Circular dichroism and the secondary structure of the ROF2 protein from *Arabidopsis thaliana*

Liliana Lighezan · David Meiri · Adina Breiman ·
Adrian Neagu

Received: 23 November 2012 / Accepted: 15 May 2013 / Published online: 19 June 2013
© Springer Science+Business Media Dordrecht 2013

Abstract The protein ROF2 from the plant *Arabidopsis thaliana* acts as a heat stress modulator, being involved in the long-term acquired thermotolerance of the plant. Here we investigate the relationship between the biological function and the structure of ROF2, inferred by circular dichroism (CD) spectroscopy. The far-UV CD spectra, analyzed with the CDPro and DICHROWEB program packages, yield the percentages of α -helices, β -sheets, unordered regions, turns and poly(Pro)II-helices in the secondary structure of ROF2. According to the analysis, the percentages of the structural elements of ROF2 are about 40% for β -sheets, 30% for unordered regions, 17% for turns, 10% for poly(Pro)II-helices and 3% for α -helices. The near-UV CD spectra suggest that ROF2 proteins can associate, forming super-secondary structures. Our CD experiments performed at temperatures between 5 °C and 97 °C indicate that the thermal denaturation of ROF2 caused by a raise in temperature up to 55 °C is followed by a thermal refolding of the protein as the temperature is raised

L. Lighezan
Faculty of Physics, West University of Timisoara, Blvd. Vasile Parvan No. 4,
300223 Timisoara, Romania
e-mail: llighezan@physics.uvt.ro

D. Meiri · A. Breiman
Department of Plant Sciences, Tel Aviv University, Tel Aviv, Israel 69978

D. Meiri
e-mail: dedimeiri1@gmail.com

A. Breiman
e-mail: adinab@tauex.tau.ac.il

L. Lighezan · A. Neagu (✉)
Department of Functional Sciences, Center for Modeling Biological Systems and Data Analysis,
Victor Babes University of Medicine and Pharmacy Timisoara,
Piata E. Murgu No. 2–4, 300041 Timisoara, Romania
e-mail: neagu@umft.ro

further. The new secondary structure, acquired around 65 °C, remains stable up to 97 °C. The structural stability of ROF2 at high temperatures might play an important role in the experimentally observed thermotolerance of *Arabidopsis thaliana*.

Keywords ROF2 • Thermal denaturation • Thermotolerance • CDPPro • DICHROWEB

1 Introduction

The protein ROF2 consists of 578 amino acids and belongs to the FK506 Binding Protein (FKBP) family from the peptidyl-prolyl *cis-trans* isomerase (PPlase) class of proteins [1–4]. Due to its molecular mass of 65 kDa, ROF2 also goes by the name of FKBP65. ROF2 is not present in plants under normal growth conditions, at 22 °C [5]. Nevertheless, after 3 h of exposure to a heat stress (37 °C), ROF2 was detected in the cytoplasm and remained present for several hours even after the cessation of the heat stress [6]. Six hours after the onset of the heat stress, ROF2 was also detected in the nucleus, being present in both the cytoplasm and the nucleus for at least 24 h [6]. It has been hypothesized that, during plant recovery following heat exposure, ROF2 translocates from the cytoplasm into the nucleus [6, 7]. Interestingly, during an exposure to 42 °C for 90 minutes, ROF2 was not detected, possibly due to the arrest of its synthesis [5].

It has been demonstrated that ROF2 acts as a heat stress modulator [6, 7], being involved in the long-term acquired thermotolerance of plants—the capacity of plants to cope with repeated heat stimulations [8, 9]. However, the underlying mechanism is unknown.

Recent studies have also revealed that intracellular acid stress in plants, generated by weak organic acids at normal external pH, induces the expression of ROF2 [4]. It has been shown that ROF2 modulates the intracellular pH homeostasis [4], inducing the tolerance of the plants to the acid stress, by increasing the proton extrusion from the cells.

The structure of the ROF2 protein is not known. To gain insight into the secondary and tertiary structures of ROF2, we recorded the absorption and circular dichroism (CD) spectra of this protein and performed a computational analysis of the CD spectra.

The motivation of the present work is to make a first step towards understanding the structure-function relationship by analyzing the secondary and super-secondary structures of ROF2 at various temperatures. To this end, we measured and analyzed the CD spectra of ROF2 purified from the plant *Arabidopsis thaliana* [10].

