THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

Vibrational Circular Dichroism Spectra of Lysozyme Solutions: Solvent Effects on Thermal Denaturation Processes

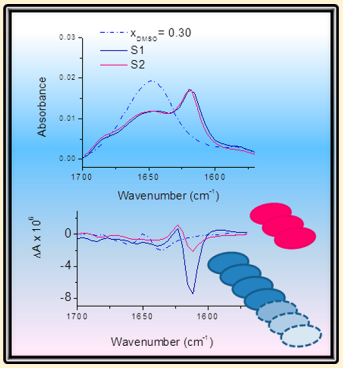
Alessandra Giugliarelli,[†] Paola Sassi,*[,†] Marco Paolantoni,[†] Assunta Morresi,[†] Rina Dukor,[‡] and Laurence Nafie[§]

[†]Dipartimento di Chimica, Università di Perugia, Via Elce di sotto 8, 06123 Perugia, Italy

[‡]BioTools, Inc., 17546 Bee Line Highway, Jupiter, Florida, 33458, United States

[§]Department of Chemistry, Syracuse University, Syracuse, New York, 13244, United States

ABSTRACT: Vibrational spectroscopy has been applied to the study of conformational variation of lysozyme during thermal denaturation. An infrared and vibrational circular dichroism (VCD) analysis of lysozyme in D_2O, D_2O/EtOD, and D_2O/DMSO at different solvent compositions and pH's was accomplished. Complete deuteration effects on amidic groups were revealed through the analysis of the amide I band of lysozyme dissolved in deuterated water. The comparison of IR absorption and VCD spectra of lysozyme in D_2O revealed the higher sensitivity of the VCD technique to the presence of partially or fully deuterated secondary structure of lysozyme. Aggregation was induced by thermalization of lysozyme at different solvent compositions and pH values. Interestingly, IR and VCD showed different sensitivities to reveal intermolecular β-sheet aggregation under thermal denaturation. The VCD intensity enhancement was observed as the aggregate dimension increased, whereas IR was sensitive to the nucleation step of the process. Moreover, chirality of supramolecular species was revealed through the analysis of the VCD sign pattern. For the first time, we demonstrate how aggregates produced under different solvent compositions but similar pH's showed the same VCD sign pattern and consequently the same sense of growth.



INTRODUCTION

The solvent effects on protein conformation are crucial in the analysis of protein conservation, activity, and also of diseases connected to protein misfunction.[1−5] Infrared vibrational spectroscopy shows high sensitivity to the conformational rearrangements of the protein system under solvent or temperature stresses; in particular, both the position and intensity of amidic bands (amide I band at 1700−1600 cm^{-1} and amide II band at 1600−1500 cm^{-1}) can be used as a probe of the secondary structure composition.[6,7] On the basis of the sensitivity of vibrational spectroscopy to protein conformations, combined VCD and IR absorption studies have been used since the 1980s.[8,9] In fact, VCD exhibits an enhanced sensitivity to protein secondary structure due to combination of structural sensitivity of infrared and stereospecificity of circular dichroism (CD).[8,10,11] Due to its biological and medical interest, one of the most studied processes involving the rearrangement of protein conformation is aggregation.[5,12,13] IR absorption is sensitive to the formation of small clusters, but it is nearly blind to the creation of larger structures such as fibrils.[14,15] In fact, the nucleation of oligomers is probed by IR absorption with the increase of a new spectral feature; on the contrary, the growing phase of the aggregation process leading to the formation of fibrillar species from small clusters does not induce any further change of IR spectrum. Thus, the IR band assigned to the presence of intermolecular contacts is not specific of fibrils: these species are usually revealed by ThT fluorescence emission, ANS (1-anilino-8-naphthalene-sulfonate) binding and microscopic techniques (AFM, SEM, and TEM).[14,16] Recently, measurements of both Raman (ROA) and IR (VCD) optical activity showed a great sensitivity in monitoring the conformational variation during both unfolding and assembling processes.[10,17,18] Moreover, these techniques also evidenced the formation of large species, since oligomers rearrange to form fibril structures whose chiroptical properties lead to the large enhancements of vibrational circular dichroism intensity.[19] Thus, traditional FT-IR spectroscopy and VCD can be used to reveal and follow the complex sequence of assembling steps from single chain to large supramolecular structures.

