Pressure versus temperature unfolding of ribonuclease A: An FTIR spectroscopic characterization of 10 variants at the carboxy-terminal site

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Abstract

FTIR spectroscopy was used to characterize and compare the temperature- and pressure-induced unfolding of ribonuclease A and a set of its variants engineered in a hydrophobic region of the C-terminal part of the molecule postulated as a CFIS. The results show for all the ribonucleases investigated, a cooperative, two-state, reversible unfolding transition using both pressure and temperature. The relative stabilities, among the different sites and different variants at the same site, monitored either through the changes in the position of the maximum of the amide I' band and the tyrosine band, or the maximum of the band assigned to the β -sheet structure, corroborate the results of a previous study using fourth-derivative UV absorbance spectroscopy. In addition, variants at position 108 are the most critical for ribonuclease structure and stability. The V108G variant seems to present a greater conformational flexibility than the other variants. The pressure- and temperature-denaturated states of all the ribonucleases characterized retained some secondary structure. However, their spectral maxima were centered at different wavenumbers, which suggests that pressure- and temperature-denaturated states do not have the same structural characteristics. Nevertheless, there was close correlation between the pressure and temperature midpoint transition values for the whole series of protein variants, which indicated a common tendency of stability toward pressure and heat.

Keywords: Ribonuclease A; protein engineering; protein folding; pressure versus heat-induced unfolding; Fourier-transform infrared spectroscopy

In the early days of protein folding, some remarkable features were discovered observing RNase A, EC 3.1.27.5. Its spontaneous refolding prompted Anfinsen (1973) to conclude that the information necessary for folding was coded

into the primary sequence, and so the field of protein folding exploded into existence. Subsequently, during the elucidation of the principles of the protein folding process, RNase A has been extensively studied structurally and functionally (Cuchillo et al. 1997; Raines 1998). Twenty-eight years later, it still serves as a paradigm of protein folding (Cuchillo et al. 1997; Neira and Rico 1997). In practically all of these studies, the unfolded state of protein was generated by high or low temperature, by pH changes, or by the use of chemical denaturants. In the case of wild-type RNase A, high-pressure unfolding has attracted widespread attention, emerging as an increasingly valuable tool for protein structure characterization (Brandts et al. 1970; Hawley 1971; Takeda et al. 1995; Tamura and Gekko 1995; Zhang et al. 1995).

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Abbreviations: CD, circular dichroism; CFIS, chain folding initiation site; C-terminal, carboxy-terminal; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared; HPLC, high-performance liquid chromatography; MES, 4-morpholine-ethanesulphonic acid; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; RNase A, bovine pancreatic ribonuclease A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UV, ultraviolet.

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Early on, Némethy and Scheraga (1979) proposed several hydrophobic amino acid regions within RNase A as likely CFISs. Residues 106–118 (of 124) are highly conserved among the RNase superfamily (Beintema et al. 1997). This hydrophobic region of the protein, which constitutes a beta-hairpin in the native state (Wlodawer et al. 1988), was predicted to be one of the most probable sites for formation of the initial structure (Matheson and Scheraga 1978). For RNase A, refolding quench flow experiments, combined with two-dimensional (2D) NMR spectroscopy, enabled identification of protons, which are protected early during the refolding reaction (Udgaonkar and Baldwin 1995). The protons of the above-mentioned CFIS are included among these. Recently a hydrogen exchange 2D NMR study of RNase A has identified the slow-exchanging protons and those that exchange by a global unfolding mechanism (Neira et al. 1999). The results of this study, compared with those of Udgaonkar and Baldwin, led the authors to suggest the presence of a putative chain-folding initiation site comprising the four-stranded region, Lys62-Ala64, Cys72-Ser75, Ile106-Cys110, and Val116-His119, together with the face of helix III containing Val54 and Val57.

Using site-directed mutagenesis to engineer RNase A, a set of mainly conservative hydrophobic variants of its 106–118 region was produced. The stability of each variant was compared by pressure- and temperature-induced unfolding and monitored by fourth-derivative UV absorbance spectroscopy (Torrent et al. 1999). The results show the contribution of I106, I107, V108, A109, V116, and V118 hydrophobic residues to overall protein stability, the 108 position being the most important site for stability and the site most destabilized by amino acid replacement. In addition, a comparison of the effect of pressure and temperature on each variant under identical experimental conditions is reported.

Recently, using staphylococcal nuclease as a model protein, pressure effects on the denaturation of variant forms have also been reported by Royer and others (Royer et al. 1993; Vidugiris et al. 1996; Frye et al. 1996; Frye and Royer 1998), showing equally interesting results for correlation studies with temperature denaturation.

Various studies have suggested that pressure and thermally denatured RNase A have compact dimensions and a nonrandom structure, but it still remains uncertain whether there are conformational differences between the two denatured states (Labhardt 1982; Privalov et al. 1989; Sosnick and Trewhella 1992; Seshadri et al. 1994; Tamura and Gekko 1995; Neira et al. 1999; Panick and Winter 2000). In the present study, we combined protein engineering methods and FTIR spectroscopy to characterize further the differences between the pressure- and temperature-unfolded states of RNase A. To this end, the unfolding process of these RNase A variants, I106A/V/L, I107A, V108G/A, V116G, V118G/A, and Y115W, induced by both denaturing agents, was compared with the process presented by

wild-type protein. In addition, the impact of amino acid substitutions on the native state of RNase A was also determined. Most of the constructed variants concern ribonucleases bearing nondisruptive deletions, with the sole exceptions of I106L and Y115W amino acid replacements.

