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Spectroscopic characterization of rhinoviral protease 2A: Zn is essential for the structural integrity

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Abstract

Recently, protease 2A of human rhinovirus 2 (HRV2 2A) was shown to require a zinc ion for the formation of an active enzyme although zinc is not involved mechanistically. The data presented clearly show that the zinc ion bound to a picornaviral-specific motif represents an essential component of the native structure, probably representing a new Zn-binding motif. This structure, containing mostly β-strand elements as shown by CD spectroscopy, changes drastically upon removal of zinc. The zinc-depleted form does represent an intermediate with mostly unchanged secondary structure, but not a fully denatured random coil as obtained by guanidinium hydrochloride. This is indicated by the blue-shifted fluorescence spectra and by CD. The native protein exhibited a cooperative phase transition at 53 °C. In contrast, the zinc-depleted form did not show any transition at all, again demonstrating the stabilizing role of the zinc ion. A structural intermediate was observed during thermal and pH denaturation that may represent a molten globule, as suggested by its ANS binding.

Keywords: folding; picornavirus; stability; structure; viral protease

Human rhinoviruses, as all other picornaviruses, encode a polyprotein by a continuous open reading frame. In the case of entero- and rhinoviruses—genera of the picornavirus family—this polyprotein is proteolytically processed into the mature proteins by the virally encoded proteases 2A, 3C, and 3CD, respectively (Rückert, 1990). In entero- and rhinoviruses, the first proteolytic step is performed by the viral protein 2A, a protease that cleaves intramolecularly at its own amino terminus (Toyoda et al., 1986; Sommergruber et al., 1989). In addition to this initial cleavage of the nascent polyprotein, 2A proteases are also responsible for the cleavage of eIF4γ (formerly p220), a subunit of the cap-binding complex eIF-4F. This results in the inhibition of the translation of capped cellular mRNA (Lloyd et al., 1987; Jewell et al., 1990). The uncapped picornavirus mRNA initiates internally and thus remains unaffected.

From a structural point of view, these viral proteases belong to the serine protease family, even though they utilize a cysteine as an active site nucleophile (Bazan et al., 1988, 1989; Gorbalenya et al., 1989; Sommergruber et al., 1989; Hellen et al., 1991). The structural relationship with serine proteases was verified recently by the determination of the 3D structure of protease 3C of hepatitis A virus and human rhinovirus 14 (Allaire et al., 1994; Matthews et al., 1994). Further sequence comparison, in combination with mutational analysis, suggested the existence of a zinc-binding site in poliovirus protease 2A (Yu & Lloyd, 1992).

Recombinant protease 2A of HRV2 (HRV2 2A) was shown to form a dimer (Liebig et al., 1993). The state of the native enzyme in infected cells cannot be studied due to the minute amounts present during viral replication (König & Rosenwirth, 1988). More recently it was shown that HRV2 2A indeed contains zinc in an equimolar ratio. This ion was shown to be essential for the formation of an enzymatically active HRV2 2A, although it is not involved in catalysis. Zinc is tightly bound to the molecule; a zinc-depleted form can only be generated by a denaturation–renaturation cycle (Sommergruber et al., 1994). However, the loss of proteolytic activity could subsequently be restored fully by Zn supplementation in a second denaturation–renaturation cycle. These recent data suggested that zinc may be an essential part of the active structure.

In this study, we applied spectroscopic methods to demonstrate that the bound zinc ion indeed is required for the formation and stabilization of the enzymatically active structure of HRV2 2A.

Results

In the following experiments, native HRV2 2A was compared with the Zn-free protein that was obtained by denaturation in

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Abbreviations: ANS, 8-anilino-1-naphtalenesulfonic acid; DSC, differential scanning calorimetry; mGuHCl, guanidinium hydrochloride; HRV, human rhinovirus.

8 M urea, followed by renaturation in the presence of EDTA. Control renaturation experiments in the presence of Zn^{2+} instead of EDTA demonstrated that this denaturation–renaturation cycle does indeed result in the formation of a fully active enzyme if Zn^{2+} is present (Sommergruber et al., 1994).

