Folding of an isolated ribonuclease H core fragment

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Abstract

Based on results from both equilibrium and kinetic hydrogen exchange studies of *Escherichia coli* ribonuclease HI (RNase H), a fragment of RNase H (eABCD) was designed. The sequence of eABCD contains less than half of the protein's primary sequence and includes the regions that were shown to be the most protected from hydrogen exchange in all previous studies of RNase H. This core fragment of RNase H encodes a well-ordered protein with native-like properties. When isolated from the full-length monomeric protein, the eABCD fragment forms a stable dimer. However, we show indirectly that the monomeric form of eABCD is folded and has an overall secondary structure similar to the dimeric form.

Keywords: ribonuclease H; protein folding; protein fragments; subdomain

Understanding how the amino acid sequence of a protein encodes its three-dimensional structure remains a major challenge for protein chemists. Because most small proteins appear to fold in a cooperative two-state manner, detailed characterization of partially folded structures or intermediates has been difficult. Recently, amide hydrogen exchange has been used to collect information about each residue in a protein as it folds or undergoes denaturation. Protein dissection, the study of smaller fragments or regions of a protein, can also provide important information about the independence or interdependence of different regions within a protein. If these fragments resemble the intermediates formed by the parent protein, they provide an avenue for obtaining high resolution information about such states.

Peptide fragments have been used to study protein structure ever since the pioneering work on Staphylococcal nuclease (Taniuchi & Anfinsen, 1969) and ribonuclease A (Brown & Klee, 1969). More recently, fragments have been used to study the folding of several proteins (Staley & Kim, 1990; Tasayco & Carey, 1992; De Sanctis et al., 1994; Peng & Kim, 1994; Yang et al., 1994; Azuaga et al., 1995; Tasayco & Chao, 1995; Gegg et al., 1997; Constans et al., 1998; Llinás & Marqusee, 1998; Oas & Kim, 1988). In many cases, these small (<100 amino acids) fragments do not fold into stable, unique structures without the assistance of disulfide bonds or oligomerization.

Recently, we have used several different hydrogen exchange methods to study the importance of individual regions to the folding and stability of *Escherichia coli* ribonuclease HI (RNase H).

RNase H is a small (155 amino acid), single-domain protein that contains five α -helices and a five-stranded β -sheet (Fig. 1) (Katayanagi et al., 1992). Like most single-domain proteins, RNase H denatures in an apparently two-state manner when monitored by global probes such as circular dichroism (CD) or fluorescence. However, we found evidence for rare partially folded conformations by measuring the amide hydrogen exchange rates in the native state as a function of low levels of guanidine (Chamberlain et al., 1996). This native state hydrogen exchange study showed that the protons most stable to exchange with solvent are in helices A and D. Protons in helix B and strand 4 have intermediate stabilities while the remainder of the protons monitored have even lower stabilities. In the acid molten globule state, the most structured regions are also helix A and helix D (Dabora et al., 1996). Finally, kinetic refolding studies (Raschke & Marqusee, 1997) show that in the kinetic intermediate formed 14 ms after dilution from denaturant, helices A and D are the most structured regions and slow hydrogen exchange up to 229-fold. All these experiments describe a specific element of RNase H, containing helices A and D, that can fold while the remainder of the protein appears unfolded. Based on the dominant role this region appears to play in the folding and stability of RNase H, we asked whether this region comprised an autonomous folding unit. In other words, is this local region alone sufficient to fold into a stable native structure?

Results and discussion

Design and initial characterization of the isolated core of RNase H

We designed and characterized a 74 amino acid fragment of RNase H that contains the most stable section of the protein, helices A and

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Fig. 1. Ribbon diagram of the crystal structure of wild-type *E. coli* ribonuclease H1 (2rn2) (Katayanagi et al., 1992). The region corresponding to eABCD is shaded. Helices are indicated: A–E. Beta-strands are labeled: $\beta 1$ – $\beta 5$.

