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Structure and Stability of a Rat Odorant-Binding Protein: Another Brick in the Wall

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The effect of temperature on the structure of the rat odorant-binding protein was investigated by spectroscopic and *in silico* methodologies. In particular, in this work, we examined the structural features of the rat OBP-1F by Fourier-transform infrared spectroscopy and molecular dynamics investigations. The obtained spectroscopic results were analyzed using the following three different methods based on the unexchanged amide hydrogens of the protein sample: (1) the analysis of difference spectra; (2) the generalized 2D-IR correlation spectroscopy; (3) the phase diagram method. The three methods indicated that at high temperatures the rOBP-1F structure undergoes a relaxation process involving the protein tertiary organization before undergoing the denaturation and aggregation processes, suggesting the presence of an intermediate state such as a molten globule-like state. Importantly, the proposed analyses represent a general approach that could be applied to the study of protein stability.

Keywords: Odorant-binding protein • Fourier transform infrared spectroscopy • Lipocalin

Introduction

To reach their membrane receptors embedded in the membrane of olfactory neurons, airborne odorants, which are commonly hydrophobic molecules, have to be conveyed through the aqueous nasal mucus. The odorant-binding proteins (OBPs), which are abundant low-molecular-weight (around 20 kDa) soluble proteins secreted by the olfactory epithelium in the nasal mucus of vertebrates, are candidates for playing such a carrier role.¹ These proteins reversibly bind odorants with dissociation constants in the micromolar range. Vertebrate OBPs belong to the lipocalin superfamily.^{2,3} Although members of this family display low sequence similarity (usually lower than 20% amino acid identity), all share a conserved folding pattern, an 8-stranded β -barrel flanked by an α -helix at the C-terminal end of the polypeptide chain. The β -barrel defines a central apolar cavity, called calix, whose role is to bind and transport hydrophobic odorant molecules.^{4–6} OBPs have been identified in a variety of species, including cow, pig, rabbit, mouse, rat and humans.^{3,7,8} Different OBP subtypes have been reported to simultaneously occur in the same animal species. Although no preferential binding was observed with the porcine and bovine OBPs, a broad specificity was revealed by the study of three rat OBPs, which are specially tuned toward distinct

chemical classes of odorants. Rat OBP-1 preferentially binds heterocyclic compounds such as pyrazine derivatives.^{9,10} OBP-2 appears to be more specific for long-chain aliphatic aldehydes and carboxylic acids,⁹ whereas OBP-3 was described to interact strongly with odorants composed of saturated or unsaturated ring structure.¹¹

Here, we examined the structural features of the rat OBP-1F (rOBP-1F) by Fourier-transform infrared (FT-IR) spectroscopy and *in silico* investigations. rOBP-1F is a recombinant protein expressed in *Pichia pastoris* in a form identical to the natural protein.¹⁰

The obtained spectroscopic results were analyzed by using three different methods based on the unexchanged amide hydrogens of the protein sample: (1) the analysis of difference spectra; (2) the generalized 2D-IR correlation spectroscopy; (3) the phase diagram method. The results obtained from the different analysis methods are discussed together with a bioinformatics investigation of the protein structure and dynamics.

Experimental Section

Materials. Deuterium oxide (99.9% $^2\text{H}_2\text{O}$), ^2HCl , NaO^2H , and deuterated ethanol (EtO^2H) were purchased from Aldrich (Sigma-Aldrich S.r.l., Milan, Italy). 2-Isobutyl-3-methoxy pyrazine (IBMP) was obtained from Sigma. All the other chemicals were commercial samples of the purest quality.

Protein Expression and Purification. Recombinant rOBP-1F was produced using the yeast *P. pastoris* according to Briand et al.¹⁰ Briefly, the supernatant containing recombinant protein was dialyzed for 4 days at 4 °C, using a dialysis tube with 12 000

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Da cutoff (Servapor, Polylabo, France) and lyophilized. The lyophilized supernatant was resuspended in eluent A (20 mM Tris-HCl pH 8.0) and purified by anion-exchange chromatography using a 5-mL HiTrap Q HP column (GE Healthcare) equilibrated with the same eluent. After sample loading, the column was extensively washed with eluent A and protein eluted using a linear gradient from 0 to 0.5 M NaCl. The flow rate was 1 mL·min⁻¹ and the absorbance was recorded at 275 nm. The fractions containing purified protein were pooled, dialyzed and used for the experiments. The purity of the protein sample was checked by SDS-PAGE.