CD studies

Native HRV2 2A exhibits a far-UV CD spectrum with a minimum around 216 nm, indicating a structure with a significant fraction of β-strand elements (Fig. 1A). The shoulder around 206 nm might indicate the presence of at least some α-helical elements. The quantitative analysis of the spectrum results in 34% β-sheet and 13.7% α-helix (Table 1).

The spectrum of the zinc-depleted HRV2 2A preparation shows the same minimum at 216 nm; however, the peak intensity is significantly increased. The shoulder at 206 nm is not visible anymore. The β-sheet content is slightly reduced; the α-helix content, however, has nearly doubled (Table 1).

The spectra of the acid-denatured forms of native and zinc-depleted HRV2 2A are nearly identical to the spectrum of nonacid-denatured zinc-depleted HRV2 2A (Fig. 1B). Heat-denatured HRV2 2A results in the same spectrum with increased intensity. The β-sheet content was not changed under these conditions (Table 1). The spectrum of urea-denatured HRV2 2A differs from the spectrum of the native enzyme only in the steep intensity increase below 211 nm. The position and intensity of the minima are identical.

Table 1. *Fraction of secondary elements as calculated from the respective CD spectra*

	α-Helix	β-Sheet	Coil	Turn
pH 7.5	13.7	34.6	43.9	7.8
pH 3.0	24.8	30.9	37.6	6.7
Zn-free pH 7.5	23.3	32.9	37.8	5.7
Zn-free pH 3.0	29.2	32.5	30.0	4.7
55 °C	24.4	29.7	39.7	6.2
Protease 3C[a,c]	5.6	42.5	–	–
Protease 3C[b,c]	0.5	50.0	–	–

[a] α-Helix and β-sheet content from Allaire et al. (1994).
[b] α-Helix and β-sheet content from Matthews et al. (1994).
[c] Data for coils and turns could not be derived from the publications.

Fluorescence spectra

An excitation wavelength of 290 nm was utilized in order to preferentially excite the single tryptophan residue, Trp-77. Removal of the zinc ion resulted in a large blue shift from 345 nm to 337 nm (Fig. 2A,B), accompanied by a reduction in fluorescence intensity. Denaturation at pH 3.0 or at 55 °C both caused a similar blue shift. The blue shift of pH 3.0-denatured zinc-depleted HRV2 2A was even more pronounced. Both heat-denatured forms of HRV2 2A exhibited the same maximum at 340 nm.

Denaturation of the native HRV2 2A with urea or GuHCl resulted in a red shift of the emission maximum to 351 nm accom-

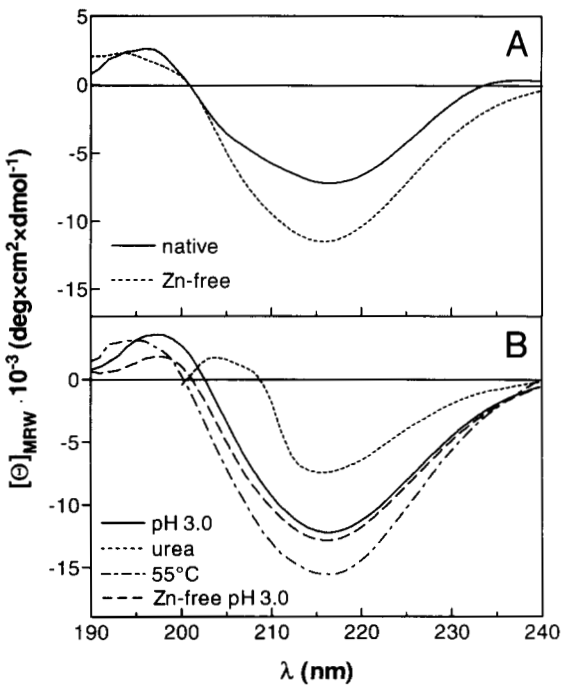

Fig. 1. CD spectra of native and zinc-depleted HRV2 2A protease 100 μg/mL at 20 °C. **A:** Comparison of native and zinc-depleted HRV2 2A protease in 10 mM phosphate, pH 7.5. **B:** Spectra of acid- (10 mM phosphate, pH 3.0), 8 M urea-, and temperature-denatured 55 °C native HRV2 2A protease and of zinc-depleted HRV2 2A protease at pH 3.0.