D, and the intervening sequence that also contains strand 4 and helices B and C (eABCD). The eABCD fragment contains less than 50% of the 155 amino acids that comprise RNase H. The isolated eABCD fragment adopts a well-ordered structure in solution. The CD spectrum shows the pronounced double minima indicative of helical structure (Fig. 2), suggesting that, like this region in the full length protein, a large proportion of the backbone is helical.

Equilibrium sedimentation revealed that unlike full-length RNase H, which is monomeric, eABCD exists in a monomer/dimer equilibrium

Fig. 2. CD spectrum of the eABCD fragment at pH 5.5, 25 °C.

librium (Fig. 3). To determine the dissociation constant, data were collected at three protein concentrations at pH 5.5, 25 °C and processed using a global fitting algorithm ($K_d = 0.3 \mu\text{M}$). Under native conditions and in the concentration range used for the CD and fluorescence spectroscopy reported here, 77–90% of the molecules are in a dimeric form. Under our NMR conditions ($\sim 1 \text{ mM}$), >98% of the molecules are in a dimeric form.

Thermal denaturation of eABCD was monitored by measuring CD signal at 222 nm as a function of temperature for a $4.4 \mu\text{M}$ sample of eABCD (Fig. 4A). The observed transition is reversible and apparently two-state, with a midpoint of 56 °C (some aggregation was observed after heating and cooling at a 10-fold higher concentration, $44 \mu\text{M}$). Urea-induced denaturation of eABCD monitored by CD and tryptophan fluorescence further suggested that eABCD folds in a cooperative, apparently two-state manner. The simplest model for cooperative unfolding of a dimer is a folded dimer to unfolded monomer scheme ($D \rightleftharpoons 2U$). Figure 4B shows the urea denaturation data processed assuming this model and a linear extrapolation (Santoro & Bolen, 1988). The free energy of unfolding, based on this model, was estimated to be $12.0 \text{ kcal mol}^{-1}$ in the absence of denaturant.

The above model ($D \rightleftharpoons 2U$) assumes that there are no populated folded monomeric species. To determine if the monomers of eABCD were structured, further equilibrium sedimentation studies were carried out in low concentrations of urea (0–1 M). Multiple initial protein concentrations were used at each urea concentration. The data from each urea concentration were fit using a global fitting algorithm. K_d 's of 0.3, 0.4, 0.5, 0.6, and $0.7 \mu\text{M}$ eABCD were determined in 0.00, 0.25, 0.50, 0.75, and 1.0 M urea. Figure 4C

Fig. 3. Equilibrium sedimentation of eABCD. A representative fit of absorbance at 280 nm vs. radius data at equilibrium (40,000 RPM, 25 °C, pH 5.5, 0.0 M urea). The fit shown is the best fit to a monomer–dimer equilibrium with data from runs at 30,000 and 40,000 RPM and initial protein concentrations of 3.5, 7.0, and $14 \mu\text{M}$.

Fig. 4. **A:** Thermal denaturation of eABCD monitored by CD. The CD signal of a 4.4 mM solution of eABCD at 222 nm is plotted as a function of temperature. eABCD unfolds cooperatively with a midpoint of 56°C at pH 5.5. **B:** Chemical denaturation of eABCD. The fraction of folded monomers in a 5.3 μ M eABCD solution (pH 5.5, 25°C) is shown as a function of urea concentration. When fit to the $D \leftrightarrow 2U$ two-state model, data from CD (squares) and fluorescence (circles) observed as a function of denaturant overlay, suggesting that tertiary and secondary structure are lost concurrently. **C:** Direct comparison of the $D \leftrightarrow 2U$ model to observed dissociation constants. The fraction of unfolded subunits as determined by the fit of data from the urea induced denaturation of 1.2 μ M eABCD to the $D \leftrightarrow 2U$ model (squares) does not agree with the calculated monomeric content as determined by equilibrium sedimentation (circles) at low urea concentrations. The dotted line is the predicted fraction monomeric assuming a linear relationship between $\ln(K_d)$ and urea concentration.