Lysozyme is an ideal model for studying protein diseases owing to its relatively simple structure. Recently, thermal denaturation processes of lysozyme in water/ethanol[15,20,21] and water/DMSO[22−25] solutions have been analyzed to evidence how the solvent composition affects the thermodynamics and the kinetics of thermal unfolding and aggregation. The use of a cosolvent allows one to observe lysozyme aggregation under mild conditions; in fact, it is well-known that lysozyme in water at low pH and 65 °C rearranges to form oligomers and fibrils after an incubation time of a few days, whereas the presence of organic cosolvents such as ethanol or DMSO induces aggregation in a few minutes and at lower temperatures.[15,21,26]

Actually, DMSO exerts a more complex effect on protein

Received: November 14, 2012
Revised: January 23, 2013
Published: February 12, 2013

Table 1. Samples Prepared as Described in the Experimental Section

lysozyme 120 mg/mL in	D_2O	D_2O^a	D_2O/EtOD[a]			D_2O/DMSO[a]			
cosolvent molar fractions	0		0.15		0.18	0.20	0.25	0.30	0.40
pH	4.05	4.65	3.57		3.63	5.55	5.80	5.75	6.30

[a]Fully deuterated samples (see the Experimental Section).

structure, since it acts as a denaturant during heating treatment but is also widely used at low molar fractions as a preservative of proteins, cells, and tissues during the cooling process.[23,25] For this reason, we decided to study the thermal denaturation of lysozyme in D_2O, D_2O/EtOD, and D_2O/DMSO solutions at different cosolvent molar fractions, protein concentrations, and pH values. We recently characterized the thermal denaturation processes of lysozyme in these solving conditions with different spectroscopic techniques.[15,20,21,25] In the present study, a combined FT-IR and VCD analysis of unfolding and aggregation processes of lysozyme is presented to give a deeper description of thermal denaturation of globular proteins.

EXPERIMENTAL SECTION

Sample Preparation. Hen egg white lysozyme, D_2O, EtOD, and DMSO were purchased from Sigma Aldrich and used without further purification. A 120 mg portion of protein was dissolved in 1 mL of deuterated water, and part of this solution was thermally treated at 70 °C for 15 min and rapidly cooled to guarantee the complete H/D exchange avoiding aggregate formation, as previously described.[20,25] A higher concentrated sample (220 mg/mL) was treated at 70 °C for 15 min and slowly cooled to check the aggregation effects on the VCD spectra. Fully deuterated samples were added to an appropriate amount of EtOD or DMSO in order to obtain the desired solvent composition (Table 1). For ethanol solutions, different pH values were adjusted by adding minute amounts of HCl (2−4 μL added to 2 mL solution).

Selected lysozyme samples in D_2O/EtOD mixtures corresponding to ethanol molar fraction x_{EtOD} = 0.15 (at the two different pH values of 4.65 and 3.57) and x_{EtOD} = 0.18 (pH 3.63) were thermalized at 55 °C for 30 min and then slowly cooled down to room temperature. The same treatment was applied to a lysozyme solution in D_2O/DMSO at DMSO molar fraction x_{DMSO} = 0.30 (pH 5.75). We chose the temperature according to our previous data on aggregation kinetics;[15] at 55 °C, we observed a fast aggregation process for these solutions. Samples of 8 μL each were placed in a BioCell (BioTools Inc.) with CaF_2 windows and a path length of ca. 4 μm. IR absorption and VCD spectra were recorded at room temperature with the ChiralIR-*2X* FT-VCD spectrometer equipped with Dual*PEM* (BioTools, Inc.) accessory. For each sample, blocks of VCD spectra were collected between 4000 and 400 cm^{-1} at 8 cm^{-1} resolution for 6 h. IR and VCD blocks were averaged to increase the spectral signal-to-noise ratio. Spectra were then normalized to the area of the IR amide I band. Second derivative spectra were calculated with the dedicated routine of Origin 8.0.