Infrared Spectroscopy. FT-IR spectra were recorded by means of a 1760-X Perkin-Elmer Fourier-transform infrared spectrometer using a deuterated triglycine sulfate detector and a normal Beer-Norton apodization function. Twenty-four hours before the experiments and during data acquisition, the spectrometer was continuously purged with dry air at a dew point of -70 °C. Prior to FT-IR spectra recording, the protein samples were equilibrated in a deuterium oxide (²H₂O) medium. Typically, 1.5 mg of rOBP-1F was dissolved in the buffer used for its purification, centrifuged in a "10 K Centricon" microconcentrator (Amicon) at 3000g and at 4 °C, and concentrated into a volume of approximately 40 µL. Then, 300 µL of 50 mM Tris/²HCl buffer, prepared in ²H₂O p²H 7.4, was added and the sample was concentrated again. The p²H value corresponds to the pH meter reading +0.4.¹² The concentration and dilution procedure was repeated several times in order to completely replace the original buffer with the Tris buffer p²H 7.4. In the last wash, the sample was brought to a volume of approximately 40 µL and then used for the infrared analysis. To study the effect of IBMP on rOBP-1F structure, 0.5 µL of a EtO²H odorant solution was added to 40 µL of the concentrated protein solution, obtaining a 3:1 ligand/protein molar ratio. The protein samples were placed in a thermostatted Graseby Specac 20500 cell (Graseby Specac, Orpington, Kent, U.K.) fitted with CaF₂ windows and a 25-µm Teflon spacer. Spectra of buffer were acquired under the same scanning and temperature conditions.

In the thermal-denaturation experiments, the temperature was raised by 5 °C steps from 20 to 95 °C. Before spectra acquisition, samples were maintained at the desired temperature for the necessary time for stabilization of the cell temperature (6 min). Spectra were processed using the SPECTRUM software from Perkin-Elmer. Subtraction of ²H₂O was adjusted to the removal of the ²H₂O bending absorption close to 1220 cm⁻¹.¹³ Second derivative spectra were calculated over a 9 data-point range (9 cm⁻¹). The deconvoluted parameters were set with a gamma value of 2.5 and a smoothing length of 60.

2D-IR Correlation Spectroscopy. Generalized 2D-IR analysis of IR band intensities of absorbance spectra was performed according to the method of Noda.¹⁴ To obtain synchronous and asynchronous plots, 2Dshige program (Shigeaki Morita, Kwansei-Gakuin University, 2004–2005) was used.^{15,16} Synchronous plots of rOBP-1F, covering 50–70, 70–95, or 50–95 °C temperature ranges were generated. For rOBP-1F in the presence of IBMP (rOBP-1F/IBMP), synchronous plots were generated from spectra in the 50–80, 80–95, or 50–95 °C temperature ranges. Asynchronous maps were obtained by the analysis of the IR absorbance spectra in the 50–95 °C temperature range for both rOBP-1F and rOBP-1F/IBMP. These temperature ranges allowed us to better describe the thermal unfolding events.¹⁷

Phase Diagram. The phase diagram method is a sensitive approach for the detection of unfolding/refolding intermediates of proteins. The essence of this method is to build up the diagram of $I(\lambda_1)$ versus $I(\lambda_2)$ dependence, where $I(\lambda_1)$ and $I(\lambda_2)$ are the spectral intensity values (e.g., fluorescence, CD, FTIR, absorbance, etc.) measured on wavelengths λ_1 and λ_2 , under different experimental conditions for a protein undergoing structural transformations.¹⁸ It is important to note that only *extensive* parameters (i.e., those parameters whose value is proportional to the amount of the analyzed matter in a system) should be used for such an analysis, whereas *intensive* parameters, characterizing system qualitatively, should be excluded from the consideration. In application to protein unfolding, the dependence $I(\lambda_1) = f(I(\lambda_2))$ will be linear if changes in protein environment lead to the all-or-none transition between two different conformations. On the contrary, the nonlinearity of this function reflects the sequential character of structural transformations.

Molecular Dynamics Simulations. The coordinates of the crystallographic structure of rOBP-1F were used to perform molecular dynamics (MD) simulations of the protein in water at increasing temperature. Since the file contains two monomeric proteins, not interacting with each others, simulations were carried out only on the first monomer, using the program GROMACS version 3.3.1^{19,20} running in parallel (MPI) on a cluster with 40 × 86_64 Opteron processors. The GROMOS96 force field²¹ was used throughout the simulations. A cubic box, containing 8592 water molecules (SPC model²²) and 10 Na⁺ ions to neutralize the net negative charge of the protein, was used to solvate rOBP-1F. Periodic boundary conditions were used to exclude surface effects. A preliminary energy minimization step with a tolerance of 500 kJ/(mol/nm) was run with the Steepest Descent method. All bonds were constrained using LINCS.²³ After minimization, a 20 ps-long MD simulation with position restraints was applied to equilibrate the water molecules around the macromolecule. Time step was set to 2 fs, and the system was coupled with a temperature bath at 27 °C using Berendsen's method.²⁴ Long-range electrostatics were handled using the PME method.²⁵ Cutoffs were set at 0.9 nm for Coulomb interactions, and at 1.4 nm for van der Waals interactions. The final MD simulations were carried out with the same settings, but without any position restraints and using NPT ensemble. Five subsequent MD simulations of 1 ns each (the final conformation of each simulation was used as input for the following simulation at higher temperature) were carried out at 27, 60, 75, 80, and 95 °C, at a constant pressure of 1 bar, with the Berendsen's method of bath coupling.²⁴ A final simulation at 100 °C was 5 ns long, for a global duration of 10 ns.