shows the fraction monomer, under the conditions of the denaturation studies, is clearly different from the fraction folded calculated with the above two-state model ($D \leftrightarrow 2U$). The CD spectra also show no significant differences in the range of 0 to 1.0 M urea (data not shown). Since the fraction folded as determined by fitting the CD data is larger than the fraction of dimers under the same conditions, the monomers must be structured. We interpret these results to mean that eABCD specifies a folded monomer that is stable relative to the unfolded state, but these monomers readily associate to form even more stable dimers. This implies there is a small population of structured but marginally stable monomeric eABCD present in the pretransition region of our CD-monitored urea denaturation, and folding is not directly linked to dimerization. Thus, the free energy of unfolding estimated with the $D \leftrightarrow 2U$ model (12.0 kcal/mol) reports a lower bound of the stability of the dimer relative to the unfolded monomer. Since dimers are the predominate species, even in 1 M urea, we were not able to fully characterize the monomers by CD or any other spectroscopic probe. While the monomeric form of eABCD shows secondary structure and is therefore folded, we have no information regarding its compactness, tertiary structure or structural uniqueness. Similarly, we know very little about the tertiary structures, packing or dynamics of the partially folded forms of RNase H that the eABCD design was based on. The cores of these partially folded forms may be less compact and structurally unique than they are in the context of the native state. Indeed, the acid state of RNase H is known to be

a molten globule (Dabora & Marqusee, 1994); it has secondary structure but lacks the specific tertiary contacts present in the native state. Therefore, it is more than a formal possibility that a fragment like eABCD, designed to mimic the kinetic and equilibrium intermediates of a protein, may fold into a monomer that is more molten globule-like and not structurally unique. If this is indeed the case for eABCD, dimerization must provide the driving force for packing specificity.

The eABCD dimer is symmetric

To compare the structure of folded eABCD to the structure of the same sequence in the full-length protein, we initiated an NMR and amide hydrogen exchange analysis of the fragment. Under NMR conditions, eABCD is essentially dimeric. The HSQC spectrum (Bax et al., 1990) of eABCD is well dispersed (Fig. 5A), and only one resonance is observed for each backbone $^1\text{H}/^{15}\text{N}$ pair, indicating the dimer is symmetric. Assignments of the backbone resonances were determined for all residues except E48 to M50 (see Materials and methods).

The secondary structure of eABCD is similar to the core of intact RNase H

Regions of secondary structure within eABCD were inferred from analysis of the C_α chemical shifts and the amide hydrogen ex-

Fig. 5. NMR analysis of eABCD. **A:** The $^1\text{H}/^{15}\text{N}$ HSQC spectrum of eABCD. Assignments of the backbone amide N/H pairs are shown. **B:** The helical regions of eABCD. Helices A, B, C, and D from RNase H are shown as thick bars between upper and lower panels. Protection factors (lower panel) and change in C_α chemical shifts from random-coil reference values are shown (upper panel). Filled circles indicate residues predicted to be helical by the C_α chemical shift index (Wishart & Sykes, 1994). Reference lines at ± 0.7 ppm indicate the chemical shift index cutoff values. Protection factors (lower panel) between 3×10^2 and 3×10^4 are observed for 25 residues. Given observed protection from hydrogen exchange and high chemical shift indices the following residues are predicted to be helices: A51–A58, I78–R87, and L104–L110.

change rates. A plot of $\Delta\delta C_\alpha$, the observed C_α chemical shifts minus random-coil C_α chemical shifts (Wishart & Sykes, 1994), for the assigned residues is shown in Figure 5B (upper panel). The

filled circles indicate the residues predicted to be helical based on chemical shift index (CSI). The correspondence of the predicted helices with helices A (43–58), C (81–89), and D (100–112) of RNase H is quite good, although they suggest that helix A may be somewhat longer than it is in RNase H. These CSI-based helical regions correlate with the regions that show the most protection from hydrogen exchange (Fig. 5B, lower panel). Taken together, the CSI analysis and the regions of protection from amide hydrogen exchange suggest that at least residues 51–58, 78–87, and 104–110 are helical. The C-terminal extension of helix A predicted by CSI is not supported by any systematic protection, so the secondary structure of these residues remains in doubt. To summarize the results of these preliminary NMR studies: eABCD contains enough sequence information to encode the A, C, and D helices of RNase H and the residues that are excluded from eABCD are not required to provide measurable protection in helices A and D in full length RNase H.

