD. However, this limit increases significantly if amino acids that are labeled with deuterium, 13C and/or 15N are incorporated into the protein. We have, for example, obtained nearly complete proton resonance assignments[4] of the protein staphylococcal nuclease complexed with thymidine 3',5'-diphosphate and Ca2+, Mr ca. 18kD, from two- and three-dimensional NMR spectra of the labeled protein. These assignments together with measurements of NOE's, coupling constants, relaxation rates and amide proton-deuterium exchange provide a significant body of information about the structure and dynamics of the protein.

Experimental

Staphylococcal nuclease was obtained from transformed E. coli grown on defined media as described previously[4]. NMR spectra were obtained using high resolution NT500, AM500 and AM600 spectrometers and a homebuilt solid state 6T instrument equipped with a Doty Scientific CPMAS probe.

Results

A ribbon diagram of the nuclease backbone structure[5], derived from the X-ray coordinates, is shown in Figure 1. The sequence of amino acids that are identified in the crystal structure is listed in Figure 2. We have assigned the backbone nitrogen and proton signals of the amino acid residues that are underlined in the figure. With the exception of protons beyond the beta-carbons in the Lys, Arg and Glx residues nearly all non-exchangable sidechain protons of the underlined residues have also been assigned.

As noted above, isotope enrichment played an essential role in our assignment strategy, particularly 15N labeling. The heteronuclear multiple quantum shift correlation, HMQC, spectrum of the fully 15N labeled protein, Figure 3, provides the chemical shifts of nearly every backbone amide nitrogen and proton in the protein. These signals are assigned to specific types of amino acids by labeling the protein with one type of 15N enriched amino acid. The inset in Figure 3 shows the HMQC spectrum of nuclease labeled with [15N]Phe, which immediately identifies the three Phe signals in the fully labeled spectrum. In addition, simultaneous labeleing with [1-13C]Thr yields the assignment of Phe34, because the Phe34 15N signal is split by the carbonyl carbon of Thr33. A variety of HMQC and isotope edited two-dimensional NMR experiments together with NOESY and HOHAHA spectra of natural abundance samples provided sequential assignments[4] of the underlined residues.

A NOESY-HMQC three-dimensional spectrum of the fully 15N labeled protein[6] confirmed the assignments obtained from the various two-dimensional spectra, and, in addition, as a consequence of the superior resolution and sensitivity of the 3-D spectrum, also provided assignments for E122, Q123, E142 and D143. The 2-D NOESY connectivities involving these residues were weak, absent or ambiguous, and therefore they could not be confidently assigned without the information contained in the 3-D spectrum, Figure 4.

The Pro residues present a challenging assignment problem because they lack amide protons. The nuclease Pro assignments were made[7] using 13C edited NOESY spectra of samples labeled with [2-13C]Pro and [4-13C]Pro.

The protein backbone assignments together with the NOESY data provide information about the protein structure in solution. Three observed[4] sets of long sequences of dNN connectivities involving residues 58-69, 101-109 and 124-137 are strong evidence for alpha helical structures in these regions of the amino acid sequence. In addition sequences of strong dαN connectivities involving residues 7-18, 22-29, 31-39, 71-79, 86-96 and 109-115, together with numerous long range dαα and dNN connectivites between these residues are strong evidence of antiparallel beta-sheet structure. These secondary structural features found in solution are also observed in the crystal structure[8]. Another feature of the crystal structure, namely, an uncommon cis peptide bond between K116 and P117, also occurs in the solution, as shown by the strong NOESY connectivity between the alpha protons of K116 and P117[7].

Additional evidence that most of the protein backbone has nearly the same structure in solution and in the solid state comes from comparisons of 13C and 15N chemical shifts measured[9] in the crystalline state with measurements made in solution. Figure 5 compares the chemical shifts of [15N]Val labeled nuclease in the crystalline and solution states. With the exception of V51, there is excellent agreement between the 15N chemical shifts in the two states. The difficulty in identifying V51 in the solid state spectrum is not too suprising because the X-ray[8] temperature factors are much larger than average, in the neighborhood of residue 50, indicating a disordered structure in this region of the protein sequence. This region of the protein is evidently more disordered and flexible in solution than in the crystal because relatively few of the NOE connectivites predicted by the crystal structure for residues 43-57 have been observed[4].