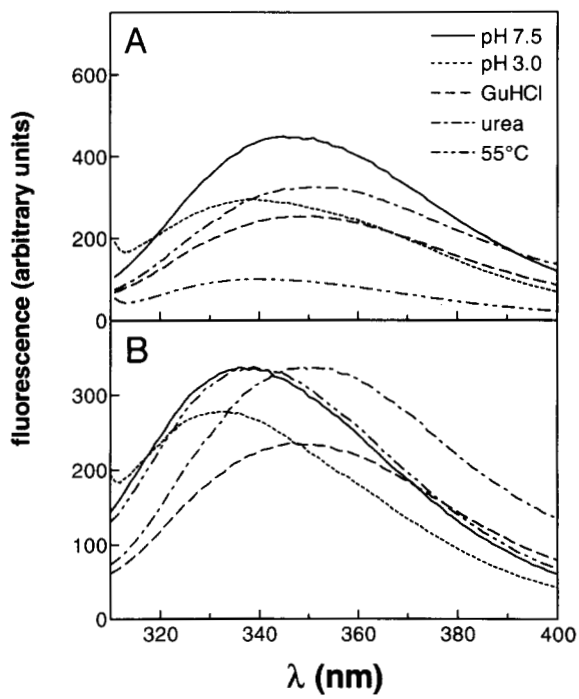

Fig. 2. Fluorescence spectra of (**A**) native and (**B**) zinc-depleted HRV2 2A protease. Spectra were recorded at 25 °C using a 290 nm excitation wavelength. Protein samples contained 70 μg/mL HRV2 2A protease. Samples were measured in 10 mM phosphate buffer at pH 7.5, pH 3.0, in 6 M GuHCl, 8 M urea, or in 10 mM phosphate buffer, pH 7.5 at 55 °C.

panied by a reduction in intensity. Zinc-depleted HRV2 2A also exhibited this red shift, however, to a lesser extent. These data are summarized in Table 2. In general, the same pattern of wavelength shifts is observed for native and zinc-depleted HRV2 2A. However, all spectra of zinc-depleted HRV2 2A are shifted toward a shorter wavelength. Fluorescence quenching under the various denaturation conditions resulted in similar emission intensities for the native and the zinc-depleted HRV2 2A (Table 2).

In order to further characterize the nature of the zinc-depleted and the acid-denatured forms, ANS binding to the various states was recorded. An increase in ANS fluorescence, accompanied by a blue shift of the maximum emission wavelength, was observed for acid denatured HRV2 2A (Fig. 3A). This ANS-binding structure was quite stable even at pH 1.0; the presence of 6 M GuHCl was required to totally denature the acid stable form (Fig. 3B). Urea- and GuHCl-denatured protein did not exhibit any significant spectral changes.

The zinc-depleted form itself showed a slightly increased ANS fluorescence. The change from pH 7.5 to pH 3.0 resulted in a dramatic increase of the ANS fluorescence (Fig. 3C). This intensity increase was 2.7-fold higher for zinc-depleted HRV2 2A compared to the native enzyme. No intensity increase was observed in any case for the GuHCl- or urea-denatured proteins.

Thermal denaturation

Figure 4A shows the thermal transition curves, measured by the ellipticity at 216 nm. The native HRV2 2A exhibits a clear transition point at 53 °C. The zinc-depleted protein does not show any significant ellipticity changes.

Furthermore, ANS fluorescence was used to monitor the thermal denaturation of native and zinc-depleted HRV2 2A. Native HRV2 2A shows a steep fluorescence increase with a half-maximum at 53 °C and a maximum at 57 °C (Fig. 4B). Above 57 °C, the intensity decreases. Zinc-depleted HRV2 2A again does not exhibit any temperature-dependent transition. The initially higher intensity drops with increasing temperature; at 75 °C, the intensity of the native HRV2 2A is reached. No precipitation was observed during these thermal denaturation experiments.