The analyses on the trajectory were conducted using programs built within GROMACS, and results were visualized and elaborated with the aid of the freely available program Grace (<http://plasma-gate.weizmann.ac.il/Grace>). The energy components were extracted from the energy files generated by the program, and analyzed to verify the stabilization of the system. The root-mean-square fluctuation (RMSF) values were obtained from a least-squares fit of the respective non-hydrogen atoms. For each temperature step, an analysis was made to identify the clusters of structures obtained, using a cutoff of 0.1 nm rmsd, and an "average" structure of the protein was calculated on the whole protein minus hydrogen atoms, for each cluster. These structures were saved in pdb format, and they were subsequently minimized with the Steepest Descent method as

Figure 1. Absorbance (A), deconvoluted (B) and second derivative (C) spectra of rOBP-1F. Spectra were obtained at 20 °C and p²H 7.4.

described above. The analysis of the variation of secondary structure elements was made using the DSSP program.²⁶ Other analyses and visualization of molecules were carried out with the Insight II package (Version 2000.1, Accelrys, Inc.; 2000).

Results and Discussion

Secondary Structure. Figure 1 shows the absorbance (A), deconvoluted (B) and second derivative (C) spectra of rOBP-1F. The resolution enhanced spectra (B and C) show the presence of amide I' (1700–1600 cm⁻¹ region) component bands allowing the description of the protein secondary structure.²⁷ The intense band at 1634 cm⁻¹ and the small band at 1648.9 cm⁻¹ indicate the predominance of β -structure with a minor content of α -helices, respectively. The 1666 cm⁻¹ band may be assigned to turns, while the other minor bands in the 1690–1670 cm⁻¹ region may be due to β -sheet and/or turns.²⁸ The bands below 1620 cm⁻¹ are due to amino acid side chain absorption.^{29,30} In particular, the 1515 cm⁻¹ band is due to tyrosine residues, whereas the 1584 and 1565 cm⁻¹ bands are due to ionized carboxyl groups of aspartic and glutamic acid residues, respectively.^{29,30} The absorbance and resolution-enhanced spectra of rOBP-1F/IBMP (not shown) do not show significant differences in the amide I' region as compared to the rOBP-1F spectra, indicating that the binding of the odorant molecule does not affect the secondary structure of the protein. A 1544 cm⁻¹ band visible in the second derivative spectrum of rOBP-1F/IBMP (see Figure 2) is due to the odorant molecule. The presence of this band does not influence the amide I' band.

Thermal Stability and Thermal Unfolding. Figure 2 displays the second derivative spectra of rOBP-1F and of rOBP-1F/IBMP in the range of temperatures where conformational changes occurred. In particular, panels A and B of Figure 2 show spectra in the 50–85 °C and in the 55–90 °C range, respectively. Spectra in the 20–50 °C temperature interval do not show significant differences (data not shown). The spectra of rOBP-1F (A) indicate that the protein is stable until 70 °C since the main β -sheet band intensity is scarcely affected by the temperature and the spectrum is similar to the spectrum obtained at 50 °C. At 80 °C, the intensity of the main β -sheet band decreases significantly and a new band at 1617 cm⁻¹ appears, indicating thermal denaturation (loss of secondary structure) and protein

Figure 2. Second derivative spectra of rOBP-1F (A) and of rOBP-1F/IBMP (B) as a function of the temperature. Panels A and B show spectra in the 50–85 °C and in the 55–90 °C range, respectively.

aggregation, respectively.³¹ The analysis of rOBP-1F/IBMP spectra (Figure 2B) indicates that the protein is more thermostable in the presence of the odorant molecule since both the decrease in intensity of the main β -sheet band and the appearance of the new band at 1617 cm⁻¹ start occurring at 85 °C. The higher thermostability of the protein in the presence of the odorant is not surprising since binding of a molecule to a protein usually increases its stability as we already showed for the porcine odorant-binding protein.³² A more detailed analysis of the spectra of rOBP-1F in the 60–70 °C temperature interval shows a downshift in wavenumber of the main β -sheet band and of the 1685.8 cm⁻¹ band. On the other hand, the spectra of rOBP-1F/IBMP indicate that the main β -sheet band shifts significantly to a lower wavenumber in the 70–80 °C temperature range. These changes in the rOBP-1F and rOBP-1F/IBMP spectra may indicate that, before denaturation (loss of secondary structure) and aggregation, the increase in temperature induces some conformational changes in the protein that may represent the formation of an intermediate structural state. To check this possibility, we applied three methods to analyze the temperature-induced protein unfolding: (a) analysis of difference spectra,^{32–34} (b) generalized 2D-IR correlation spectroscopy (2D-IRCOS),^{16,33} and (c) phase diagram method.^{18,34}