Quite similar solution and crystal backbone structures are also found[10] by comparing measured NH-αH J couplings with J couplings derived from the Karplus equation[2] and the crystallographic[8] phi angles. Overall the RMS difference in the NMR and crystallographic J values for nuclease is nearly as good as found[11] for the BPTI protein that was used for parameterization of the Karplus equation. One interesting result is that the major contributions to the RMS difference originate from K70, K78, Y85, E135 and S141. The first three residues are involved in intermolecular contacts in the nuclease crystal, K78 is in a sharp turn immediately adjacent to the major interprotein contact domain in the crystal, and S141 is the last residue observed in the nuclease crystal structure[8]. A second interesting result is that energy minimization of the crystal structure followed by a 100ps molecular dynamics simulation of the fully hydrated energy minimized protein crystal structure significantly decreased agreement between NMR and X-ray calculated J values. Evidently 100 ps of dynamics is insufficient to move the structure out of the local energy minimum that resulted from straightforward energy minimization of the good crystal structure.

In addition to structural informantion, NMR measurements provide useful information about nuclease molecular dynamics. Measurements of T1, T2 and NOE have been made[12] for ca. 100 of the assigned backbone amide nitrogens in nuclease. Using the model independent approach[13] we find an overall correlation time of 9.1 ns for nuclease and order parameters in the range 0.78 to 0.92 for the backbone nitrogens. The large order parameters show that residues in the non-terminal portions of the protein exhibit only small amplitude backbone motions on the picosecond timescale. However, linewidth measurements show that residues E52 and K53, located in the disordered loop region discussed above, also undergo significant additional motions on the ms timescale.

While relaxation measurements provide information about motions on the millisecond to picosecond timescales, amide hydrogen-deuterium exchange experiments provide information about motions on a much slower time scale. For instance, twenty four hours after dissolving nuclease in deuterium oxide, about forty five amide protons remained unexchanged with solvent[4]. The locations of these slowly exchanging

amides are indicated by the shaded regions in Figure 1. As expected, all slowly exchanging amide protons are observed to form hydrogen bonds in the crystal structure; however not all hydrogen-bonded amide protons exchange slowly, i.e. about one-half of the hydrogen bonded protons have exchange lifetimes less than ca. 8hr. For instance only three amide protons in the helix spanning residues 124-137 exchange slowly, and only amides in the C-terminal half of helix 58-69 exchange slowly. After maintaining the sample at 8°C for ca. 8 months, it was found that helix 99-109 and the beta-strand 86-96 were the regions having the greatest stabilities against hydrogen exchange. This beta-strand also contains Y91, the only tyrosine in nuclease whose ring flips slowly in solution[4]. In addition the Y91 hydroxyl proton is not exchange broadened in water. This result enabled us to identify NOE connectivities between this proton and its adjacent ring protons as well as amide protons of T120 and H121. The Tyr91-OH is the only nuclease hydroxyl proton that we have observed.

In addition to studying the structure and dynamics of wild type nuclease, we are, in collaboration with Professor John Gerlt, comparing the spectra of wild type nuclease with that of the mutant E43D. The latter has ca. 0.1% the activity of the wild type enzyme. The wild type and mutant protein spectra show many small differences in chemical shifts; however their NOESY spectra are qualitatively the same. A careful comparison of the NOESY spectra is currently underway to determine if structural differences between the wild type and mutant proteins can be identified.