In order to predict the fractions of various architectural motifs in the secondary structure of ROF2, we used the software packages DICHROWEB [11] and CDPPro [12]. Using the experimental CD spectra and comparing these with a large set of reference CD spectra of proteins with known structure, the programs from DICHROWEB and CDPPro compute the secondary structure content, expressed as fractions of α -helices, β -sheets, turns and unordered structures, present in the investigated protein.

2 Materials and methods

The ROF2 protein samples have been purified from *Arabidopsis thaliana* at the Department of Plant Science of Tel Aviv University, Israel, using the method developed by Meiri and coworkers [7].

2.1 Purification of recombinant ROF1 and ROF2

In order to study the structure–function relationship of the ROF proteins, we produced recombinant proteins containing a His-tagged tail.

ROF1 and ROF2 coding sequences were amplified from 2-week-old seedlings cDNA library with specific primers. The PCR products were cloned into a pET- 28a vector (Novagen). This vector contains a histidine-tagged site and a cleavage site for the TEV protease, which enable removal of the histidine-tagged protein.

The His-tagged ROF1 and ROF2 were sequenced to confirm lack of mutations and overexpressed in *Escherichia coli* strain BL21. For protein expression, single transformed colonies were grown at 37 °C to an OD600 and the cells were harvested by centrifugation. The cells were ruptured using a French press and the supernatant and pellet were separated by centrifugation. The crude supernatant was loaded on a nickel-nitrilotriacetic acid-agarose column that was then washed to get rid of non-specifically bound proteins. The ROF1/ROF2 proteins were eluted with a higher imidazole concentration. The proteins were further purified using a MonoQ ion-exchange column (Bio-Rad, Hercules, CA, USA) in an FPLC system (Bio-Rad). The third and final purification step was gel filtration chromatography on a Superdex 200 gel filtration column.

2.2 Absorption spectroscopy measurements

The absorption spectrum was recorded at a temperature of 5 °C using a Varian CARY-300 UV-VIS Spectrophotometer, version 9. The spectrum was collected using double-beam mode. The spectral bandwidth was 2 nm, the average time was 0.2 s, and the scan rate was 150.25 nm/min.

2.3 CD spectroscopy measurements

Solutions of the protein ROF2 with molar concentrations of 1.24, 6.2, and 62 μM were prepared and maintained at pH 7.8 by a 10 mM Na₂HPO₄ buffer along with 1 mM C₄H₁₀O₂ S₂.

The CD measurements were performed using a JASCO J710 spectropolarimeter (JASCO Corporation, Tokyo, Japan) equipped with a Peltier-based temperature-controlled chamber. The experimental setup measures the *ellipticity*, θ , of the electromagnetic wave that traversed the sample. We express the CD spectra in terms of *molar ellipticity per residue*, defined by the formula [13]:

$$[\theta]_r = \frac{\theta}{c \cdot l \cdot N_r}, \quad (1)$$

where c is the molar concentration of the protein in the solution, l is the length of the light beam's path through the sample and N_r is the number of amino acid residues in the protein.

The CD spectra were collected with a 2-nm bandwidth. The scanning speed was 20 nm/min and the spectral resolution was 0.2 nm. In both the far-UV (190–250 nm) and near-UV (250–310 nm) domains, we used 10-mm-path-length cuvettes. The CD spectrum of the empty cuvette was recorded and subtracted from sample spectra.

For variable temperature measurements, the samples were heated from 5 °C to 97 °C at a rate of 1 °C/min, with a resolution of 0.1 °C; the CD signal was recorded at a fixed wavelength.

2.4 Computational analysis of the spectra

To gain information on the secondary structure of the ROF2 protein, we analyzed the CD spectra of ROF2 using the program packages DICHROWEB [11] and CDPPro [12].

DICHROWEB is a Web server set up at the Department of Crystallography of the Institute of Structural and Molecular Biology, Birkbeck College, University of London, UK [11, 14]. It is publicly available for non-profit organizations at <http://dichroweb.cryst.bbk.ac.uk>. CDPPro has been developed at the Department of Biochemistry and Molecular Biology of Colorado State University, Fort Collins, CO, USA [12]. It is freely available for non-profit organizations at <http://lamar.colostate.edu/~sreeram/CDPro/>. Starting from a reference set of proteins with known structure and known CD spectra, using the singular value deconvolution algorithm and variable selection procedures, the programs calculate the fraction of each secondary structure motif (α -helix, β -sheet, turn and unordered structure) that contributes to the protein's spectrum.