Analysis and Discussion. *Lysozyme in D_2O.* In Figure 1, the IR and VCD spectra of the amide I band of partially deuterated (PD) and fully deuterated (FD) samples of lysozyme (120 mg/mL aqueous solution) are shown. The effect of H/D exchange on IR absorption signals is well-known;[20,27,28] in Figure 1a, the amide I band of FD lysozyme is centered at lower wavenumbers with respect to the PD sample due to the complete deuteration of inner NH groups. On the

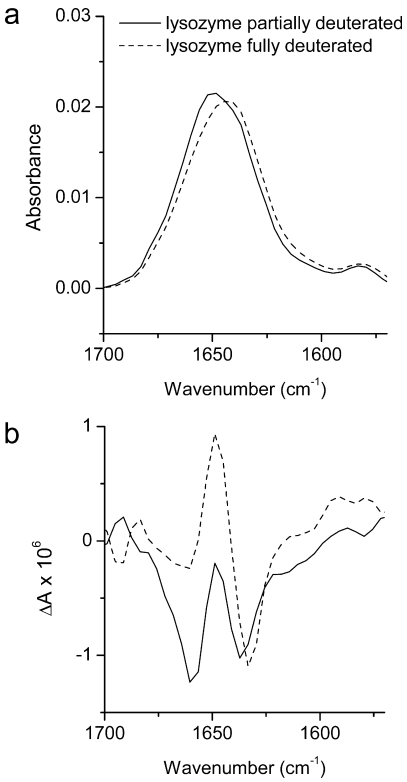


Figure 1. IR (a) and VCD (b) spectra in the amide I band region of 120 mg/mL partially (thin line) and fully (dashed line) deuterated lysozyme in D_2O.

contrary, nothing is known about the effect of complete deuteration on VCD profiles of globular proteins. The VCD spectrum of partially deuterated lysozyme shows the presence of two negative components at 1635 and 1660 cm^{-1}, in perfect agreement with literature data.[9,29] The full H/D substitution of amide groups shifts the frequency of the whole signal to lower wavenumbers, completely changing the band profile. The spectrum of the fully exchanged sample shows two main components at 1635 cm^{-1} (negative) and 1650 cm^{-1} (positive) and a less intense negative component at 1660 cm^{-1}; thus, large modifications are observed especially on the high frequency side of the final spectral shape.

In order to identify the reason of such differences, we decided to use a curve fitting procedure to analyze the amide I band profile of VCD spectra. As shown in Figure 2, both experimental profiles are reproduced using 3 Gaussian components (Figure 2) as described in the literature.[8,9]

The intense negative component at 1635 cm^{-1} is usually associated with β-sheet structures.[30−32] Upon deuteration, it slightly red shifts while its intensity remains almost the same. According to the depicted band decomposition, spectral variation is mainly ascribed to the relative intensity change of the two higher frequency components at ca. 1660 and 1650 cm^{-1}. In fact, in the spectrum of the partially deuterated sample (Figure 2a), the negative component at 1660 cm^{-1} prevails.

dx.doi.org/10.1021/jp311268x | J. Phys. Chem. B 2013, 117, 2645−2652

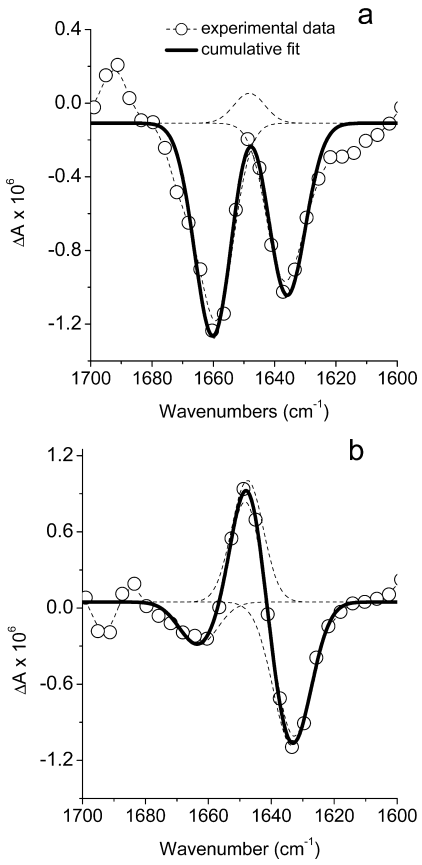


Figure 2. Results of the curve fitting procedure for 120 mg/mL partially (a) and fully (b) deuterated lysozyme in D_2O. Circles, experimental points; thick line, total fitting curve; dashed curves, Gaussian fitting components.