Differential scanning calorimetry was applied to further analyze the thermal unfolding transition. The CD and fluorescence data presented above demonstrated a rather sharp transition for native HRV2 2A, indicating a cooperative process that should be accompanied by an enthalpy change. The zinc-depleted protein, in contrast, did not exhibit a cooperative transition.

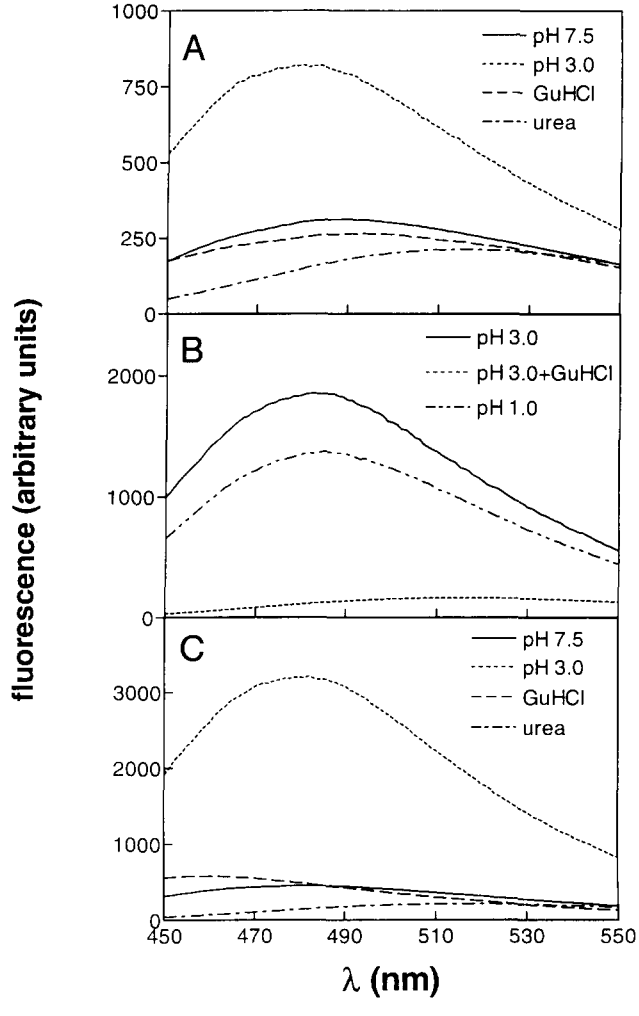

Fig. 3. Fluorescence spectra of 50 μM ANS in the presence of 50 μg/mL native and zinc-depleted HRV2 2A protease. Excitation wavelength was 390 nm. **A:** Native, 10 mM phosphate buffer, pH 7.5, pH 3.0, 6 M GuHCl, 8 M urea. **B:** Native, 10 mM phosphate buffer, pH 3.0, pH 1.0, pH 3.0 + 6 M GuHCl. **C:** Zinc-depleted, 10 mM phosphate buffer, pH 7.5, pH 3.0, 6 M GuHCl, 8 M urea.

Table 2. *Summary of observed fluorescence emission maxima (nm) and intensity at the maximum (arbitrary units) of native and zinc-depleted HRV2 2A protease derived from the fluorescence spectra shown in Figure 2*

	Native HRV2 2A		Zn-depleted HRV2 2A	
	Maximum	intensity	Maximum	intensity
pH 7.5	345	448	336	336
pH 3.0	338	295	332	277
6 M GuHCl	351	251	346	235
8 M urea	351	22	349	337
55 °C	340	100	339	337

The thermal unfolding of native HRV2 2A shows a distinct heat absorption peak (ΔH = 253.1 kJ/mol; Fig. 5). The peak temperature of 52.5 °C is in good accordance with those values determined above. No significant heat absorbance is observed for the acid denatured and the zinc-depleted form.