It is generally accepted that FTIR spectroscopy is one of the most powerful techniques for determining conformational changes in proteins under equilibrium conditions (Jackson and Mantsch 1995). It has already been used to determine pressure- and temperature-induced changes in the secondary structure of wild-type RNase A (Takeda et al. 1995; Panick and Winter 2000). In the present study, peptide backbone, with its unique signature for β -sheets and side-chain infrared “marker” bands (Fabian et al. 1993, 1994), were used as conformation-sensitive monitors for following the induced reversible changes in the secondary structure of RNase A wild type and variants. Additionally, marginal changes in protein conformation that may arise from the amino acid substitutions in the hydrophobic 106–118 region were examined. Furthermore, bandfitting of the Fourier-deconvoluted amide I' spectrum (Smeller et al. 1995a) allowed us to follow the variation of the secondary structure elements for the unfolding process of the proteins under study.

The present results show that for RNase A, the two-state assumption is valid for the analysis of either the temperature or the pressure-induced unfolding. They also provide a structural basis for our previous findings concerning the contribution to the stability of RNase A of the hydrophobic interactions in its 106–118 region (Torrent et al. 1999). Further, they provide new insights into the differences between pressure- and temperature-induced changes, and lay the basis for kinetic investigations to probe the role of the region in protein folding.

Results

The amide I' band under native conditions

Under native conditions, the amide I' region of RNase A wild type and its variants shows a broad and asymmetric outline in the infrared (IR) spectrum. Spectral features are emphasized under Fourier self-deconvolution (Fig. 1A,B). Deconvolution of the original spectrum of the amide I' band showed, within the precision of the method, the presence of component bands at different wavenumbers. The band positions for RNase A wild type after partial hydrogen-deuterium (H-D) exchange are given in Table 1, along with secondary structure assignments based on the work of Byler and Susi (1986). A minor band at 1610 cm^{-1} is attributable to the amino acid side-chain vibration (Matsuura et al. 1986). It is worth noting that β -sheets and turns were each assigned to multiple bands. The broad band at about 1651 cm^{-1} could not be resolved, leaving superimposed the com-

Fig. 1. (A) Original and (B) Fourier-deconvoluted and fitted IR spectra in the amide I' region (solid line) with individual Gaussian components (broken lines) of RNase A wild type; (C) normalised Fourier-deconvoluted IR spectra of RNase A wild type (solid line) and V108G variant (broken line). Solution conditions: RNase A and V108G at 75 mg/mL in sodium acetate buffer, 50 mM, pH 5.0, P = 0.1 MPa, T = 12°C.

ponent bands of α -helices and unordered structures, because of the small wavenumber difference between the amide I' C = O stretch of these two structural components (Dong et al. 1990). Bandfitting of the deconvoluted spectra (Fig. 1B) allowed a semiquantitative estimate of the fractional composition of the secondary structure elements in the native

Table 1. Band positions and secondary structure assignments for the amide I' band of RNase A at 12°C and 0.1 MPa after partial hydrogen-deuterium exchange

Position (cm ⁻¹)	Assignment	Band area (%)
1610	Side chains	1
1624	β -sheet	5
1637	β -sheet	45
1651	α -helix + unordered	22
1664	turns	20
1678	β -sheet	5
1686	turns	2

state of wild-type RNase A and variants. The resulting fractional areas (listed in Table 1) of the bands assigned to different types of secondary structure were assumed to represent percentages of these structures in the protein. Percentages of secondary structure content of wild-type protein, from both natural and recombinant sources, are 55% β -structure, 22% turns and bends, and 22% α -helix and unordered. This agrees with the generally accepted secondary structure derived from X-ray crystallographic studies (Wlodawer et al. 1988) and 2D NMR spectroscopic studies (Rico et al. 1989), and supports the assignments made.

Qualitative analysis of the deconvoluted spectra suggests that wild-type protein and its variants show structural similarity in the native state, although small effects are observed on amino acid replacement at V108 (Fig. 1C) and V118 sites. Accordingly, the results from curve fitting show, in general, no significant differences in the secondary structure content, under native conditions, of wild-type and variant proteins. Only the small effects of replacements at positions 108 and 118 prove large enough for some secondary structural changes to be discerned with FTIR. The impact on the polypeptide backbone conformation increases in the order, V118G < V108A < V108G. Therefore, we focused on the spectral changes observed in the characterization of the V108G variant, although similar variations apply to the other two variants. As shown in Figure 1C, in the V108G variant there is a small shift of the amide I' band toward a lower wavenumber than that of the wild-type protein. This is especially remarkable for the component band assigned to turn structures, which is shifted from 1686 cm⁻¹ to 1684 cm⁻¹, suggesting a more complete initial hydrogen-deuterium exchange. In particular, the broader amide I' component band at 1637 cm⁻¹ shown for the V108G variant may indicate that fine differences distinguish the hydrogen-bonding pattern of the β -sheet structures from that of the wild-type protein. Moreover, the increases in intensity at 1651 and 1684 cm⁻¹ bands, assigned to α -helix + unordered and turns, respectively, point to a higher percentage of irregular and turn structures in this variant.

We noticed differences, although these were not systematically studied, in the rate and extent of H-D exchange between the variants, which suggested differences in the dynamics of the structures.