The situation is reversed for the fully deuterated sample where the component at 1650 cm^{-1} becomes prevalent. Unfortunately, the molecular origin of these two components has not been clearly understood.

The VCD spectrum of a simple polypeptide (poly-L-lysine) with α-helical structure shows a couplet with a negative band at higher frequency (1660−1665 cm^{-1}) and a positive band at lower frequency (1652−1648 cm^{-1}).[33−35] After dissolution in a deuterated solvent, the corresponding spectrum shows a negative−positive−negative (− + −) sign pattern, with peak positions at 1666, 1650, and 1636 cm^{-1}, respectively, which is commonly assigned to completely deuterated α-helices.[33] It is worth noting that the three-peaked feature characterizing α-helices can be more or less symmetrical, depending on the solvent.[33,36−38] In any case, assuming that the negative peak at 1636 cm^{-1} is overlapped to the β-sheet contribution (at the same frequency in our sample), we can assign the two higher frequency components of the VCD spectrum in Figure 2b to the fully deuterated right-handed α-helices of lysozyme. On the other hand, a similar spectral shape has been observed for other secondary structures, namely, 3_{10}-helix[35] and random coil (extended helix),[34] but the expected peak frequencies do not correspond to those of Figure 2b. Moreover, the presence of such structures in FD and not in the PD sample would imply a strong conformational rearrangement induced by the isotopic substitution, which is not in line with the results obtained by previous IR findings.[20] It is noteworthy that at a lysozyme concentration of FD and PD samples the protein aggregation is not likely to occur. Thus, the spectral change caused by isotopic exchange is not attributed to a change in the protein conformation but to a modulation of vibrational properties of the samples. In this respect, the VCD spectrum of Figure 2b represents a standard reference spectrum of a fully deuterated lysozyme sample in its native state. To the best of our knowledge, this is the first time that this VCD comparison is made for a complex protein system as lysozyme, instead of simple peptides.

To check the sensitivity of VCD to the thermal denaturation of lysozyme, we thermalized a sample with higher protein concentration (ca. 220 mg/mL) in D_2O at 70 °C for 15 min and cooled it slowly to room temperature; after this treatment, both IR and VCD spectra show significant differences (Figure 3). In particular, the band broadening of the IR profile with a decrease of intensity at 1650 cm^{-1} probably suggests the partial loss of native structures. VCD measurement shows a larger variation of profile with the evident increase of a negative peak at 1660 cm^{-1} (see Figure 3c), and the decrease of intensity at 1650 cm^{-1}: this is probably due to the subtle conformational rearrangement monitored by IR absorption. Moreover, it also shows the developing of a new feature at ca. 1620 cm^{-1} recently observed for insulin and lysozyme aggregates in water.[19] Aggregation of lysozyme in water is usually achieved after incubation at 65 °C and pH 2 for 2 days.[19,39] This suggests that VCD is very efficient in detecting the presence of aggregates: it reveals the formation of intermolecular bonds after mild thermal treatment of a concentrated sample, even though IR absorption does not show evident features characteristic of these structures.[15]

Lysozyme in Mixed Solvent: Ethanol−Water Solutions. In order to gain more insights into the effects of unfolding and aggregation on IR and VCD spectral profiles, protein solutions in water/EtOD and water/DMSO have been analyzed, since the presence of organic cosolvent favors denaturation.[15,23]

Figure 4 shows the IR (a) and VCD (b) spectra in the amide I band region of fully deuterated lysozyme at x_{EtOD} = 0.15 and 0.18 at room temperature. In our previous studies,[15,20,21] we evidenced both different melting temperatures and different thermal stability of aggregates on changing the ethanol molar fraction from 0.15 to 0.18. On this basis, the present analysis of VCD profiles as compared with IR absorption suggests further details to the comprehension of chemico-physical properties of this ternary system. According to IR band shapes of Figure 4a and to literature data,[15,20] ethanol addition at these molar fractions does not modify the native structure of lysozyme: at room temperature, it is mainly composed of α-helix and β-sheet arrangements. Nevertheless, a sensitive intensity variation is observed for the 1660 cm^{-1} VCD component. We suggest that this band-shape modification is not due to a conformational change because the change is not supported by other experimental evidence; it is rather due to changes in protein−solvent interactions as previously evidenced for different protein systems.[11,33,37]