Difference Spectra. Figure 3 shows the difference spectra from 50 to 95 °C. These spectra were obtained from the series

Figure 3. Difference spectra of rOBP-1F (A) and of rOBP-1F/IBMP (B) in the 50–95 °C temperature range. Each trace (difference spectrum) was obtained by subtracting the original absorbance spectrum recorded at the lower temperature from the one recorded at higher temperature (e.g., 85–80 °C difference spectrum corresponds to the spectrum recorded at 85 °C, after subtraction of the spectrum recorded at 80 °C).

of absorbance spectra recorded at stepwise increasing temperatures, by subtracting each one of them from the spectrum recorded at the next higher temperature (e.g., the 85 °C – 80 °C difference spectrum corresponds to the spectrum recorded at 85 °C, after subtraction of the spectrum recorded at 80 °C). Both negative and positive peaks reflect the total changes in a particular band which is present in the two IR spectra to be subtracted, and their characteristics depend on several factors.³⁵ Negative bands in the amide I' region reflect protein denaturation (loss of secondary structure)³³ or band-shifts in case that positive adjacent bands of similar intensity are also present.³² It must be pointed out that band-shift to lower wavenumbers is caused by further $^1\text{H}/^2\text{H}$ exchange, which can be visualized by the decrease in intensity of amide II band.^{32–34} Indeed, the intensity of amide II band (1600–1500 cm^{-1} spectral range) is particularly sensitive to $^1\text{H}/^2\text{H}$ exchange: the larger the extent of $^1\text{H}/^2\text{H}$ exchange, the larger the decrease in intensity of amide II band.³⁶ It is also important to note that proteins at room temperature in $^2\text{H}_2\text{O}$ medium do not exchange completely the amide hydrogen with deuterium and that complete $^1\text{H}/^2\text{H}$ exchange can be reached at high temperatures.³⁷

Band-shift can be observed in (60–55 °C), (65–60 °C) and (70–65 °C) difference spectra (panel A) and in (75–70 °C) and (80–75 °C) difference spectra (panel B). In fact, a 1637 cm^{-1} negative band and a 1625 cm^{-1} positive band of similar intensity are found within the above-mentioned temperature ranges. Since the band at 1637 cm^{-1} belongs to β -sheet, the band-shift to 1625 cm^{-1} indicates that the β -sheets became more exposed to the solvent ($^2\text{H}_2\text{O}$), allowing the protein to exchange residual amide hydrogens that were not exchanged during the preparation of protein sample in $^2\text{H}_2\text{O}$ buffer.^{32–34} In fact, further $^1\text{H}/^2\text{H}$ exchange is supported by the appearance of a negative broadband close to 1550 cm^{-1} due to a decrease in intensity of the residual amide II band. These findings are in agreement with the shift of β -sheets observed in Figure 2.

In summary, the difference spectra indicate that before protein denaturation there are not significant changes in the secondary structure of rOBP-1F and rOBP-1F/IBMP (see also Figure 2), whereas the tertiary structures of the proteins undergo a relaxation process with an additional $^1\text{H}/^2\text{H}$ exchange. This phenomenon could describe a protein less-folded state in which the secondary structural elements of the protein are still preserved. We might associate this protein less-folded state with the presence of molten globule-like state.³⁸

2D-IR Correlation Spectroscopy. Figure 4 shows synchronous (A–F) and asynchronous (G and H) spectra of dynamic spectral intensity variations induced by the increase in temperature for rOBP-1F (left column) and rOBP-1F/IBMP (right column). Synchronous maps of rOBP-1F (A, C, and E) were obtained analyzing the IR absorbance spectra in the 50–70 °C, 70–95 °C, and 50–95 °C temperature ranges, respectively. Synchronous maps of rOBP-1F/IBMP (B, D, and F) were obtained analyzing the IR absorbance spectra in the 50–80 °C, 80–95 °C, and 50–95 °C temperature ranges, respectively. This separation of IR spectra in different temperature sets and in a specific spectral range was done because it allows a more detailed description of the spectral events.¹⁷ Asynchronous maps (G and H) were obtained analyzing the IR absorbance spectra in the 50–95 °C temperature range. Multiple lines in the spots reflect the intensity of the peaks. Gray and white spots represent negative and positive peaks, respectively.