Pressure and temperature dependence of the amide I' FTIR spectrum

Pressure- and temperature-induced changes in the deconvoluted amide I' region of the IR spectrum were followed to obtain information about protein structure changes. Typical alterations in the amide I' region of the V118G variant on increases in pressure or temperature are shown in Figure 2, panels A and B, respectively. For all the proteins analyzed, cooperative spectral changes are observed when pressure or

Fig. 2. (A) Fourier-deconvoluted IR spectra of V118G variant as a function of pressure between room pressure and 1200 MPa. Solution conditions: V118G at 75 mg/mL in MES buffer, 50 mM, pH 5.0, $T = 20^{\circ}\text{C}$. (B) Deconvoluted infrared spectra of V118G as a function of temperature between 12°C and 85°C . Solution conditions: V118G at 75 mg/mL in sodium acetate buffer, 50 mM, pH 5.0, $P = 0.1\text{ MPa}$. Note that the Y axis (not shown) corresponds to absorbance.

temperature is increased, although gradual absorbance changes occurred before the unfolding transition. At low pressures or temperatures, a general shift of the amide I' band around 1637 cm^{-1} toward a lower wavenumber is observed. As no changes in intensity of this main compositional band are observed under these conditions, these alterations may be assigned to enhanced H-D exchange in the interior of the protein. In contrast, the V108G variant showed only a very small shift, suggesting a more complete initial H-D exchange. As pressure or temperature is increased for all the variants and wild-type protein, a cooperative decrease in band intensities is observed, indicating the disappearance of native structures. There is also an increase in band intensity around 1645 cm^{-1} , indicating an increase in the amount of unordered structure. At high pressure or temperature, it seems that the unordered structure dominates in the final stage of protein unfolding, although the proteins nevertheless retain some secondary structure. This is consistent with previous results on the wild-type protein monitored using CD (Labhardt 1982; Yang et al.

1986; Seshadri et al. 1994), dynamic light scattering (Nicoli and Benedek 1976), small-angle X-ray scattering (Sosnick and Trewhella 1992), FTIR (Anderle and Mendelsohn 1987; Sosnick and Trewhella 1992; Panick and Winter 2000), compressibility (Tamura and Gekko 1995), and NMR H-D exchange data (Talluri and Scheraga 1990; Neira et al. 1999). Interestingly, comparison between IR spectra of the pressure-denatured proteins and of the corresponding thermally denatured proteins (Fig. 3A,B) shows similar amide I' band profiles, but with the spectral maxima centered at different wavenumbers of the amide I' vibrational mode.

Effect of amino acid substitutions on the stability of RNase A determined by monitoring spectral changes

Stability information on pressure- and temperature-induced unfolding of RNase A wild type and variants was obtained by following the shift of the maximum of the amide I' band, which reflects variations in composition of various secondary structural elements of the protein. Protein structure changes were also followed by observing the tyrosine side-chain band at 1515 cm^{-1} . This band indicates changes in the environment of these side-chain groups, which may result from changes in tertiary and secondary structure.

According to the changes observed in the amide I' band, secondary structure elements are disrupted cooperatively, and there is one main transition, preceded and followed by

Fig. 3. Normalised Fourier deconvoluted IR spectra of the pressure (1200 MPa, 20°C) (A) and temperature (0.1 MPa, 85°C) (B). Denatured RNase A wild type (solid line) and V108G variant (broken line).

(near linear) curves, when the pressure or temperature is increased. This is also observed in the small shifts that take place in the Tyr side bands (Fig. 4).

With increasing pressure along the pretransition region, the wavenumber maximum of the amide I' band shifts to lower wavenumbers, resulting in a negative slope and indicating a strengthening of the hydrogen bonds. This effect is known as the elastic shift (Frauenfelder et al. 1990). A further increase in pressure, just below the main cooperative conformational changes, results in a sudden wavenumber decrease, which is assumed to be because of a stimulation of the H-D exchange as a result of enhanced solvent accessibility, as mentioned above. This enhanced H-D exchange is also observed in the unfolding curves obtained on temperature denaturation. Following this effect, a pressure and temperature increase results in a wavenumber maximum increase that reflects the unfolding of the protein. These induced changes, in the wild type and in all variants, show fairly good reversibility as measured through the recovery of the wavenumber maximum of the amide I' band and the Tyr band on releasing pressure or decreasing temperature. The incomplete recovery of the wavenumber maximum value of the amide I' band probably arises from the amide protons, which were incompletely H-D exchanged at native conditions. The transition in the Tyr bands around 1515 cm^{-1} coincides with the above-mentioned unfolding transition and not with the changes corresponding to H-D exchange in the pretransition region.

All unfolding transition curves were fitted to a two-state thermodynamic model combined with sloping functions for the native and denatured states as described previously (Pace 1990). The results show that all equilibrium-unfolding profiles are shifted to lower pressures or temperatures than in the wild-type protein. The amino acid substitutions decreased the stability of the protein by different amounts. The midpoint transition values, $P_{1/2}$ and $T_{1/2}$, obtained from the induced changes in the amide I' region on increasing pressure and temperature, are listed in Table 2. They give a consistent idea of the relative stabilities among the different sites and among different variants at the same site. The figures range from 151 MPa for V108G to 743 MPa for wild-type RNase A, and from 37°C for V108G to 62°C for wild-type protein, respectively.

These midpoint values, corresponding to the secondary structure decrease for a given protein, are very consistent with values found when the changes of the conformation of the protein on unfolding were followed by observation of the tyrosine band at 1515 cm^{-1} (summarized in Table 2). They correlate well with those recently found under comparable experimental conditions (at 1 mg/mL) using fourth-derivative UV absorbance spectroscopy (Torrent et al. 1999). Both Tyr band and UV spectroscopy were used as local monitors that detect changes in the tertiary structure along the denaturation process. These data indicate a simul-

Fig. 4. Pressure- and temperature-unfolding curves for the V118G variant. The wavenumber maxima of the amide I' and Tyr band were plotted upon increasing (filled circles) and decreasing (open circles) pressure (A) and temperature (B). The solid line represents the non-linear least-square fit of the data based on a two-state model.