As a goodness-of-fit parameter, we used the normalized root mean square deviation (σ), defined as:

$$\sigma = \sqrt{\frac{\sum_{\lambda} (\theta_{\text{exp}} - \theta_{\text{calc}})^2}{\sum_{\lambda} \theta_{\text{exp}}^2}}, \quad (2)$$

where θ_{exp} and θ_{calc} are the ellipticities of the experimental and calculated spectra, respectively; the sums run over all wavelengths of the spectra [11].

3 Results

3.1 Purified ROF2 proteins

We produced and purified recombinant ROF2 proteins containing a His-tagged tail (Fig. 1). ROF2 coding sequences were amplified from 2-week-old *Arabidopsis* seedlings, cloned, expressed, and purified as described previously [7].

Fig. 1 Purification of the ROF2 protein. Coomassie staining of: (a) First step of ROF2 purification on a nickel-agarose column. Lanes 1–8—samples of eluted fractions (3 ml each). (b) The eluted fractions from the first step of ROF2 purification (nickel-agarose column) concentrated and loaded on a MonoQ ion-exchange column. Lanes 1–8—samples of eluted fractions from the ion-exchange column (3 ml each)

Fig. 2 The near-UV absorption spectrum of the ROF2 protein solution with a molar concentration of 6.2 μM , at a temperature of 5 $^{\circ}\text{C}$

3.2 Near-UV absorption spectrum

Figure 2 presents the near-UV absorption spectrum of the ROF2 protein with a concentration of 6.2 μM . The spectrum displays a large absorption band with a maximum at about 279 nm and a shoulder at about 290 nm. No vibrational structure can be seen in the spectrum.

3.3 Far-UV circular dichroism spectra

In order to investigate the secondary structure of the ROF2 protein, the molar ellipticity per residue, $[\theta]_r$, given by (1), was measured in the far-UV spectral domain, for a 1.24 μM solution of ROF2, at a temperature of 5 $^{\circ}\text{C}$. The obtained spectrum (Fig. 3) consists in a negative band between 200 and 245 nm, with two minima at approximately 212 and 217 nm and a positive band below 200 nm, with a maximum at about 190 nm.

Fig. 3 The far-UV CD spectrum (molar ellipticity per residue vs. wavelength) of a ROF2 protein solution with a molar concentration of 1.24 μM , at a temperature of 5 $^{\circ}\text{C}$

3.4 Computational analysis of the circular dichroism spectra using DICHROWEB and CDPro

To calculate the percentages of the ROF2 protein's secondary structure motifs, we used the program packages DICHROWEB and CDPro. Shown in Fig. 4 are the computed spectra (solid disks) compared with the experimental spectrum (empty squares) obtained for the ROF2 protein sample with a concentration of 1.24 μM .

Using the DICHROWEB on-line server, the best fits were obtained by the CDSSTR program [15], with the reference sets of proteins SP175 (Fig. 4a) and 3 (Fig. 4b), taking in the analysis CD data in the spectral range 190–240 nm and 185–240 nm, respectively. We also obtained a good fit using the CONTIN program with the reference set SP175 [15]. The corresponding secondary structure contents of ROF2 are listed in Table 1.

Using the CDPro package, we have taken in the analysis the data at wavelengths down to 195 nm. The best fit of the experimental spectrum of ROF2 was obtained by the CONTINLL program with the reference set 5 (Fig. 4c), composed of proteins with different types of secondary structure elements, including poly(Pro)II helices [16, 17]. Good fits were also

Fig. 4 On each panel, *empty squares* plot the CD spectrum of a 1.24 μM ROF2 solution, measured at a temperature of 5 °C. **(a)** *Solid disks* plot the best calculated spectrum given by the CDSSTR program of the DICHROWEB package [11] using the reference set of proteins SP175. **(b)** *Solid disks* plot the best calculated spectrum given by the CDSSTR program of the DICHROWEB package [11] using the reference set of proteins 3. **(c)** *Solid disks* plot the best calculated spectrum given by the CONTINLL program from the CDPro package [12], using the reference set of proteins 5. **(d)** *Solid disks* plot the best calculated spectrum given by the CONTINLL program from the CDPro package [12], using the reference set of proteins 10

Table 1 The percentages of various secondary structural elements in the ROF2 protein, resulted from the best fits of the experimental CD spectrum of ROF2 by using the software packages DICHROWEB [11] and CDPro [12]. The indices R and D refer to regular and distorted structures, respectively; regular structures have a precise geometry, whereas distorted structures result from the finite size of the respective motifs. The PP2 fractions refer to the poly(Pro)II helical elements. The experimental and computed spectra are shown on Fig. 4a and b (for DICHROWEB) and Fig. 4c and d (for CDPro). The last column contains the normalized root mean square deviation, σ (2)