Discussion

Recent studies on denaturation/renaturation of highly purified HRV2 2A suggested that the equimolarly bound zinc ion is essential for the stabilization of the native structure (Sommergruber et al., 1994). Furthermore, mutational analysis of poliovirus protease 2A support this idea (Yu & Lloyd, 1992). According to this study, zinc should be tightly bound by a highly conserved zinc-binding motif comprising the residues Cys-55, Cys-57, Cys-115, and His-117 in protease 2A of poliovirus type 1; in HRV2 2A,

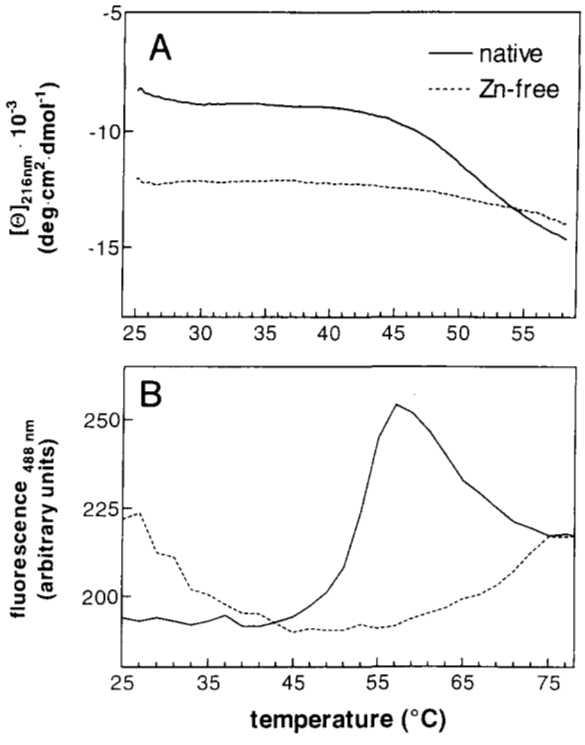

Fig. 4. A: Thermal unfolding transition of 100 μg/mL native and zinc-depleted HRV2 2A protease in 10 mM phosphate buffer, pH 7.5, measured by the ellipticity at 216 nm. **B:** Temperature dependence of ANS (50 μM) fluorescence in the presence of 50 μg/mL native and zinc-depleted HRV2 2A protease in 10 mM phosphate buffer, pH 7.5. Fluorescence was recorded at 488 nm, excitation was 390 nm.

this putative motif is represented by residues Cys-52, Cys-54, Cys-112, and His-114.

This putative zinc-binding site is built up by two separate loops in poliovirus 2A (Yu & Lloyd, 1992). The active site nucleophile (Cys-109 in poliovirus 2A and Cys-106 in HRV2 2A) is located directly at the beginning of the second loop. These loops seem to be held together and may thus be stabilized by the zinc ion. Thus, it is likely that the zinc ion will—by holding in place these two loops—act in a stabilizing manner on the entire structure.

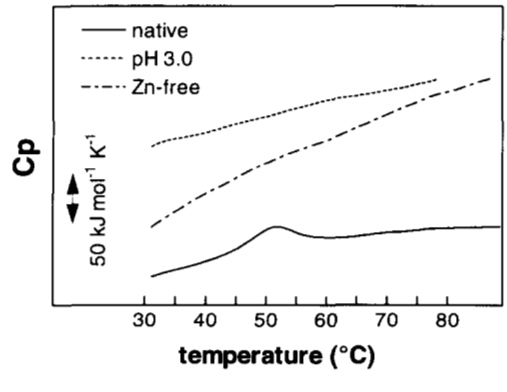

Fig. 5. DSC curves of the unfolding transition of 0.25 mg/mL native and 0.36 mg/mL zinc-depleted HRV2 2A protease in 10 mM phosphate buffer, pH 7.5, and 0.22 mg/mL phosphate buffer, pH 3.0-denatured HRV2 2A protease.

This motif with the sequence pattern C-X-C/H differs from the C-X-X-C/H motifs found in other Zn-binding proteins (Schwabe & Klug, 1994). It thus seems to be a new, unique motif found only in the picornaviral proteases 2A.

The experimental data presented here clearly demonstrate the essential role of the zinc ion for the structural integrity of native HRV2 2A.