Table 2. Midpoint transition pressures and temperatures of wild-type RNase A and its variant forms

Protein	P _{1/2} (MPa)		T _{1/2} (°C)	
	Amide I'	Tyr	Amide I'	Tyr
Natural wild type	743.3	779.9	61.5	63.6
Recombinant wild type	743.2	758.1	59.4	62.8
Y115W	694.0	716.0	57.5	59.8
I106A	337.5	377.5	46.6	47.9
I106L	525.6	575.8	53.6	55.7
I106V	612.0	603.5	56.2	59.0
I107A	505.7	548.9	53.7	52.9
V108A	274.6	298.1	46.0	49.0
V108G	150.7	183.2	37.3	37.5
V116G	569.1	566.8	53.9	56.4
V118A	652.0	649.0	55.7	57.8
V118G	533.0	534.1	47.6	51.7

taneous breakdown of secondary structure elements and pronounced changes in the environment of Tyr side-chain groups, suggesting a highly cooperative unfolding process. However, as is to be expected, the $T_{1/2}$ values obtained in D₂O buffer were higher than those derived from the UV data in H₂O buffer. It is well known that proteins in D₂O solutions show higher $T_{1/2}$ values than those found in H₂O solutions (Talluri and Sheraga 1990; Yamamoto and Tsumi 1991; Fabian et al. 1994). The data given in Table 2, consistent with our previous results, also suggest that among the variants at each site, stability decreases in the order Ile>Val>Leu>Ala>Gly, and that two particular residues, I106 and V108, are the ones most destabilized by amino acid replacement.

Amino acid replacements that produce the greatest protein destabilization (i.e., V108G) seem to affect the residual secondary structure of pressure and temperature unfolded states in a different way.

The spectra of the pressure and temperature denaturated states of RNase A wild type and V108G variant are compared in Figure 3, panels A and B, respectively. From the temperature-denaturated spectral outlines, the amino acid change seems to lead to a more Gaussian amide I' band and to the disappearance of the shoulder at around 1670 cm⁻¹. In contrast, the pressure-denaturated spectral outlines for the two proteins are practically identical.

To determine the trends in the conformational changes of the protein backbone for RNase A wild type and its variants in terms of variation of secondary structure elements on increasing pressure and temperature, the deconvoluted spectral progressions with both agents were curve fitted (Smeller et al. 1995a). FTIR spectroscopy is particularly sensitive to changes in β -strands of proteins (Fabian et al. 1994). As listed in Table 1, three component bands (at 1624, 1637, and 1678 cm⁻¹) account for this secondary structural element. Among them, the intensity of the 1637 cm⁻¹ band is the

strongest. In fact, about half of the whole fitted area of the deconvoluted spectrum of native RNase A belongs to this β -sheet band. Because the amino acid substitutions engineered in this work were performed in a region of β -sheet structure of RNase A, most significant changes in the recorded IR spectra were expected to arise from this secondary structural element. Therefore, we focused on the percentage variation of the main β -sheet component of the amide I' band at 1637 cm⁻¹ and the broad band at 1651 cm⁻¹ that indicates the appearance of unordered structures. Both bands show abrupt and simultaneous changes of intensity on increases in pressure and temperature. On analysis of the whole set of variants, the stability results obtained basically confirm those obtained from the shape of the amide I' band.

Discussion

In our previous paper (Torrent et al. 1999), the stabilities of wild-type RNase A and a set of predominantly conservative hydrophobic variants of the protein were compared by pressure- and temperature-induced equilibrium unfolding monitored by fourth-derivative UV absorbance spectroscopy. In the current study, we examined the structural response of RNase A wild type and several of the above-mentioned variant forms to pressure and thermal perturbation, under equilibrium conditions, using FTIR spectroscopy.

Native and denatured states

Judging from the amide I' band contour at 12°C and 0.1 MPa, all the variants have a similar folded conformation to the wild-type protein, with the exception of variants V108G, V108A, and V118G, in which subtle conformational differences seem to be present. This observation is consistent with data from CD measurements, although significant structural differences were only found in the V108G variant (Torrent et al. 1999).

The pressure- and temperature-denatured states of all the ribonucleases characterized in this study show some secondary structure. However, it is noteworthy that their spectral maxima are centered at different wavenumbers of the amide I' vibrational mode. Whereas the spectrum at 1200 MPa is centered at 1640 cm⁻¹, the spectrum at 85°C is centered at 1650 cm⁻¹ (Fig. 3). A previous study involving wild-type RNase A offers some insight into the significance of this spectral shift (Takeda et al. 1995). The authors suggest a difference in hydration of the backbone peptide groups between the pressure- and temperature-unfolded states. Apparently, pressure or temperature leads to different unfolded states of the protein. The higher intensity at 1630 cm⁻¹ in the pressure-unfolded state suggests a higher β -sheet content. The higher intensity at 1670 cm⁻¹ in the temperature-unfolded state suggests a higher content of

turns. This seems to corroborate numerous studies in which the pressure-induced protein unfolding appears to lead to a molten globule state (Zhang et al. 1995; Yamaguchi et al. 1995) in which native-like secondary structure is retained but solvent is admitted to the core regions of the protein. In contrast, the thermally unfolded state appears to be more extensively unfolded (Zhang et al. 1995).

Stability

The pressure and temperature stabilities of the variants are indicated by comparison of the $P_{1/2}$ and $T_{1/2}$ values (Table 2). Unfolding midpoint values obtained from monitoring unfolding transitions using both peptide backbone and Tyr side-chain infrared "marker" bands (Arrondo et al. 1988; Fabian et al. 1993, 1994) are consistent with each other and are also consistent with the hypothesis that the pressure- and temperature-induced unfolding of RNase A, under equilibrium conditions, is an overall two-state and highly cooperative process, involving all structural components between folded and unfolded species. This is in line with data obtained by fourth-derivative UV absorption spectroscopy (Torrent et al. 1999) and by DSC analysis (Coll et al. 1999). The Tyr band shift observed in the IR and the UV absorbance change seems to reflect the same molecular events during protein unfolding. Monitoring of RNase A wild-type unfolding gave similar results (Reinstädler et al. 1996). The fact that the spectral changes occur at about the same pressure and temperature using low and high protein concentration regime techniques, UV and FTIR respectively, is a strong indication that the observed unfolding events are intramolecular.