Software package	Program	Basis set of proteins	α helices		β sheets		Turns		Unordered (%)	PP2 (%)	σ
			α_R (%)	α_D (%)	α_{tot} (%)	β_R (%)	β_D (%)	β_{tot} (%)			
DICHROWEB	CDSSTR	SP175	-3	3	0	35	16	51	8	35	-
		3	-3	5	2	29	14	43	24	28	-
	CONTIN	SP175	0	7.3	7.3	26.2	14.1	40.3	12.3	40.1	-
	Average results		-2	5.1	3.1	30.1	14.7	44.8	14.8	34.4	-
CDPro	CONTIN LL	5	-	-	4.4	-	-	29.6	12.1	42.6	11.2
		10	0	4	4	28.3	13.9	42.2	20.7	33	-
		4	0	3.7	3.7	28.5	13.3	41.8	21.2	33.2	-
		9	0	4	4	27.8	14.2	42	21.2	32.7	-
		3	0.1	3.9	4	27.9	14.2	42.1	20.8	33	-
		6	0.1	3.4	3.5	27.6	14	41.6	20.2	34.7	-
		7	0.1	2.9	3	27	13.2	40.2	18.5	38.3	-
		8	0.1	2.9	3	27	13.2	40.2	18.5	38.3	-
		1	0.1	2.4	2.5	34.7	17.1	51.8	19.1	26.7	-
	Average results ^a		0.06	3.4	3.46	28.6	14.14	42.74	20.02	33.74	-
Global average results ^a			-0.97	4.25	3.28	29.35	14.42	43.77	17.41	34.07	-

^a Average results given by CDPro and the global average results have been calculated by omitting the results obtained using the reference set 5, which includes proteins with poly(Pro)II helices in their secondary structures

obtained using CONTINLL with the reference set 10 (Fig. 4d), which combines soluble proteins with membrane proteins [18] and with the reference set 4, which contains soluble proteins [12]. Other results and the predicted secondary structure content are given in Table 1.

In order to obtain information on the tertiary structure of ROF2, we analyzed the far-UV CD spectrum of the ROF2 sample with a concentration of 1.24 μM , using the CLUSTER program from the CDPro package. The CLUSTER program creates a reference set of a certain number of proteins with known tertiary structure, selected by the program in order to obtain the best fit [12]. The program returned the result “All β ”.

3.5 Variable temperature circular dichroism measurements

To gain insight into the thermal denaturation of ROF2, we recorded the temperature dependence of the circular dichroism of ROF2 at a wavelength of 212 nm, which is the location of the minimum of the negative band.

As shown in Fig. 5a, the molar ellipticity per residue increases as the temperature rises to 55 $^{\circ}\text{C}$ and decreases as the temperature is raised further.

To answer the question of whether the thermal denaturation of ROF2 is reversible or not, we recorded the CD spectrum of a ROF2 solution that was heated up to 97 $^{\circ}\text{C}$ and, subsequently, cooled down to 5 $^{\circ}\text{C}$ (black line in Fig. 5b).

3.6 Near-UV circular dichroism spectra

To investigate the tertiary structure of ROF2, we recorded the CD spectrum in the near-UV domain. According to Fig. 6 (black curve), the near-UV CD spectrum of the sample with a ROF2 concentration of 6.2 μM , at a temperature of 5 $^{\circ}\text{C}$, displays a large negative band, with two minima at 286 and 294 nm. We can also observe some shoulders at about 263, 270, and 277 nm.

In order to identify concentration-dependent phenomena, such as protein cluster formation, we also recorded the near-UV spectrum of a sample with a higher ROF2 concentration of 62 μM . The obtained CD spectrum (Fig. 6, light gray curve) presents only one minimum,

Fig. 5 **a** Molar ellipticity recorded as a function of temperature for a 1.24 μM solution of ROF2 at a wavelength of 212 nm. **(b)** The far-UV CD spectrum of ROF2 at 5 $^{\circ}\text{C}$ before (gray line) and after a thermal treatment composed of heating up to 97 $^{\circ}\text{C}$ and cooling down to 5 $^{\circ}\text{C}$ (black line)

Fig. 6 The near-UV CD spectra of ROF2 at low concentration (6.2 μM , black line) and high concentration (62 μM , gray line). In both experiments, the temperature was maintained at 5 $^{\circ}\text{C}$

at about 300 nm, the absolute value of the CD being smaller than the absolute value obtained for the less concentrated sample.