The CD spectrum of native HRV2 2A indicates a mostly β-strand structure with some α-helical elements (Table 1). This is in accordance with the related proteases 3C from hepatitis virus and rhinovirus that contain about 50% β-strands (Allaire et al., 1994; Matthews et al., 1994).

Removal of zinc as well as acid-denaturation results in drastic structural changes. These are reflected by a blue shift of 10 nm in the fluorescence spectra (Fig. 2; Table 2), indicating a more hydrophobic environment of the single Trp-77. The emission is significantly quenched relative to the native state. This suggests an increased mobility of the tryptophan side chain, thus resulting in dynamic quenching of the excited state (Goto & Fink, 1989). The changes in secondary structure content observed by CD spectroscopy are less pronounced (Fig. 1; Table 1). The overall content of the major structural element, the β-sheet, is fairly unchanged under all conditions, the α-helicality seems to have increased.

The species generated by Zn removal or acid denaturation must represent related structural intermediates with more or less preserved secondary structure elements. These structures are different from the fully denatured, random coil state. This is obtained upon denaturation with urea or GuHCl, as indicated by the red shift of the tryptophan fluorescence to 351 nm, being indicative for a completely denatured molecule. Urea denaturation, however, does not seem to break up all secondary structure elements because the CD spectrum of urea denatured HRV2 2A clearly does not exhibit the features of a random coil spectrum.

The temperature denaturation profiles recorded by either CD or fluorescence spectroscopy (Fig. 4) demonstrate a clear, cooperative phase transition around 53 °C. Denaturation occurs via an ANS-binding intermediate observed as a fluorescence emission peak at 488 nm. This intermediate disappears above 57 °C. The cooperative transition expectedly results in a clear heat absorption peak at 53 °C (DSC, Fig. 5). Zinc-depleted HRV2 2A, in contrast, does not exhibit any significant phase transition in the fluorescence and DSC spectra, indicating the absence of a cooperative stabilization of the tertiary structure. A rather continuous, but small ellipticity change may indicate the initial presence of some native-like secondary structure elements in the zinc-depleted molecule.

The ANS-fluorescence of the structures observed during temperature denaturation or at pH 3.0 is indicative for a molten globule, which is characterized by the binding of ANS to water-accessible hydrophobic surfaces (Ptitsyn, 1992). The notion of these structures representing a molten globule is further supported by the nearly unchanged β-sheet content (Table 1). The molten globule is considered to be less densely packed. This is reflected by the increased mobility of the tryptophan side chain, as indicated by the quenched fluorescence intensity.

Recombinant HRV2 2A does form a dimer (Liebig et al., 1993), as shown by gel electrophoresis under native conditions.

The Zn-free HRV2 2A exhibits the identical band pattern, indicating its dimeric nature (data not shown). The homologous Zn-binding protease 2A of Coxsackie B4, in contrast, behaves as a monomer under identical conditions. Thus, the Zn ion is not involved in the dimer formation. HRV2 2A can be converted into a monomer by mild detergent treatment without losing enzymatic activity (data not shown). Thus, the characteristics of the Zn-free or acid-denatured structures (changed secondary structure content, fluorescence blue shift, lack of cooperative phase transition) clearly demonstrate drastic changes within the molecule. These data do not simply reflect a dimer dissociation.

The thermal unfolding of a molten globule has been assumed to be noncooperative. Hydrophobic interactions were assumed to be of minor importance for its stabilization (Kuvajima, 1989; Yutani et al., 1992). Thus, the molten globule state is enthalpically equivalent to the unfolded state, or $\Delta C_{p,U}$ and ΔH_U are zero. More recent data, however, report small, but significant $\Delta C_{p,U}$ and ΔH_U data for the molten globule of cytochrome *c* (Hagihara et al., 1994), and apomyoglobin (Nishii et al., 1994). Theoretical studies support these data (Haynie & Freire, 1993), proposing a $\Delta C_{p,U}$ that is smaller than that of the native molecule. The DSC data, showing no temperature transition for the acid-denatured molecule (thus demonstrating the lack of a cooperative transition), are in contrast to the published data. In the DSC experiments, rather low concentrations of HRV2 2A had to be used due to the fact that the enzyme aggregates at higher concentrations. The small $\Delta C_{p,U}$ may have been missed due to these low concentrations.