It is notable that all variants have lower transition midpoint values than wild-type protein. Of particular interest is the dramatic pressure and temperature destabilization of the V108G variant. Its pressure- and temperature-unfolding midpoint values decrease by 592 MPa and 25°C, respectively, compared to wild-type protein. These results suggest that V108 is an important residue for the structural integrity of the RNase A fold. The exchange of the amide and side-chain hydrogens with solvent deuterons observed for native V108G, possibly higher than for wild-type protein, could be associated with greater conformational flexibility and a more open or dynamic structure. Both observations (lower pressure and temperature stability and an increased protein flexibility) suggest that this amino acid substitution could potentially leave a cavity in the variant protein, which several investigators (Buckle et al. 1996; Fusi et al. 1997), using similar point amino acid substitutions in other proteins, have addressed.

Recently, we followed the thermal denaturation of wild-type RNase A and its variants by DSC (Coll et al. 1999) and found a ratio between calorimetric and van't Hoff enthalpies (R value) near 1 for wild-type RNase A and most of the

variants. The sole exception was the variant at position 108, which showed R values lower than unity. This has been interpreted as a proof of existence of self-association between molecules during denaturation, specifically in the case of V108G RNase A variant. This effect is reversible on cooling. Surprisingly, when the same variants at position 108 were analyzed, both by pressure- and temperature-induced unfolding, no aggregation process was found. Although this may be because of the different techniques used to monitor the unfolding process, it stresses the particular dynamic nature attributed to the V108G RNase A variant.

In our previous study (Torrent et al. 1999), we showed that part of the observed destabilization of the different variants is accounted for largely by the change in the nature of the side chain (i.e., the change in core residue volume and hydrophobicity). The remaining effect on stability was found to result from the environment surrounding the different residues, primarily the role of van der Waals interactions, which are generally accepted to have a strong dependence on the packing density and the solvent exposure in the native structure. These results are consistent with the greater effects found at the Val108 and Ile106 sites, which are the most destabilized by amino acid substitution and thus the two most important residues for stability. Their importance is emphasized by their high conservation in the RNase sequences (Beintema et al. 1997).

As determined by 2D NMR spectroscopic studies of native wild-type RNase A, the backbone amide protons buried or involved in hydrogen-bonded structures, such as β -sheets and α -helices, are protected strongly against exchanges with solvent deuterons (Zhang et al. 1995; Talluri and Scheraga 1990; Robertson and Baldwin 1991; Nash et al. 1996; Neira et al. 1999). Exchange-induced band shift has been shown at pressure or temperature below the cooperative unfolding of secondary structure for almost all variants. The major amide I' band at 1637 cm^{-1} shifts toward lower wavenumbers when a pressure or temperature increase leads to a complete H-D exchange. This band is skewed pronouncedly in all variants, except for V108G, in which a marked difference in the rate of exchange seems to be present, probably because of slightly higher flexibility. Backbone amide protons associated with turns and random coil structures appear to exchange more under conditions that favor the native state, as small shifts of the corresponding amide I' component bands are observed. The results are consistent with recent H-D exchange experiments in RNase A, which have posited that some amide protons located within a putative folding initiation site, primarily comprising a four-stranded β -sheet region, may account for the slow exchange in the unfolded protein observed (Neira et al. 1999). Similarly, Talluri and Scheraga (1990) reported that temperature-denatured RNase A has a residual structure in the vicinity of the C-terminal CFIS, which indicates the presence of short- and long-range interactions. Thus, because it appears that the pressure and

thermally denatured states of RNase A have substantial secondary structure, probably primarily in the CFIS region, it is reasonable to assume that amino acid substitution in this region will lead to a further loss of the remaining structure, as mentioned above. The results fit well the hypothesis that the persisting protons participate constitutively in protein structure stabilization (Backmann et al. 1996), and also fit the conclusion that the hydrophobic residues of the postulated CFIS appear to promote secondary structural contacts, which are important during folding of the β -sheet region. It seems likely that this region consolidates around the weak secondary structure of the β -hairpin under study. The importance of these hydrophobic residues to the stability and structure of the whole protein reinforces the idea that the β -hairpin in RNase A is an important structural element for attaining a native-like fold.

Comparison of pressure and temperature unfolding

Measurements of effects of amino acid substitutions on RNase A stability have been studied to date by thermal and chemically induced unfolding. We compare here how the changes in amino acid hydrophobicity and volume have affected the pressure- and temperature-induced denaturation of the different RNase A variants. It is well known that pressure affects the volume of the system, leads to changes in protein and solvent organization, and drives protein equilibrium toward the lower volume unfolded state. However, temperature increase affects both thermal energy and the volume of protein and solvent. The results reveal a surprising similarity between both processes of denaturation (Fig. 2).

In line with our previous equilibrium studies, the relative stability of the various RNase A variants is virtually the same for pressure- and heat-induced unfolding. That is, a plot of $P_{1/2}$ against $T_{1/2}$ can be fitted by a straight line (Fig. 5). The linear correlation between $T_{1/2}$ and $P_{1/2}$ follows from the assumption that the unfolding is a two-state process. In this case the change in enthalpy is a linear function of $T_{1/2}$ (Elwell and Schellman 1977). For ribonuclease this relationship holds for a large number of experimental approaches (Pace et al. 1999):

$$\Delta C_p = d(\Delta H)/d(T_{1/2})$$

A similar relationship should hold for the change in volume and $P_{1/2}$, although this relationship is not extensively documented in the literature:

$$\Delta \beta = d(\Delta V)/d(P_{1/2})$$

Here, β stands for the compression. From these relationships it can be seen that the slope of the line $T_{1/2}/P_{1/2}$ is given by:

Fig. 5. Correlation between the pressure and temperature midpoint transition values of each ribonuclease characterized. The solid line shows the best fit to a linear equation ($r = 0.954$).

$$T_{1/2}/P_{1/2} = (\Delta H \Delta \beta)/(\Delta V \Delta C_p)$$

The strong correlation between $T_{1/2}/P_{1/2}$ indicates that the two-state assumption for temperature unfolding also applies to pressure-induced unfolding.