In Figure 5b, the VCD profiles at different pH values are also compared: in this case, a noticeable change in the relative intensities at 1630 and 1650 cm^{-1} can be seen. This evidence suggests the presence of small variations on the protein conformation at the different pH values. In fact, as stated above, the negative peak at 1630 cm^{-1} is mainly associated to the intramolecular β-sheet arrangement and the component at 1650 cm^{-1} to α-helix; thus, the relative change of intensity evidenced in Figure 5b can be qualitatively described as a

dx.doi.org/10.1021/jp311268x | J. Phys. Chem. B 2013, 117, 2645−2652

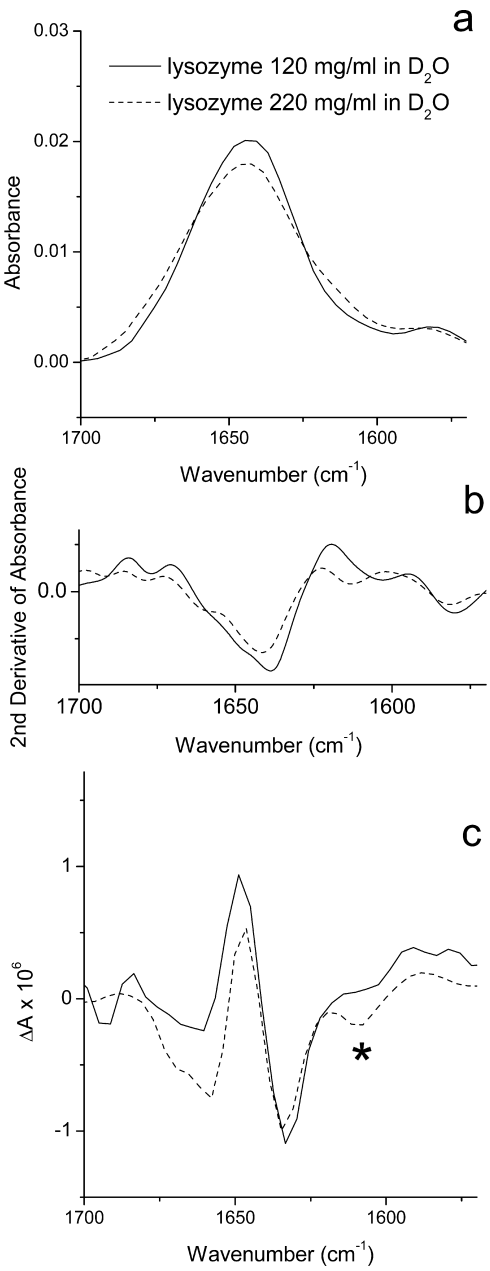


Figure 3. IR (a), second derivative (b), and VCD (c) spectra in the amide I band region of lysozyme 120 mg/mL (thin line) and 220 mg/mL (dashed line) in D_2O. The second derivative of the absorption spectrum helps in recognizing the components of the amide I band. The aggregate peak at 1620 cm^{-1} (*) is revealed with feasible intensity in the VCD profile and not in the absorption measurement.

change of single chain conformation leading to the increase of β-sheet structures on increasing pH. Such observation is in agreement with dielectric spectroscopic data on the conformational effects of alcohols on the protein structure.[40] The sensitivity of the VCD technique in revealing this subtle modification of the protein structure prompted us to investigate also the effects of protein aggregation. Aggregated lysozyme in ethanol was produced as described in the Experimental Section; in particular, both pH and temperature were fixed in order to compare at room temperature the effect induced by the different amounts of ethanol. The corresponding IR and VCD spectra are depicted in Figure 6.