The putative molten globule state of HRV2 2A is quite stable up to 57 °C and even at pH 1.0. Renaturation is not possible for temperature-denatured HRV2 2A (data not shown) and for HRV2 2A denatured at pH below 2.5 (Sommergruber et al., 1992).

In summary, these data clearly show that the zinc ion bound to Zn-binding motif specific for picornaviral protease 2A is an integral part of the native structure of HRV2 2A. Removal of the ion results in the formation of a structural intermediate reminiscent of a molten globule. Detailed studies have to be carried out to evaluate the role of the putative molten globule in the folding pathway of HRV2 2A.

Materials and methods

Protein expression and purification

The expression of HRV2 2A in *Escherichia coli* BL21 (DE3) pLysE cells and its purification was carried out as described earlier (Liebig et al., 1993; Liebig & Skern, 1994). For the studies presented here, only fresh preparations were used in order to prevent aggregation.

Sample preparation for spectroscopy

HRV2 2A was stored in 150 mM NaCl, 5 mM DTT, and 50 mM Tris/HCl, pH 8.5. A solution containing approximately 0.25 mg/mL HRV2 2A was concentrated 10 times by centrifugation (Beckman J2-21, JA21 rotor, 5000 rpm) in Centricon-10 tubes (10-kDa cut off; Amicon) and diluted with 10 mM Na-phosphate, pH 7.5, 6 M GuHCl or 8 M urea to the original concentration of the HRV2 2A solution. This was repeated two times. For comparison of spectra at pH 7.5 and 3.0, the sample was divided in two equal parts. One half was adjusted to pH 3.0 with 5 M HCl, resulting in a volume increase of less than 3%.

The preparation of zinc-depleted HRV2 2A was performed essentially as described (Sommergruber et al., 1994). Briefly, 1 mL of an HRV2 2A stock solution (2 mg/mL) in buffer B (50 mM Tris/HCl, pH 8.0, and 150 mM NaCl) was mixed with 9 mL of a saturated urea solution in buffer B. After an incubation period of 4 h at room temperature, 100 μL of 0.5 M EDTA was added and the HRV2 2A solution was dialyzed once against 5 L of buffer B.

The protein concentration was determined using the Biorad protein assay or by UV absorption at 280 nm (ϵ_{280} = 20,000 M^{-1} cm^{-1}).

CD-spectroscopy

Spectra were measured on a JASCO 600 spectrometer with a scan rate of 50 nm/min at 0.5-nm intervals. Spectra were obtained using a thermostated cell with 0.1-cm pathlength at a concentration of 100 μg/mL. Thermal unfolding was monitored by the ellipticity at 216 nm. The temperature was increased at a rate of 1 °C/min and monitored with a thermocouple (Luton DH-802C temperature adapter and a NiCr-Ni flexible probe, BBC-Goerz recorder) inserted directly into the cell. The data are expressed as residue ellipticity (molecular weight of 16,209 Da, 142 amino acid residues). The number of secondary structure elements was calculated from the CD spectra using the Antheprot software (Deléage & Geourjon, 1993).

Fluorescence spectroscopy

Fluorescence spectra were measured with a Hitachi F-2000 fluorescence spectrometer controlled by the F-2000 IC software via a NEC personal computer. Measurements were carried out in a thermostated cell holder. Thermal unfolding was carried out as described above.

Differential scanning calorimetry

The differential scanning calorimeter MC-2 and the DA-2 data acquisition and analysis system were from Microcal Inc. (Massachusetts, USA) equipped with an IBM PC AT. Into the sample cell of the calorimeter was placed 1.35 mL of the protein-containing solutions. The scan rate was adjusted to 1.5 °C/min for all measurements. Samples were measured at concentrations of 0.25 mg/mL (pH 7.5), 0.36 mg/mL (pH 3.0), and 0.22 mg/mL (zinc-depleted).

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