At the molecular level the correlation between $P_{1/2}$ and $T_{1/2}$ found in the pressure and temperature experiments may be explained by the particular parameter we are varying, that is, the hydrophobicity of the CFIS. In other words, this suggests that pressure and heat have comparable effects on the hydration of hydrophobic residues. In addition, the multiplicity of weak noncovalent interactions often leads to nonequivocal conclusions as to the predominant role of one type of interaction (Cooper 1999).

A comparative analysis of the amide I' region of the infrared spectrum of pressure- and temperature-denatured RNase A wild type and variants indicates that they are very similar. Indeed, whereas heat denaturation leads in most proteins to the formation of irreversible intermolecular β -sheet structure and molecular aggregation, our data clearly indicate the absence of aggregation in all the thermally denatured RNase A variants. This is shown by the absence of an increase in intensity of the amide I' band around $1615\text{--}1620\text{ cm}^{-1}$. It has been reported previously that RNase A does not aggregate on unfolding at high temperature (Clark et al. 1981). Consequently, both pressure and thermally denatured RNase A lead to a reversible protein state that contains a predominance of unordered structure and some residual structure.

Materials and methods

Purification of wild-type ribonuclease A

RNase A (type I-A) purchased from Sigma Chemical was further purified by cation exchange chromatography, as described by Alonso and coworkers (1986).

Mutagenesis, protein expression, and purification

The gene that encodes RNase A (DelCardayré et al. 1995) was used as a template for mutagenesis by PCR following a method described elsewhere (Juncosa-Ginestà et al. 1994). The mutated and wild-type RNase A genes were transferred into an expression vector, expressed, and purified as described previously (Torrent et al. 1999). Protein purity and homogeneity were confirmed by SDS-PAGE and by cation exchange as well as by reversed-phase HPLC, using a Mono S HR 5/5 and a 214TP Vydac C4 column, respectively.

Preparation of samples and FTIR studies

Lyophilised pure proteins were dissolved to a concentration of 75 mg/mL in 50 mM MES buffer at pD 5.0 for pressure experiments, and in 50 mM sodium acetate buffer pD 5.0 for temperature experiments. pD was measured by adding 0.4 units to the pH value (Glasoe and Long 1960). These buffers were selected for their relatively small pressure and thermal pD dependencies, respectively (Kitamura and Itoh 1987). Samples were dissolved and stored overnight at room temperature to ensure that the H-D exchange reached a static regime. IR spectra were recorded as a function of temperature at atmospheric pressure, or as a function of pressure at 20°C. Spectra were recorded using a Bruker IFS66 FT-IR spectrometer (Karlsruhe) equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector. Infrared light was focused on the sample by a NaCl lens. The sample compartment was purged continuously with dry air. 250 interferograms were coadded at a resolution of 2 cm⁻¹. For pressure measurements, the protein solution was mounted in a stainless steel gasket of a diamond anvil cell obtained from Diacell Products. The initial gasket thickness was 0.05 mm and the hole diameter 0.65 mm. Pressure was measured with BaSO₄ by following the shift of the 983 cm⁻¹ band (Wong and Moffat 1989). Pressure was increased at a rate of about 100 MPa per hour. For the temperature experiments, protein solutions were placed between a pair of CaF₂ windows separated by a gasket of 0.03 mm. The cell was controlled by a Graseby/Specac low-voltage heating system. Temperature was increased at a rate of 0.2°C/min. Secondary structure prediction was based on a combination of Fourier self-deconvolution with band fitting (Byler and Susi 1986; Harris and Chapman 1995). Fourier self-deconvolution, a mathematical technique of bandnarrowing, was performed with the Bruker software. A half bandwidth of 21 cm⁻¹ and an enhancement factor (K) of 1.7 were used (Smeller et al. 1995b).

To compare qualitatively the spectra of different variants, we normalized each spectra by use of the corresponding minimum and resulting maximum absorbance. First, each native or denatured spectrum was subtracted from its minimum absorbance. Second, each resulting spectrum was then divided by its maximum absorbance, yielding a normalized spectrum from 0 to 1.