The characterization of eABCD demonstrates that the eABCD subsequence of RNase H specifies a stable helical domain, and therefore, eABCD is an autonomously folding unit. By many criteria, cooperative unfolding, well-dispersed NMR spectra, and CD spectroscopy, eABCD has been shown to behave like a well-ordered native protein.

Comparison of eABCD and the partly folded forms of RNase H

The largest structural difference between eABCD and the known kinetic and equilibrium intermediates of RNase H is the fact that eABCD dimerizes readily. The molten globule state of RNase H exists as a monomer in solution (Dabora & Marqusee, 1994) and the refolding kinetics of RNase H are independent of protein concentration (T.M. Raschke, pers. comm.). Our data suggest, therefore, that the unprotected β -sheet regions in the intermediates of RNase H (which were not included in eABCD) play an important role in blocking dimerization. Hence, the least stable and slowest folding region of RNase H prevents the formation of oligomers during refolding experiments and in the rare partially unfolded forms present under native conditions.

Dimerization of eABCD

The location of the dimerization surface of eABCD is unclear. One apparent difference between the intermediates of RNase H and the eABCD fragment is found in the region comprising helix B and helix C. In eABCD, much of helix C is protected, while only 1 out of the 10 residues in helix B shows protection. In the partially folded conformation of RNase H populated under native conditions (Chamberlain et al., 1996) helix B appears structured while helix C does not (there is no hydrogen exchange data available on the folding kinetics of helices B and C or on helix C in the molten globule state). These differences suggest that this region may be involved in dimerization of the eABCD fragment. Alternatively, helix A may form the dimer interface. We see variations of peak intensities of helix A in the HSQC spectrum and cannot detect resonances for E48 to M50 in the NMR spectra used for assignments. This is indicative of dynamic behavior in helix A, which could be linked to the oligomerization process. Furthermore, substitution of a glutamic acid for valine at position 54, in helix A, decreases dimerization by 10-fold (S. Robic & S. Marqusee, pers.

comm.). It is unclear, however, whether this is a direct effect at the dimerization surface. Indeed, the structure of RNase H suggests that the most likely dimerization surfaces for the fragment are the hydrophobic surface of helix A that interacts with the β -sheet or the orphaned β -strand in eABCD (Fig. 1, β 4). Identification of the exact location of the dimerization surface will have to await the complete structure of the dimeric fragment.

Previously characterized protein fragments provide no definitive patterns to guide our expectations when 50% or more of a protein is removed. Mini-myoglobin (~70% of the parent protein) retains heme-binding activity and native helical structure (De Sanctis et al., 1994). A subdomain of villin headpiece, HP-35, is less than half of the parent protein but maintains the secondary structure present in the domain (McKnight et al., 1997) and melts cooperatively at a temperature of 70 °C. The C-terminal 205–316 fragment of thermolysin is stable as a monomer or a dimer, depending on solvent conditions (Conejero-Lara & Mateo, 1996). A shorter but related fragment, thermolysin 255–306 folds only as a dimer (Rico et al., 1994). Though the goal of protein dissection studies is usually to determine the structural independence of various regions in a protein, changes in oligomerization state seriously complicate the analysis.