4 Discussion

4.1 Near-UV absorption spectrum of the ROF2 protein

It is known that the absorption bands of proteins in the spectral region between about 250 and 350 nm (near-UV) are mainly due to the aromatic amino acids Trp, Tyr, and Phe [19]. The absorption bands often present fine vibrational structure, due to the transitions between electronic states with vibrational structure. Trp presents a maximum around 290 nm, with fine vibrational structure between 290 and 305 nm. Tyr displays a maximum between 275 and 282 nm, with a shoulder at higher wavelengths that can often be covered by the Trp bands. Phe produces weaker and narrower bands, with fine vibrational structure between 255 and 270 nm [19].

Making a comparison between the absorption spectrum of the ROF2 protein (Fig. 2) and the known absorption spectra of Trp and Tyr [19], we can say that the absorption spectrum of the ROF2 protein is mainly due to the Tyr and Trp amino acids, present in the protein's sequence, their bands being overlapped. The absence of the observable vibrational structure indicates a low mobility of these aromatic amino acids.

4.2 Secondary structure of the ROF2 protein

The features of the far-UV spectrum of the ROF2 protein (Fig. 3) allow for a rough estimation of the protein's secondary structure.

It is known that the CD spectrum of an α -helix displays two negative bands between 203 and 240 nm, with minima positioned between 209 and 222 nm; below 203 nm, the α -helix spectrum also displays positive bands with a strong maximum close to 192 nm [13, 20, 21]. The CD spectra of β -sheets show a greater variety and less intense bands, with a minimum

positioned between 210 nm and 225 nm and a maximum between 190 nm and 200 nm [13, 20–22].

A comparison between the ROF2 experimental spectrum (Fig. 3) and spectra of known α -helices and β -sheets suggests that the secondary structure of ROF2 includes both α -helix and β -sheet conformations, the proportion of the latter being significantly higher. It seems that the minimum of the β -sheet spectrum has a relatively large intensity, so it partially overlaps with the two minima of the α -helix spectrum. It is possible, however, that β -sheets associate with α -helices to form a super-secondary structure with predominantly β -sheet conformations, in agreement with the computational results (Section 4.4).

4.3 Percentages of the secondary structure motifs in the ROF2 protein

Using the program packages DICHROWEB and CDPro, we calculated the percentages of secondary structure motifs (Table 1). Our computational analysis reconfirms the intuitive conclusion drawn by inspecting the far-UV CD spectra (Fig. 3) in comparison with known pure spectra of the motifs that a large proportion of ROF2 is composed of β -sheets. On average (Table 1, last row), the secondary structure of ROF2 consists predominantly of β -sheets (43.77%), a considerable percentage of unordered structures (34.07%), turns (17.41%), and a small proportion of α -helices (3.28%). Even though the calculated spectra are based on different basis sets of proteins, their errors (σ) and outputs are quite similar (Table 1). A σ value lower than about 0.1 indicates an accurate calculation of the secondary structure; values higher than 0.5 suggest that the characteristics of the analyzed protein are not well represented in the reference set [11].

Using the DICHROWEB on-line server, the best fits were obtained by the CDSSTR program, with the reference sets of proteins SP175 (Fig. 4a) and 3 (Fig. 4b), taking in the analysis CD data in the spectral range of 190–240 nm and 185–240 nm, respectively. The reference set S175 is composed of 70 globular soluble proteins [15, 22] with data collected down to 175 nm but that can also be used with data down to 190 nm, giving excellent results. The reference set 3 contains 37 globular soluble proteins [15, 22] and needs the data at wavelengths down to 185 nm. We also obtained a good fit using the CONTIN program with the reference set SP175 (not shown).

Due to the high level of noise observed in the CD spectra at wavelengths below 195 nm while using the CDPro package, we included in the analysis only the data obtained at wavelengths above 195 nm (Fig. 3). The best fit of the experimental spectrum of ROF2 was obtained by the CONTINLL program with the reference set 5 (Fig. 4c), composed of 37 proteins with different types of secondary structure elements, including poly(Pro)II helices [16, 17].

The results listed in Table 1 show that poly(Pro)II helices represent 11.2% of the secondary structure of ROF2; β -sheets represent 29.6%, whereas turns represent 12.1%. The presence of poly(Pro)II helices is expected due to the high amount of proline present in the ROF2 sequence. It is known that poly(Pro)II helices are shorter (4–8 amino acids) and more flexible than α -helices, serving as bridges between different secondary structure elements or being included in other secondary structure elements. So, our results indicate that the poly(Pro)II helices present in ROF2 are mainly attached to β -sheets, unordered structures and turns.