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References

- Alonso, J., Nogués, M.V., and Cuchillo, C.M. 1986. Modification of bovine pancreatic ribonuclease A with 6-chloropurine riboside. *Arch. Biochem. Biophys.* **246**: 681–689.
- Anderle, G. and Mendelsohn, R. 1987. Thermal denaturation of globular proteins. Fourier transform-infrared studies of the amide III spectral region. *Biophys. J.* **52**: 69–74.
- Anfinsen, C.B. 1973. Principles that govern the folding of protein chains. *Science* **181**: 223–230.
- Arrondo, J.L.R., Young, N.M., and Mantsch, H.H. 1988. The solution structure of conalbumin A probed by FT-IR spectroscopy. *Biochim. Biophys. Acta* **952**: 261–268.
- Backmann, J., Schultz, C., Fabian, H., Hahn, U., and Saenger, W. 1996. Thermally induced hydrogen exchange processes in small proteins as seen by FTIR spectroscopy. *Proteins* **24**: 379–387.
- Beintema, J.J., Breukelman, H.J., Carsana, A., and Furia, A. 1997. Evolution of vertebrate ribonucleases: Ribonuclease A superfamily. In *Ribonucleases: Structures and functions* (eds. G. D'Alessio and J.F. Riordan), pp. 245–269. Academic Press, New York.
- Brands, J.F., Oliveira, R.J., and Westort, C. 1970. Thermodynamics of protein denaturation. Effect of pressure on the denaturation of ribonuclease A. *Biochemistry* **9**: 1038–1047.
- Buckle, A.M., Cramer, P., and Fersht, A.R. 1996. Structural and energetic responses to cavity-creating mutations in hydrophobic cores: Observation of a buried water molecule and the hydrophilic nature of such hydrophobic cavities. *Biochemistry* **35**: 4298–4305.
- Byler, D.M. and Susi, H. 1986. Examination of the secondary structure of proteins by deconvoluted FTIR spectra. *Biopolymers* **25**: 469–487.
- Clark, A.H., Saunderson, D.H.P., and Suggett, A. 1981. Infrared and laser-Raman spectroscopic studies of thermally-induced globular protein gels. *Int. J. Pept. Protein Res.* **17**: 353–364.
- Coll, M.G., Protasevich, I.I., Torrent, J., Ribó, M., Lobachov, V.M., Makarov, A.A., and Vilanova, M. 1999. Valine 108, a chain-folding initiation site-belonging residue, crucial for the ribonuclease A stability. *Biochem. Biophys. Res. Commun.* **265**: 356–360.
- Cooper, A. 1999. Thermodynamic analysis of biomolecular interactions. *Curr. Opin. Chem. Biol.* **3**: 557–563.
- Cuchillo, C.M., Vilanova, M., and Nogués, M.V. 1997. Pancreatic ribonucleases. In *Ribonucleases: Structures and functions* (eds. G. D'Alessio and J.F. Riordan), pp. 271–304. Academic Press, New York.
- DelCardayré, S.B., Ribó, M., Yokel, E.M., Quirk, D.J., Rutter, W.J., and Raines, R.T. 1995. Engineering ribonuclease A: Production, purification and characterization of wild-type enzyme and mutants at Gln11. *Protein Eng.* **8**: 261–273.
- Dong, A., Huang, P., and Caughey, W.S. 1990. Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochemistry* **29**: 3303–3308.
- Elwell, M.L. and Schellman, J.A. 1977. Stability of phage T4 lysozymes. I. Native properties and thermal stability of wild type and two mutant lysozymes. *Biochim. Biophys. Acta* **494**: 367–383.
- Fabian, H., Schultz, C., Naumann, D., Landt, O., Hahn, U., and Saenger, W. 1993. Secondary structure and temperature-induced unfolding and refolding of ribonuclease T1 in aqueous solution. *J. Mol. Biol.* **232**: 967–981.
- Fabian, H., Schultz, C., Backmann, D., Hahn, U., Saenger, W., Mantsch, H.H., and Naumann, D. 1994. Impact of point mutations on the structure and thermal stability of ribonuclease T1 in aqueous solution probed by Fourier transform infrared spectroscopy. *Biochemistry* **33**: 10725–10730.
- Frauenfelder, H., Alberding, N.A., Ansari, A., Braunstein, D., Cowen, B.R., Hong, M.K., Iben, I.E.T., Johnson, J.B., Luck, S., Marden, et al. 1990. Proteins and pressure. *J. Phys. Chem.* **94**: 1024–1037.
- Frye, K.J. and Royer, C.A. 1998. Probing the contribution of internal cavities to the volume change of protein unfolding under pressure. *Protein Sci.* **10**: 2217–2222.
- Frye, K.J., Perman, C.S., and Royer, C.A. 1996. Testing the correlation between delta A and delta V of protein unfolding using m value mutants of staphylococcal nuclease. *Biochemistry* **35**: 10234–10239.
- Fusi, P., Goossens, K., Consonni, R., Grisa, M., Puricelli, P., Vecchio, G., Vanoni, M., Zetta, L., Heremans, K., and Tortora, P. 1997. Extreme heat- and pressure-resistant 7-kDa protein P2 from the archaeon *Sulfolobus sol-*