It has been observed in all proteins studied by amide hydrogen exchange that protection from exchange is not uniform (Englander et al., 1996), rather some regions are more protected than others. These patterns of protection are often used to identify kinetic and equilibrium intermediates. However, it is not known if the local sequence of these protected regions contain enough information to define their structures. Interestingly, a fragment of cytochrome b5, "abridged b5," which excludes the heme-binding site shows that a core of the protein, distinct from the heme-binding site can fold independently (Constans et al., 1998). Although the RNase H core eABCD dimerizes, abridged b5 is a structured population of monomers that is in slow exchange between at least two monomeric forms. Both oligomerization and structural heterogeneity of protein core fragments may simply be caused by the exposure of new hydrophobic surface and the loss of folding context. These phenomena may be explained in terms of their presumed role as folding intermediates. Such intermediates would fold before the rest of the chain presenting surfaces that aid the folding of still unstructured parts of the protein. At the very least, an intermediate must have surfaces that are competent to interact with other parts of the protein. Hence, oligomerization of well-folded core fragments is consistent with their being models of folding intermediates.

The overall organization of the dimer will be of further interest since it may reveal features of domain swapping (Bennett et al., 1995). Three-dimensional domain swapping provides a mechanism for monomers to dimerize: dimers are formed by exchanging equivalent regions or domains between monomers. Aberrant domain swapping could lead to protein precipitates and even abnormal aggregates such as amyloid plaques (Schlunegger et al., 1997). Further studies of eABCD may help us understand why some partly folded proteins aggregate while others avoid this fate.

Conclusions

In summary, these studies show that the most stable region of RNase H, which is also the first region to fold, encodes enough local information for autonomous folding. Moreover, the structure of this fragment appears to be similar to the equivalent region in the parent protein. If these results prove general, such folded sub-

domains may provide ideal scaffolds for use in designing novel proteins.

Materials and methods

Expression and purification of eABCD

eABCD consists of an initial methionine followed by residues R41–Q113 of RNase H. The fragment was chosen to include the most stable secondary structural elements of RNase H (helices A, D, and B, and strand 4). Residues in helix C and the C-D loop were included to improve solubility.

A plasmid encoding eABCD, pDR101, was created by polymerase chain reaction amplification of the region corresponding to residues R41–Q113 of RNase H from pSM101 (Dabora & Marqusee, 1994). Unlabeled eABCD expression and purification were performed as previously described (Goedken et al., 1997). ^{15}N and $^{15}\text{N}/^{13}\text{C}$ labeled eABCD were expressed in *E. coli* grown in M9 media as described for RNase H (Chamberlain et al., 1996). The mass of eABCD was verified by electrospray mass spectrometry. Concentrations of eABCD solutions were determined by absorbance at 280 nm using an extinction coefficient of $24,000 \text{ cm}^{-1} \text{ M}^{-1}$ (Edelhoch, 1967).

CD and fluorescence

All CD measurements were performed on an Aviv 62DS spectrometer in a 1 cm pathlength cuvette. The spectra were collected in 2 mM sodium phosphate, pH 5.4 at 25 °C, 4.4 μM eABCD. Data points were averaged for 2 s at 0.4 nm intervals from 280 to 193 nm. Thermal denaturation of 4.4 μM eABCD was observed in 50 mM sodium acetate pH 5.5 at 222 nm from 2 to 85 °C with 2 °C increments, 2.5 min equilibration time, and 45 s averaging time at each temperature. Chemical denaturation was monitored in 5 mM sodium acetate, 50 mM NaCl, pH 5.5, 25 °C, 5.3 μM eABCD. Individual solutions were made from an 8.11 M urea stock solution measured by refractive index, equilibrated for >1 h. The CD signal at 222 nm was averaged for 60 s. Fluorescence emission was measured for 0.5 s with excitation and emission wavelengths of 295 and 322 nm, respectively, using an ISS K2 digital lifetime fluorimeter in steady-state mode with 8 nm bandwidths. The model used to determine the free energy of unfolding and fraction folded eABCD assumes a two-state transition of folded dimer to unfolded monomers (Bowie & Sauer, 1989) and a linear dependence of the free energy on denaturant concentration (Santoro & Bolen, 1988). Curve fitting was done using a nonlinear least-squares fitting program, Sigmaplot (Jandel Scientific, Austin, Texas). Urea-induced denaturation of the 1.2 μM eABCD (same buffer as the previous urea denaturation) solution was carried out with an automated titrator connected to the AVIV 62 DS, with 5 min mixing and 60 s averaging of the CD signal at 222 nm.