Good fits were also obtained using the CONTINLL program with the reference set 10 (Fig. 4d) (which includes 43 soluble proteins and 13 membrane proteins [18]) and with the reference set 4 (which contains 43 soluble proteins [12]).

Other results listed in Table 1 were obtained for reference sets 9 (37 soluble proteins and 13 membrane proteins), 3 (37 soluble proteins), 6 (42 soluble proteins with unordered structure), 7 (48 soluble proteins with unordered structure), 8 (variable number of proteins with known tertiary structure) and 1 (29 soluble proteins) [12].

In order to obtain information on the super-secondary (tertiary) structure of ROF2, we analyzed its far-UV CD spectrum using the CLUSTER program from the CDPro package, which creates a reference set of specific proteins with known tertiary structure. The obtained result, “All β ”, indicates that the super-secondary structure of ROF2 consists in domains formed by β -strands arranged in a predominantly antiparallel manner [23]. It is known that these β -strands form two β -sheets packed against each other, which determine a barrel-like structure. Inside the barrel, the structure presents a hydrophobic core, formed by residues with hydrophobic side-chains [23].

4.4 Thermostability of the ROF 2 protein

As shown in Fig. 5a, the molar ellipticity per residue slightly increases as the temperature rises to 55 °C and decreases as the temperature is raised further, showing that, as the temperature rises, the ROF2 protein suffers two transitions. The first transition starts from the native (folded) state and leads to a denatured, metastable (partially unfolded) state. This transition starts at about 35 °C and has its middle point at $T_{m1} = 45$ °C. The protein’s denaturation reaches a maximum in the metastable state, at about 55 °C. The second transition, which starts from the metastable state, has its middle point temperature $T_{m2} = 60$ °C. The decrease of the molar ellipticity (Fig. 5a) suggests that, as the temperature rises above T_{m2} , thermal refolding leads to a new secondary structure, which is stable up to 97 °C and remains unchanged while the system is cooled down to 5 °C.

Comparing the CD spectrum of a ROF2 solution that has been heated up to 97 °C and, subsequently, cooled down to 5 °C (black line in Fig. 5b) with the spectrum of a ROF2 solution of native structure at 5 °C (gray line on Fig. 5b), we observe that the two spectra are almost similar, suggesting that the new secondary structure that emerges by thermal refolding is slightly different from the native structure.

Several factors may be responsible for this behavior. First, the high number of hydrophobic amino acid residues in the ROF2 protein tends to reduce the accessible surface area of the protein. This tendency may overcome the effect of charged or polar amino acid residues, which tend to hydrate the protein surface by interacting with water dipoles. If hydrophobic interactions stabilize the protein, it remains compact even if its inner C–N terminal bindings are broken due to heating [24, 25]. Second, the protein could be stabilized by its poly(Pro)II helices, which are hydrophilic structures with no internal hydrogen bonds, capable to form hydrogen bonds with water. Third, since ROF2 is a member of the peptidyl-prolyl *cis-trans* isomerase class of proteins, its structure might be stabilized by its own activity (by catalyzing the *cis-trans* isomerization of the peptide bonds of proline [26]) and by its chaperone-like function (the capability to bind to other proteins, favoring their folding and preventing their denaturation [27, 28]). Peptidyl-prolyl isomerase proteins can accelerate the folding of other proteins in vitro [27, 28]. Moreover, it is also known that the folding of peptidyl-prolyl isomerase proteins can be autocatalytic [29]. So, the recovery of ROF2’s structure in spite of an increase in temperature could be favored by a tendency of ROF2 proteins to associate in vitro, thereby assuring proper conditions for autocatalytic activity.

The remarkable thermostability of ROF2 (Fig. 5a) might contribute to the long-term acquired thermotolerance of *Arabidopsis thaliana* [6, 7, 30].

4.5 Tertiary structure of the ROF2 protein

In order to investigate the tertiary structure of ROF2, we also analyzed the near-UV CD spectrum of the protein (Fig. 6).

The aromatic moiety of isolated Trp, Tyr, and Phe molecules is not chiral, so that the CD signal produced by these amino acids is negligible. Only if they are bound to environmental chiral molecules, forming together with them local chiral complexes, they can induce an observable CD signal [13]. For folded proteins, there exists a great variety of chiral environments, which stabilize the protein's tertiary structure. The most important interactions that can lead to such a complex formation in proteins are hydrogen bonds and electrostatic forces [20, 21]. Thus, in the near-UV spectral region, the CD signal is mainly given by aromatic amino acid (Trp, Tyr, and Phe) side chains, bound into local chiral complexes [13, 20, 21].