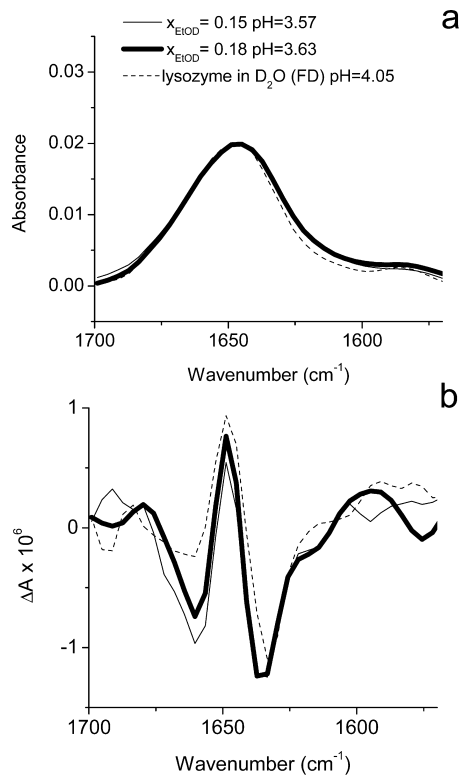


Figure 4. IR (a) and VCD (b) spectra in the amide I band region of lysozyme 120 mg/mL in D_2O and D_2O/EtOD, x_{EtOD} = 0.15 and 0.18.

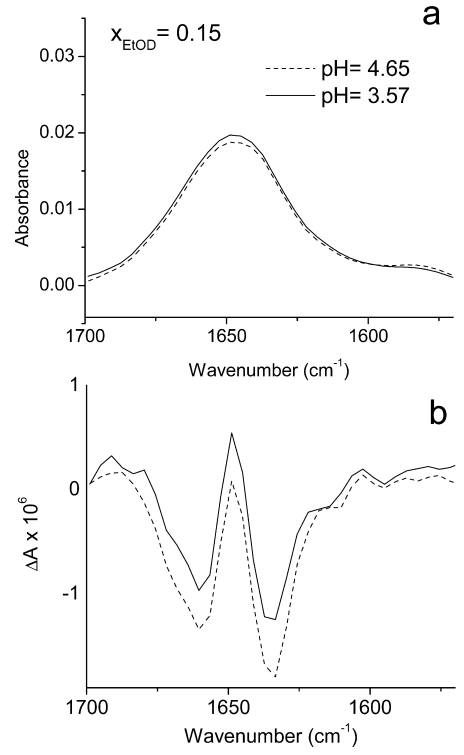


Figure 5. IR (a) and VCD (b) spectra in the amide I band region of lysozyme 120 mg/mL in x_{EtOD} = 0.15 at two different pH values.

IR and the corresponding second derivative spectra show the characteristic signals of intermolecular β-sheet structures[18] at 1618 and 1690 cm^{-1} (Figure 6a and b). The corresponding VCD spectra (Figure 6c) exhibit the negative peak at ca. 1620

dx.doi.org/10.1021/jp311268x | J. Phys. Chem. B 2013, 117, 2645−2652

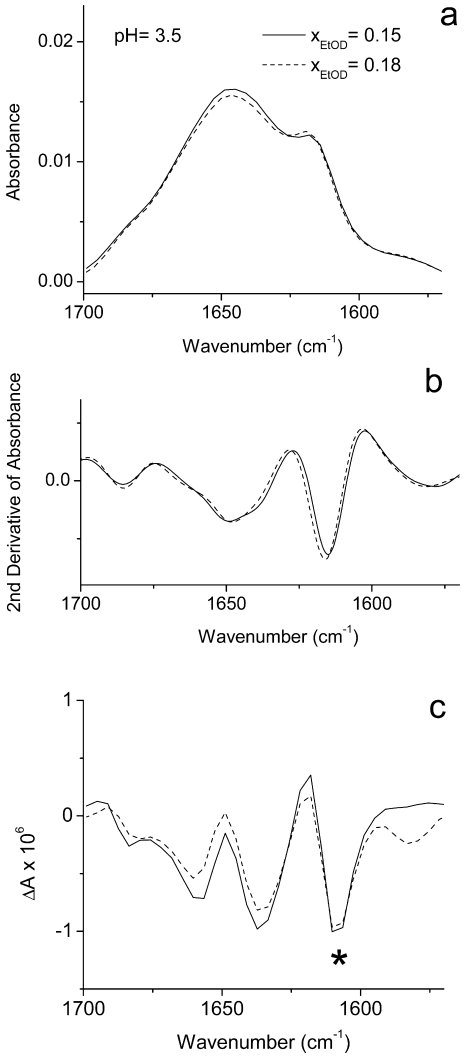


Figure 6. IR (a), second derivative (b), and VCD (c) spectra in the amide I band region of thermally treated lysozyme 120 mg/mL in D_2O/EtOD, x_{EtOD} = 0.15 and 0.18. pH 3.57 and 3.63 for x_{EtOD} = 0.15 and 0.18, respectively. *: VCD signal of aggregates.