Ultracentrifugation

The oligomerization of eABCD in 0.0–1.0 M urea was characterized by sedimentation equilibrium in a Beckman Optima XL-A ultracentrifuge. Measurements were performed in 0.0–1.0 M urea at 25 °C, 30,000 and 40,000 RPM, with 3.5, 7.0, and 14 μM eABCD in 5 mM sodium acetate, 50 mM NaCl, pH 5.5. The data

were analyzed using the manufacturer supplied software, a partial specific volume of 0.74 g mL^{-1} and solvent densities of 1.000, 1.004, 1.008, 1.011, and 1.016 g mL^{-1} for urea concentrations of 0.00, 0.25, 0.50, 0.75, and 1.00 M, respectively. Dissociation constants at each urea concentration were determined by fitting all the data at a given urea concentration to a single K_d , assuming a monomer–dimer equilibrium and the monomer molecular weight of $8,603 \text{ g mol}^{-1}$, calculated from the amino acid sequence. Fraction monomer was calculated using the relationship $F_m = (-K_d + (K_d^2 + 8PK_d)^{1/2})/(4P)$, where P is the subunit concentration, K_d the dissociation constant, and F_m the fraction of monomeric subunits.

NMR assignments

Data collection for assignments was done on 1–1.7 mM uniformly $^{13}\text{C}/^{15}\text{N}$ labeled eABCD in 0.01% sodium azide, 90% H_2O , 10% D_2O at 302 K on a 600 MHz Bruker DMX spectrometer. The sample was adjusted to pH 5.5 with sodium hydroxide. Main-chain amide nitrogen, proton, C_α , and C_β resonances were assigned using three-dimensional CBCA(CO)NNH (Grzesiek & Bax, 1992a) and CBCANH (Grzesiek & Bax, 1992b) and $^1\text{H}/^{15}\text{N}$ HSQC (Bax et al., 1990) spectra. Resonances for three amino acids, E48–M50, could not be assigned unambiguously.

All spectra were acquired with 8,090.615 Hz in the directly detected ^1H dimension, 800 Hz in the ^{15}N dimension, and 10 kHz in ^{13}C . For the HSQC, acquisition times were 63.3 ms (^1H) and 80 ms (^{15}N). For the CBCA(CO)NNH, acquisition times were 63.3 ms (^1H), 12.5 ms (^{15}N), and 5.9 ms (^{13}C) and for the CBCANH, 63.3, 25, and 4.9 ms, respectively. Data were processed within the program AZARA (W. Boucher, unpubl. results) using maximum entropy to extend the indirect dimensions. Assignments were made in ANSIG (Kraulis, 1989).

Hydrogen exchange measurements

Hydrogen exchange measurements were initiated by dissolving 9.2 mg ^{15}N -labeled eABCD in 550 mL D_2O and adjusting the pH to 5.5 with NaOD. HSQC spectra were collected from 0.8 to 17.6 h and processed with AZARA. Both dimensions were multiplied by a shifted sine function and zero filled prior to Fourier transformation. Peak heights were determined with a version of Priism (Chen et al., 1996) modified by Dr. David A. Agard for NMR data analysis. The observed rate of hydrogen exchange k_{obs} was determined for each proton by fitting the peak height as a function of time to an exponential decay using KaleidaGraph (Abelbeck Software, Reading, Pennsylvania). Protection factors $P = k_{\text{rc}}/k_{\text{obs}}$ were calculated using estimates of the random coil rates k_{rc} from model dipeptides (Bai et al., 1993).

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