The high intensity of the CD signal obtained for a 6.2 μM ROF2 solution (Fig. 6, black curve) indicates that, at this concentration, most of the Tyr and Trp amino acids present in the ROF2 sequence are bound into chiral complexes.

The spectrum of the ROF2 protein with a higher concentration of 62 μM (Fig. 6) indicates that, at high-protein concentrations, the local environments of the aromatic amino acids change. This concentration-dependent shape of the near-UV spectrum suggests that, at high concentrations, the ROF2 protein molecules might associate, forming aggregates that contain a smaller number of aromatic amino acid side chains bound into chiral complexes. This hypothesis is supported also by our CD study of the thermal denaturation of ROF2 discussed in the previous subsection. Nevertheless, these experiments should be treated as preliminary evidence that aggregation might take place in a ROF2 solution.

5 Conclusions

Using CD spectroscopy, combined with a computational analysis by the DICHROWEB online server and the CDPro package, we investigated the structure of the ROF2 protein from the plant *Arabidopsis thaliana*.

By using DICHROWEB and CDPro, we performed a computational analysis of the far-UV CD spectra of ROF2 to obtain quantitative estimates of its secondary structure. The analysis revealed that a high percentage of β -sheets, unordered structures, turns, poly(Pro)II-helices and a small percentage of α -helices are present in the secondary structure of ROF2 (Table 1).

In CD studies performed at different temperatures, we observed that, due to heating, the secondary structure of ROF2 suffers two transitions. Thermal denaturation starts at about 35 $^{\circ}\text{C}$, and reaches a maximum at about 55 $^{\circ}\text{C}$, whereas around 65 $^{\circ}\text{C}$ the protein adopts a new, slightly different secondary structure, which remains stable as the system is heated up to 97 $^{\circ}\text{C}$ and cooled down to 5 $^{\circ}\text{C}$. The mechanisms responsible for the thermal refolding of ROF2 represent an interesting topic for future computational studies.

The near-UV CD spectra suggested that in the native state of ROF2 a significant amount of aromatic amino acids are bound in chiral complexes, which stabilize the tertiary structure of the protein. The near-UV CD spectra pointed out a dramatic change in the tertiary structure of ROF2 at high concentrations, a result that might be explained by protein association.

Both the concentration-dependence of the near-UV CD spectrum and the thermal refolding of ROF2 at high temperatures are consistent with the hypothesis that ROF2

molecules tend to associate in vitro, forming aggregates. Further studies are needed to prove this hypothesis.

The stability of the secondary structure of ROF2 at high temperatures could play an important role in the thermotolerance of the plant *Arabidopsis thaliana*.

Acknowledgements These studies were partially supported by a fellowship awarded to L.L. by the Federation of European Biochemical Societies (FEBS). Part of the work was supported by CNCIS-UEFISCSU, project number PNII – IDEI code ID 76/2010. During this work, L.L. benefitted significantly from the hospitality and guidance of the late Dr. Constantin Crăescu from the Curie Institute INSERM U759, Paris, France. We dedicate this work to his memory.