A synchronous spectrum represents the simultaneous or coincidental changes of spectral intensities measured at two discrete and independent wavenumbers ν_1 and ν_2 (on x - and y -axes, respectively). An asynchronous spectrum represents sequential or unsynchronized changes of spectral intensities measured at ν_1 and ν_2 .^{14,16} Auto-peaks are present only on the diagonal of a synchronous map; these peaks represent the main changes in spectral intensity as a consequence of an external perturbation. Cross-peaks are present in either synchronous or asynchronous maps at the off-diagonal positions and they can be positive or negative. The sign of synchronous cross-peaks becomes positive if the spectral intensities at corresponding wavenumbers are either increasing or decreasing together. Negative synchronous cross-peaks indicate that one of the spectral intensities is increasing while the other is decreasing. The sign of asynchronous cross-peaks becomes positive if the intensity change at ν_1 occurs predominantly before ν_2 . On the other hand, it becomes negative if the change occurs after ν_2 . This rule is reversed if the synchronous cross-peak at ν_1 and ν_2 is negative. The combination of synchronous and asynchronous plots provides useful information on the sequential order of the events following a perturbation on protein structure.^{14,16}

Figure 4. Synchronous and asynchronous spectra for rOBP-1F and rOBP-1F/IBMP in the 1650–1500 cm^{-1} spectral range. Left and right columns refer to rOBP-1F and to rOBP-1F/IBMP, respectively. Synchronous spectra, maps (A–F); asynchronous spectra, maps (G and H). Synchronous maps (A, C, and E) were obtained analyzing the IR absorbance spectra in the 50–70, 70–95, and 50–95 $^{\circ}\text{C}$ temperature ranges, respectively. Synchronous maps (B, D, and F) were obtained analyzing the IR absorbance spectra in the 50–80, 80–95, and 50–95 $^{\circ}\text{C}$ temperature ranges, respectively. Asynchronous maps (G and H) were obtained analyzing the IR absorbance spectra in the 50–95 $^{\circ}\text{C}$ temperature range. Gray and white spots represent negative and positive peaks, respectively. Multiple lines in the spots reflect the intensity of the peaks.

Table 1 summarizes the information contained in Figure 4. In particular, in map (A) the auto-peaks (1 and 2) represent the shift of the 1637 cm^{-1} band to 1625 cm^{-1} . The auto-peak (3) represents the decrease in intensity of the residual amide II band at 1551 cm^{-1} ($^1\text{H}/^2\text{H}$ exchange) that occurs concomi-

tantly to the shift. The positive cross-peak (4) (1551, 1637) indicates that the 1637 cm^{-1} and the 1551 cm^{-1} bands intensities both decrease. The negative cross-peak (5) (1551, 1625) indicates that the 1625 cm^{-1} band intensity increases while the 1551 cm^{-1} band intensity decreases. Similarly, the negative

Table 1. Data Obtained from Synchronous and Asynchronous Maps Reported in Figure 4^a

rOBP-1F			rOBP-1F/IBMP		
map	auto-peaks (cm ⁻¹)	cross-peaks at ν_1 , ν_2 (cm ⁻¹)	map	auto-peaks (cm ⁻¹)	cross-peaks at ν_1 , ν_2 (cm ⁻¹)
Synchronous Maps					
A	1 (1637) ^a	4 (1551 ^{↓b} , 1637 ^{↓a})+	B	1 (1637) ^a	4 (1551 ^{↓b} , 1637 ^{↓a})+
50–70 °C	2 (1625) ^a	5 (1551 ^{↓b} , 1625 ^{↑a})–	50–80 °C	2 (1625) ^a	5 (1551 ^{↓b} , 1625 ^{↑a})–
	3 (1551) ^b	6 (1625 ^{↑a} , 1637 ^{↓a})–		3 (1551) ^b	6 (1625 ^{↑a} , 1637 ^{↓a})–
C	1 (1632) ^c	3 (1617 ^{↑d} , 1632 ^{↓c})–	D	1 (1632) ^c	3 (1617 ^{↑d} , 1632 ^{↓c})–
70–95 °C	2 (1617) ^d		80–95 °C	2 (1617) ^d	
E	1 (1632) ^c	4 (1556 ^{↓b} , 1632 ^{↓c})+	F	1 (1632) ^c	4 (1556 ^{↓b} , 1632 ^{↓c})+
50–95 °C	2 (1617) ^d	5 (1556 ^{↓b} , 1617 ^{↑d})–	50–95 °C	2 (1617) ^d	5 (1556 ^{↓b} , 1617 ^{↑d})–
	3 (1556) ^b	6 (1617 ^{↑d} , 1632 ^{↓c})–		3 (1556) ^b	6 (1617 ^{↑d} , 1632 ^{↓c})–
Asynchronous Maps					
G	1 (1617 ^d , 1625 ^a)–		H	1 (1617 ^d , 1625 ^a)–	
50–95 °C	2 (1617 ^d , 1637 ^a)+		50–95 °C	2 (1617 ^d , 1637 ^a)+	
	3 (1550 ^b , 1617 ^d)–			3 (1550 ^b , 1617 ^d)–	
	4 (1550 ^b , 1632 ^c)+			4 (1550 ^b , 1632 ^c)+	