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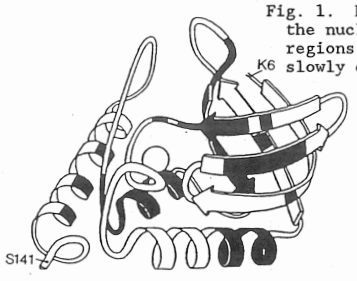

Fig. 1. Ribbon representation of the nuclease backbone; shaded regions show locations of the slowly exchanging amide protons.

6 11 21 31 41 51 61
KLHKEPATLIKAIDGDTVKLMYKGQPMTFRLLLVDTPETKHPKKGVEKYGPEASAFTKKMVENAK

71 81 91 101 111 121 131 141
KIEVEFDKGQRTDKYGRGLAYIYADGKMVNEALVRQGLAKVAYVYKPNNTHEQHLRKSEAQAKKEKLNIWS

Fig. 2. Sequence of amino acids identified in the crystal structure of nuclease. Backbone proton and/or nitrogen signals of underlined residues are assigned.

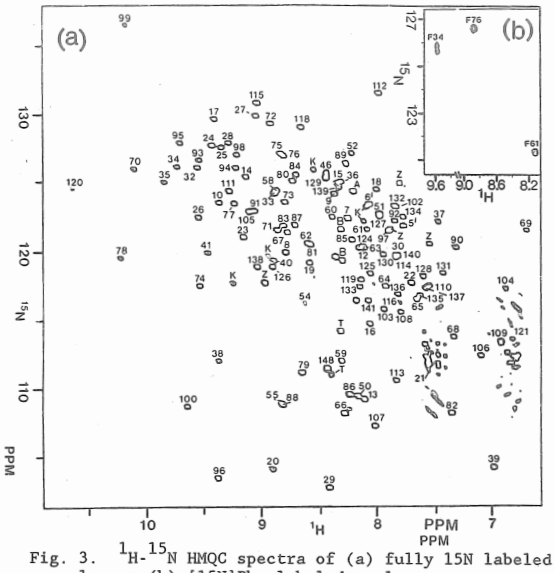

Fig. 3. ^{1}H-^{15}N HMQC spectra of (a) fully 15N labeled nuclease; (b) [15N]Phe labeled nuclease.

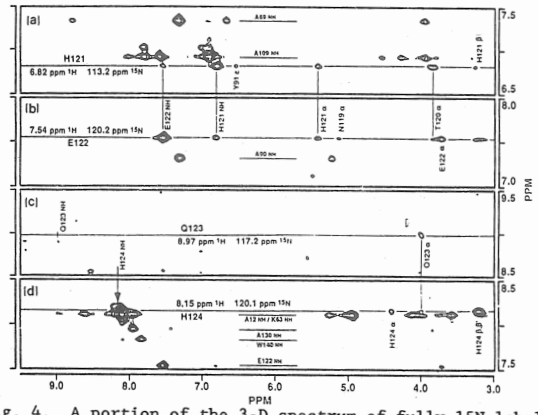

Fig. 4. A portion of the 3-D spectrum of fully 15N labeled nuclease showing dNN connectivities of H121-E122 the $d_{N\alpha}(i,i)$ connectivity of Q123 that were obscured by other connectivities in the 2-D spectra.

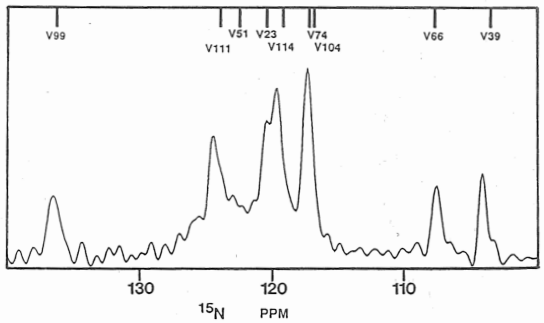

Fig. 5. Comparison of solution chemical shifts, indicated by the short vertical bars, with the CPMAS spectrum of crystalline nuclease labeled with [15N]Val. The broad spectral component centered at 118 ppm is primarily due to amorphous material.

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