References

- Geisler, M., Bailly, A.: Tête-à-tête: the function of FKBP in plant development. *Trends Plant Sci.* **12**(10), 465–473 (2007)
- Harrar, Y., Bellini, C., Faure, J.-D.: FKBP: at the crossroads of folding and transduction. *Trends Plant Sci.* **6**(9), 426–431 (2001)
- Magiri, E.N., Farchi-Pisanty, O., Avni, A., Breiman, A.: The expression of the large rice FK506 binding proteins (FKBPs) demonstrates tissue specificity and heat stress responsiveness. *Plant Sci.* **170**(4), 695–704 (2006)
- Bissoli, G., Niñoles, R., Fresquet, S., Palombieri, S., Bueso, E., Rubio, L., García-Sánchez, M.J., Fernández, J.A., Mulet, J.M., Serrano, R.: Peptidyl-prolyl *cis-trans* isomerase ROF2 modulates intracellular pH homeostasis in *Arabidopsis*. *Plant J.* **70**(4), 704–716 (2012)
- Aviezer-Hagai, K., Skovorodnikova, J., Galigniana, M., Farchi-Pisanty, O., Maayan, E., Bocovza, S., Efrat, Y., Koskull-Döring, P., Ohad, N., Breiman, A.: *Arabidopsis* immunophilins ROF1 (AtFKBP62) and ROF2 (AtFKBP65) exhibit tissue specificity, are heat-stress induced, and bind HSP90. *Plant Mol. Biol.* **63**(2), 237–255 (2007)
- Meiri, D., Tazat, K., Cohen-Peer, R., Farchi-Pisanty, O., Aviezer-Hagai, K., Avni, A., Breiman, A.: Involvement of *Arabidopsis* ROF2 (FKBP65) in thermotolerance. *Plant Mol. Biol.* **72**(1–2), 191–203 (2010)
- Meiri, D., Breiman, A.: *Arabidopsis* ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs. *Plant J.* **59**(3), 387–399 (2009)
- Kotak, S., Larkindale, J., Lee, U., von Koskull-Döring, P., Vierling, E., Scharf, K.-D.: Complexity of the heat stress response in plants. *Curr. Opin. Plant Biol.* **10**(3), 310–316 (2007)
- Schöffl, F., Prändl, R., Reindl, A.: Regulation of the heat-shock response. *Plant Physiol.* **117**, 1135–1141 (1998)
- Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D., Koornneef, M.: *Arabidopsis thaliana*: a model plant for genome analysis. *Science* **282**, 662–682 (1998)
- Whitmore, L., Wallace, B.A.: DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res.* **32**(suppl 2), W668–W673 (2004)
- Sreerama, N., Woody, R.W.: Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal. Biochem.* **287**(2), 252–260 (2000)
- Serdyuk, I.N., Zaccari, N.R., Zaccari, J.: *Methods in Molecular Biophysics*. Cambridge University Press, Cambridge (2007)
- Wallace, B.A.: Protein characterisation by synchrotron radiation circular dichroism spectroscopy. *Q. Rev. Biophys.* **42**, 317–370 (2009)
- Lees, J.G., Miles, A.J., Wien, F., Wallace, B.A.: A reference database for circular dichroism spectroscopy covering fold and secondary structure space. *Bioinformatics* **22**(16), 1955–1962 (2006)
- Johnson, W.C.: Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins* **35**(3), 307–312 (1999)
- Sreerama, N., Woody, R.W.: Poly(pro)II helices in globular proteins: identification and circular dichroic analysis. *Biochemistry* **33**(33), 10022–10025 (1994)
- Sreerama, N., Woody, R.W.: On the analysis of membrane protein circular dichroism spectra. *Protein Sci.* **13**(1), 100–112 (2004)
- Wetlaufer, D.B.: Ultraviolet spectra of proteins and amino acids. In: Anfinsen, C.B., Bailey, K., Anson, M.L., Edsall, J.T. (eds.) *Advances in Protein Chemistry*, vol. 17, pp. 303–390. Academic Press (1962)

20. Kelly, S.M., Jess, T.J., Price, N.C.: How to study proteins by circular dichroism. *BBA - Proteins Proteom.* **1751**(2), 119–139 (2005)
21. Kelly, S.M., Price, N.C.: The use of circular dichroism in the investigation of protein structure and function. *Curr. Protein Pept. Sci.* **1**, 349–384 (2000)
22. Whitmore, L., Wallace, B.A.: Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. *Biopolymers* **89**(5), 392–400 (2008)
23. Branden, C., Tooze, J.: *Introduction to Protein Structure*, 2nd edn, pp. 67–88. Garland, New York (1999)
24. Duan, J., Nilsson, L.: Thermal unfolding simulations of a multimeric protein—transition state and unfolding pathways. *Proteins* **59**(2), 170–182 (2005)
25. Sanchez-Ruiz, J.M.: Protein kinetic stability. *Biophys. Chem.* **148**(1–3), 1–15 (2010)
26. Fischer, G., Tradler, T., Zarnt, T.: The mode of action of peptidyl prolyl *cis/trans* isomerases in vivo: binding vs. catalysis. *FEBS Lett.* **426**(1), 17–20 (1998)
27. Fischer, G., Schmid, F.X.: The mechanism of protein folding. Implications of in vitro refolding models for de novo protein folding and translocation in the cell. *Biochemistry* **29**(9), 2205–2212 (1990)
28. Kern, G., Kern, D., Schmid, F.X., Fischer, G.: Reassessment of the putative chaperone function of prolyl-*cis/trans*-isomerases. *FEBS Lett.* **348**(2), 145–148 (1994)
29. Veeraraghavan, S., Holzman, T.F., Nall, B.T.: Autocatalyzed protein folding. *Biochemistry* **35**(33), 10601–10607 (1996)
30. Song, L., Jiang, Y., Zhao, H., Hou, M.: Acquired thermotolerance in plants. *Plant Cell, Tissue and Organ Cult. (PCTOC)* **111**(3), 265–276 (2012)