- fataricus* is dramatically destabilized by a single-point amino acid substitution. *Proteins* **29**: 381–390.
- Glasoe, P.K. and Long, F.A. 1960. Use of glass electrodes to measure acidities in deuterium oxide. *J. Phys. Chem.* **64**: 188–190.
- Harris, P.I. and Chapman, D. 1995. The conformational analysis of peptides using Fourier transform IR spectroscopy. *Biopolymers* **37**: 251–263.
- Hawley, S.A. 1971. Reversible pressure-temperature denaturation of chymotrypsinogen. *Biochemistry* **10**: 2436–2442.
- Jackson, M. and Mantsch, H.H. 1995. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol.* **30**: 95–120.
- Juncosa-Ginestà, M., Pons, J., Planas, A., and Querol, E. 1994. Improved efficiency in site-directed mutagenesis by PCR using a *Pyrococcus* sp. GB-D polymerase. *Biotechniques* **16**: 820–823.
- Kitamura, Y. and Itoh, T. 1987. Reaction volume of protonic ionization for buffering agents - Prediction of pressure-dependence of pH and pOH. *J. Sol. Chem.* **16**: 715–725.
- Labhardt, A.M. 1982. Secondary structure in ribonuclease I. Equilibrium folding transitions seen by amide CD. *J. Mol. Biol.* **157**: 331–355.
- Matheson, R.R. and Scheraga, H.A. 1978. A method for predicting nucleation sites for protein folding based on hydrophobic contacts. *Macromolecules* **11**: 819–829.
- Matsuura, H., Hasegawa, K., and Miyazawa, T. 1986. Infrared and raman spectra of N-acetyl-L-amino acid methylamides with aromatic side groups. *Spectrochim. Acta* **42A**: 1181–1192.
- Nash, D., Lee, B.S., and Jonas, J. 1996. Hydrogen-exchange kinetics in the cold denatured state of ribonuclease A. *Biochim. Biophys. Acta* **1297**: 40–48.
- Neira, J.L. and Rico, M. 1997. Folding studies on ribonuclease A, a model protein. *Fold. Des.* **2**: R1–R11.
- Neira, J.L., Sevilla, P., Menéndez, M., Bruix, M., and Rico, M. 1999. Hydrogen exchange in ribonuclease A and ribonuclease S: Evidence for residual structure in the unfolded state under native conditions. *J. Mol. Biol.* **285**: 627–643.
- Nicoli, D.F. and Benedek, G.B. 1976. Study of thermal denaturation of lysozyme and other globular proteins by light-scattering spectroscopy. *Biopolymers* **15**: 2421–2437.
- Pace, C.N. 1990. Measuring and increasing protein stability. *Trends Biotechnol.* **8**: 93–98.
- Pace, C.N., Grimsley, G.R., Thomas, S.T., and Makhatadze, G.I. 1999. Heat capacity change for ribonuclease A folding. *Protein Sci.* **8**: 1500–1504.
- Panick, G. and Winter, R. 2000. Pressure-induced unfolding/refolding of ribonuclease A: Static and kinetic Fourier transform infrared spectroscopy study. *Biochemistry* **39**: 1862–1869.
- Privalov, P.L., Tiktopulo, E.I., Venyaminov, S.Yu., Griko, Yu.V., Makhatadze, G.I., and Khechinashvili, N.N. 1989. Heat capacity and conformation of proteins in the denatured state. *J. Mol. Biol.* **205**: 737–750.
- Raines, R.T. 1998. Ribonuclease A. *Chem. Rev.* **98**: 1045–1065.
- Reinstädler, D., Fabian, H., Backmann, J., and Naumann, D. 1996. Refolding of thermally and urea-denatured ribonuclease A monitored by time-resolved FTIR spectroscopy. *Biochemistry* **35**: 15822–15830.
- Rico, M., Bruix, M., Santoro, J., Gonzalez, C., Neira, J.L., Nieto, J.L., and Herranz, J. 1989. Sequential ¹H-NMR assignment and solution structure of bovine pancreatic ribonuclease A. *Eur. J. Biochem.* **183**: 623–638.
- Robertson, A.D. and Baldwin, R.L. 1991. Hydrogen exchange in thermally denatured ribonuclease A. *Biochemistry* **30**: 9907–9914.
- Royer, C.A., Hinck, A.P., Loh, S.N., Prehoda, K.E., Peng, X., Jonas, J., and Markley, J.L. 1993. Effects of amino acid substitutions on the pressure denaturation of staphylococcal nuclease as monitored by fluorescence and nuclear magnetic resonance spectroscopy. *Biochemistry* **32**: 5222–5232.
- Seshadri, S., Oberg, K.A., and Fink, A.L. 1994. Thermally denatured ribonuclease A retains secondary structure as shown by FTIR. *Biochemistry* **33**: 1351–1355.
- Smeller, L., Goossens, K., and Heremans, K. 1995a. The determination of the secondary structure of proteins at high pressure. *Vib. Spectrosc.* **8**: 199–203.
- . 1995b. How to minimize certain artifacts in Fourier self-deconvolution. *Appl. Spectrosc.* **10**: 1538–1542.
- Sosnick, T.R. and Trehwella, J. 1992. Denatured states of ribonuclease A have compact dimensions and residual secondary structure. *Biochemistry* **31**: 8329–8335.
- Takeda, N., Kato, M., and Taniguchi, Y. 1995. Pressure and thermally-induced reversible changes in the secondary structure of ribonuclease A studied by FTIR spectroscopy. *Biochemistry* **34**: 5980–5987.
- Talluri, S. and Scheraga, H.A. 1990. Amide H/D exchange in the thermal transition of bovine pancreatic ribonuclease A. *Biochem. Biophys. Res. Co.* **172**: 800–803.
- Tamura, Y. and Gekko, K. 1995. Compactness of thermally and chemically denatured ribonuclease A as revealed by volume and compressibility. *Biochemistry* **34**: 1878–1884.
- Torrent, J., Connelly, J.P., Coll, M.G., Ribó, M., Lange, R., and Vilanova, M. 1999. Pressure versus heat-induced unfolding of ribonuclease A: The case of hydrophobic interactions within a chain-folding initiation site. *Biochemistry* **38**: 15952–15961.
- Udgaonkar, J.B. and Baldwin, R.L. 1995. Nature of the early folding intermediate of ribonuclease A. *Biochemistry* **34**: 4088–4096.
- Vidugiris, G.J.A., Truckses, D.M., Markley, J.L., and Royer, C.A. 1996. High-pressure denaturation of staphylococcal nuclease proline-to-glycine substitution mutants. *Biochemistry* **35**: 3857–3864.
- Wlodawer, A., Svensson, L.A., Sjölin, L., and Gilliland, G.L. 1988. Structure of phosphate-free ribonuclease A refined at 1.26 Å. *Biochemistry* **27**: 2705–2717.
- Wong, P.T.T. and Moffat, D.J. 1989. A new internal pressure calibrant for high-pressure infrared spectroscopy of aqueous systems. *Appl. Spectrosc.* **43**: 1279–1281.
- Yang, J.T., Wu, C.S., and Martínez, H.M. 1986. Calculation of protein conformation from circular dichroism. *Method. Enzymol.* **130**: 208–269.
- Zhang, J., Peng, X., Jonas, A., and Jonas, J. 1995. NMR study of the cold, heat and pressure unfolding of ribonuclease A. *Biochemistry* **34**: 8631–8641.