^a (a) Peaks involved in β -sheet band shift; (b) ¹H/²H exchange; (c) denaturation; (d) aggregation. The arrows ↑ and ↓ indicate the increase and decrease of peak intensity, respectively. (+) and (–) represent positive and negative peaks, respectively.

cross-peak (6) (1625, 1637) indicates that the 1625 cm⁻¹ band intensity increases while the 1637 cm⁻¹ band intensity decreases. Map (B) of rOBP-1F/IBMP describes the same phenomena, that is, band shift and ¹H/²H exchange, occurring in a different temperature range (50–80 °C) with respect to map (A) (50–70 °C). The auto-peaks (1 and 2) in the map (C) and map (D) are due to denaturation and aggregation, respectively, and the negative cross-peak (3) (1617, 1632) indicates that the 1632 cm⁻¹ band intensity decreases while the 1617 cm⁻¹ band intensity increases. In maps (E) and (F), the auto-peaks (1), (2), and (3) indicate denaturation, aggregation, and ¹H/²H exchange, respectively. The cross-peaks (4), (5), and (6) indicate the relative changes in intensity occurring at two discrete and independent wave numbers ν_1 and ν_2 .

The asynchronous maps (G) and (H) together with synchronous maps (E) and (F) allowed us to calculate the sequential order of the events occurred upon the increase of temperature up to 95 °C.

In the asynchronous maps, four asynchronous cross-peaks are present. From the sign of each cross-peak and by using the rule previously described, we can state if the intensity change at ν_1 occurs predominantly before ν_2 . Combining the information obtained from all the cross-peaks, we can obtain the sequential order of the events. As an example, the negative asynchronous cross-peak (3) in map (G) indicates that the change in intensity of the 1550 cm⁻¹ band occurs before the change in intensity of the 1617 cm⁻¹ band. In fact, a negative asynchronous cross-peak indicates that the intensity change at ν_1 occurs after ν_2 , but in this case, the rule is reversed because the corresponding synchronous cross-peak at ν_1 and ν_2 is negative (see synchronous cross-peak 5 in map E). The complete sequential order of the heating-induced events and their description is described in Table 2 which indicates that in both rOBP-1F and rOBP-1F/IBMP the increase of temperature causes as a first event the ¹H/²H exchange with a concomitant band shift of the main β -sheet band. After that, the protein undergoes denaturation and aggregation concomitantly.

Hence, the data indicate that before denaturation (loss of secondary structure) and aggregation, the protein assumes an intermediate state characterized by a native-like secondary

structure and by a less-folded state (relaxed tertiary structure) that may be associated to a molten globule-like state.³⁸

Phase Diagram. Figure 5 represents the thermal unfolding of rOBP-1F and rOBP-1F/IBMP as obtained by the phase diagram method.¹⁸ With this approach, we can validate that the temperature-induced unfolding of rOBP-1F and rOBP-1F/IBMP involves intermediate states. The diagram indicates that between the native state (N) and the unfolded state (U) of both rOBP-1F and rOBP-1F/IBMP are present two intermediated states. One of them (MG) corresponds to the molten globule-like state that has been detected by both difference spectra (Figure 3) and by 2D-IR correlation spectroscopy. Indeed, the temperatures at which the MG is detected in rOBP-1F and rOBP-1F/IBMP (70 and 80 °C, respectively) correspond to the higher temperature of existence of the MG as shown by the difference spectra (Figure 3). The other intermediate state, that we have indicated as (N*), is detected at 55 and 70 °C for rOBP-1F and rOBP-1F/IBMP, respectively. At these temperatures, the MG like-state has not been detected by difference spectra, and the secondary structure of the proteins is the same or very similar to that of native protein (see Figure 2). Hence, as the tertiary and secondary structure are the same or very similar to that of native state (N), it is possible to hypothesize that the intermediate state (N*) detected by the phase diagram might represent an excited native state that precedes the MG-like state (MG). Each straight line of the diagram represents an all-or-none transition between two conformers,¹⁸ denoted as N, N*, MG, and U. As the diagrams show, the straight line N* → MG covers the 55–70 °C and the 70–80 °C temperature ranges in panels A and B, respectively, which are the ranges of temperature where a molten globule-like state was suggested to be present by difference spectra (Figure 3).

In conclusion, the three different approaches used to analyze the infrared spectra indicate that, when raising the temperature, rOBP-1F undergoes relaxation in the tertiary structure before undergoing denaturation and aggregation. This behavior indicates the presence of an intermediate state that fits with a molten globule-like state as proposed by Ptitsyn.³⁸

The three methods are based on the unexchanged amide hydrogens of the protein sample. As previously mentioned,

Figure 5. Phase diagram for rOBP-1F (A) and rOBP-1F/IBMP (B). The diagrams were obtained from deconvoluted spectra recorded at different temperatures by plotting, for each spectrum, the intensity at 1631 cm⁻¹ (β -sheet) versus the intensity at 1550 cm⁻¹ (residual amide II band). Each straight line represents an all-or-none transition between two conformers, denoted as N (native), N* (excited native), MG (molten globule), and U (unfolded). Temperature values (°C) at which each spectrum was obtained and at which the conformers are present are indicated in the phase diagrams.

when a protein is dissolved in ²H₂O medium, the hydrogens of solvent-exposed protein backbone are exchanged with deuterium. As a result, the amide II band of the infrared spectrum decreases in intensity. This process, however, is not complete under the conditions used in this work to prepare the protein sample for infrared analysis. Hence, with the rise in temperature, further ¹H/²H exchange takes place and it may be due to (a) increase of molecular dynamics, (b) denaturation (loss of secondary structure), and (c) relaxation of the tertiary structure. In concomitance of the latter two phenomena, a rapid decrease of the amide II band intensity occurs. If no denaturation takes place, a rapid decrease in the amide II band intensity is attributable to a relaxation of the tertiary structure, which is accompanied also by a downshift in wavenumber of the β -sheet band. Hence, the first (difference spectra) and second method (2D-IR correlation spectroscopy), used to detect a molten globule-like state, monitor the concomitant shift of the β -sheet band and the decrease in intensity of the amide II band. The third method (phase diagram) monitors the amide

II band intensity as a function of the intensity of the β -sheet band. Concerning this method, each linear portion of the $I(\lambda_1) = fI(\lambda_2)$ dependence describes the individual all-or-none transition. In principle, λ_1 and λ_2 are arbitrary wavelengths of the spectrum. However, in practice, such diagrams will be more informative if λ_1 and λ_2 are on different slopes of the spectrum. If the wavelengths are from one slope or near the maximum, some transitions may remain undetected.¹⁸ Hence, intermediate state(s) can be detected or missed depending on the parameter chosen to create a phase diagram. Therefore, one should be cautious in interpretation and usually it is advisable to construct several different phase diagrams using different parameters and arrive at a conclusion. Comparing the three methods, one can argue that difference spectra give information on the presence on heating-induced molten globule-like state in a easy and direct way, while the other two methods require more efforts. It must be pointed out, however, that the phase diagram and 2D-IR correlation spectroscopy are powerful tools to study protein conformation and they can give additional information on conformational changes with respect to the difference spectra.

Molecular Dynamics Simulations. The aim of the simulations was to analyze the effect of temperature on structure and dynamics of rOBP-1F at the molecular level. Despite the time scale of the simulation which does not allow the investigation of the complete denaturation process of the protein, it allows us to identify possible early traces of destabilization of the protein structure or clues about the formation of intermediate states at longer time scales.

The analysis of the radius of gyration and of the solvent accessible surface area of the protein at different temperatures shows that these two parameters are quite constant during the entire simulation (data not shown). Therefore, the compactness of rOBP-1F and the exposure of its hydrophobic and hydrophilic residues are not affected during simulations, indicating no dramatic perturbations on the overall structure of rOBP-1F. The analysis with DSSP²⁶ reveals a substantial resistance of the secondary structures of the protein, with only a minimal decrease of the total amount of residues included in some kind of ordered secondary structures and a slight increase of the amount of residues in random coil conformation during the simulations (Figure 6). In particular, the amount of residues included in β -sheet is stable during simulation, whereas the α -helices seem slightly more perturbed by temperature. These results are in agreement with experimental data showing that, before denaturation, there are not significant changes in the secondary structure of this protein, and confirm that also in rOBP-1F, like in bOBP and in pOBP, secondary structures are not largely affected by thermal stress.

The evaluation of the RMSF of the residues in rOBP-1F during the simulation can be used as an indicator of motility of the various segments of the protein. The analysis performed on rOBP-1F indicates that the most mobile zones of the protein correspond to several β -strands composing the central β -sheet, and to the C-terminal portion of the protein. The amplitude of fluctuations of these segments is generally increasing as temperature increases, and this is especially evident for segments 19–22 (in strand S2), 42–45 (in strand S3), 70–72 (in strand S5), 77–86 (strand S6), 90–93 (in strand S7), 100–110 (strand S8 and loop between S8/S9), and 133–141 (the C-terminal part of the long α -helix and of the following loop) (Figure 7). Note that strands S2, S9, S8, and S7 compose the portion of β -barrel nearest to the α -helix in the structure of

Table 2. Sequence of Temperature-Induced Events for rOBP-1F and rOBP-1F/IBMP^a

temperature (°C)	rOBP-1F	rOBP-1F/IBMP
	bands involved in intensity changes (cm ⁻¹)	bands involved in intensity changes (cm ⁻¹)
50–95 °C	A 1637↓ ^a , 1625↑ ^a , 1550↓ ^b (band shift ^a and ¹ H/ ² H exchange ^b)	A 1637↓ ^a , 1625↑ ^a , 1550↓ ^b , (band shift ^a and ¹ H/ ² H exchange ^b)
	B 1632↓ ^c , 1617↑ ^d , (denaturation ^c , aggregation ^d)	B 1632↓ ^c , 1617↑ ^d , (denaturation ^c , aggregation ^d)

^a Symbols (↑) and (↓) mean the increase and the decrease of the band intensity, respectively. The temperature-induced sequence of events for rOBP-1F and rOBP-1F/IBMP is the same: (A) followed by (B). The events reported in (A) or (B) occur concomitantly. (a) Peaks involved in β-sheet band shift; (b) ¹H/²H exchange; (c) denaturation; (d) aggregation.

Figure 6. Analysis of the variation of secondary structures during molecular dynamics simulation. The program DSSP has been applied to evaluate the variation of secondary structure elements during the 10-ns long simulation at 100 °C. Different colors represent different types of secondary structures, as shown in the legend on the right. The black line is the sum of the residue inserted in α helix, β-sheet, β-bridge and turns.

rOBP-1F. Therefore, the segments with the highest fluctuation at higher temperatures are grouped together in the structure of rOBP-1F, and this could suggest that this zone is the core of destabilization of the tertiary structure of the protein.

The analysis of structures during the simulations showed other interesting information. Simulations at lower temperatures (until 75 °C) showed that the different structures composing the trajectory of the protein can be grouped in a single cluster, with a single representative structure. Instead, at higher temperatures, the structures composing the trajectory can be differentiated into several clusters: in particular, at 80 and 95 °C, the structures can be grouped in two distinct clusters with two representative structures, and at 100 °C, it is possible to find at least 10 different clusters of structures. The presence of several different clusters of structures might indicate that several different tertiary organizations of rOBP-1F, with conserved secondary structures, are formed at temperatures above 75 °C. The comparison of the average structures representatives for each cluster, at different temperatures, show that at 80 °C the differences between the two representative structures reside mainly in segments 55–64, 77–83, and 89–96, and at 95 °C, in segment 100–110. At 100 °C, it is possible to note that the

Figure 7. RMSF of the residues of rOBP-1F at different temperatures. Different colors represent different temperatures: black, 27 °C; blue, 60 °C; green, 75 °C; yellow, 80 °C; orange, 95 °C; red, 100 °C. RMSF has been calculated on the whole atoms excluding hydrogens.

major differences between all the representative structures are grouped in segments 77–82 (strand S6), 100–110 (strand S8), and 133–141 (α-helix). Furthermore, three structures representative of three different clusters show additional differences in segments 16–23, 38–51, 65–91, 94–115, and 122–145, creating at least two distinct groups of cluster structures. The comparison with the crystallographic structure of rOBP-1F (Figure 8) shows that at high temperature the tertiary organization of the protein is perturbed, with a significant displacement of the position of the α-helix and of several β-strands. This could represent the evidence for a preliminary phase of formation of a molten globule state, involving the most flexible portions of rOBP-1F structure. It is interesting to compare these data with those obtained in the past for wt-bOBP, for which the evidence of the formation of a molten globule state in analogous experimental conditions was not proved.³⁹ Analyzing the simulations obtained for that protein, for each step of temperature, a single cluster of structures is obtained, with the exception of the last step at 100 °C, when two clusters of structures are found. On the contrary, in the search for clusters of structures in the trajectories of molecular dynamics simulations obtained for the triple deswapped mutant bOBP⁴⁰ for which the presence of the molten globule state was inferred from experimental data, 7 clusters of structures were found at 95 °C in the protein in the absence of ligand, and only one cluster of structures in the protein with ligand bound. This would support the hypothesis that the presence of more

Figure 8. Comparison between the crystallographic structure of rOBP-1F and the average structures representative of ten different clusters obtained at 100 °C. The crystallographic structure of rOBP-1F is represented with a white ribbon; each other color represents a different average structure. Two groups of clusters are visible: one including average structures in green, light green and yellow-green, and another one with all the rest of structures. The representation of secondary structures is referred to the crystallographic structure of rOBP-1F only. α -Helices are represented as red cylinders, and β -strands as yellow arrows.

clusters of structures is a signal of the presence of different tertiary forms of the protein.

Therefore, despite the differences in time scale between the simulation and the formation of partially unfolded or molten globule state, these data can provide an early signal of relaxation of the tertiary structure, preceding the protein denaturation and aggregation processes.

Abbreviations: FT-IR, Fourier-transform infrared spectroscopy; OBP, odorant-binding protein; rOBP-1F, rat odorant-binding protein-1F.

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