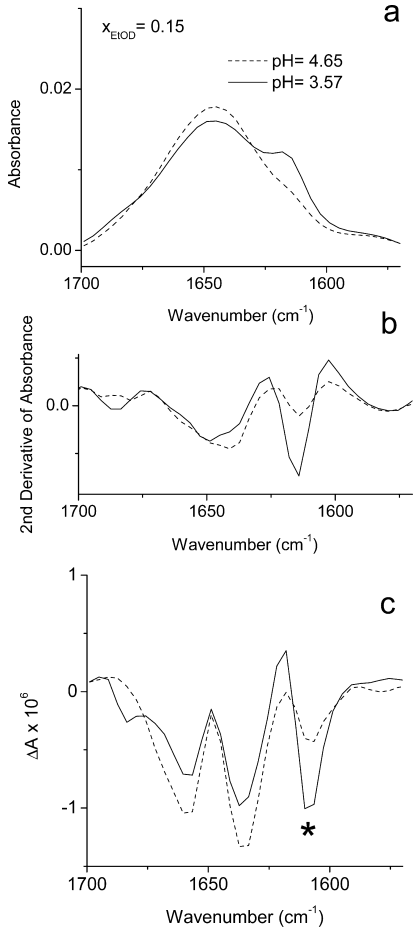


Figure 7. IR (a), second derivative (b), and VCD (c) spectra in the amide I band region of thermally treated lysozyme 120 mg/mL at x_{EtOD} = 0.15 and two different pH values. The intermolecular amide I components of IR spectra (a) are also evidenced in the second derivative profiles (b) at 1690 and 1620 cm^{-1}. *: VCD signal of aggregates.

cm^{-1}, further confirming the presence of aggregated species.[19] Pure fibrils show the (++−++) sign pattern where the negative peak is centered at ca. 1624 cm^{-1} in H_2O.[19] In our samples, the five-peak pattern is less visible probably due to the strong contribution from native lysozyme (Figure 4) overlapped to the VCD aggregate signals. The small relative intensity of the 1618 cm^{-1} component in Figure 6a suggests a low concentration of oligomers with respect to monomeric species.

A perfect agreement between IR and VCD data is also evidenced in the spectral evolution on increasing pH (Figure 7): aggregation is favored at pH 3.57 compared to pH 4.65, in line with literature data.[41] In fact, Yan and co-workers have evidenced the higher production of ordered polymers for lysozyme at low pH, whereas alkaline pH leads to an observation of amorphous aggregates. It is noteworthy how lysozyme solutions at different solvent compositions (x_{EtOD} = 0.15 and 0.18) but the same pH show similar IR and VCD spectra; this evidences the crucial role of pH in the aggregation process.

Lysozyme in Mixed Solvent: DMSO–Water Solutions. In order to analyze lysozyme in a different environment, we also prepared fully deuterated lysozyme in water/DMSO solutions at different DMSO molar fractions. Figure 8 shows IR and VCD spectra at room temperature for x_{DMSO} = 0.20, 0.25, and 0.30. IR spectra of the different samples practically overlap, and also, VCD profiles show the same sign patterns as observed for lysozyme in EtOD (Figure 5).

Literature data evidenced that the stability of the native lysozyme is reduced at room temperature when x_{DMSO} > 0.30, and this can induce both unfolding and assembling of single chains.[42,43] Therefore, two different protein samples in DMSO were prepared: the first one at x_{DMSO} = 0.30 thermalized at 50 °C for 30 min and then cooled to room temperature (S1), and the second one at room temperature and x_{DMSO} = 0.40 (S2). Both samples formed transparent gels. IR and VCD spectra are presented in Figure 9 together with the spectra of native lysozyme in the D_2O/DMSO solvent (x_{DMSO} = 0.30) for comparison.

The IR absorption spectra of both gelified samples evidence the 1620 cm^{-1} component, indicating the presence of a large amount of ordered aggregates (Figure 9a and b).

The intensity of this component in the IR spectra is similar in the two cases; on the contrary, a strong variation is observed in the VCD profiles. In fact, this component is more intense (about 4 times) for the sample in which aggregates are

dx.doi.org/10.1021/jp311268x | J. Phys. Chem. B 2013, 117, 2645−2652

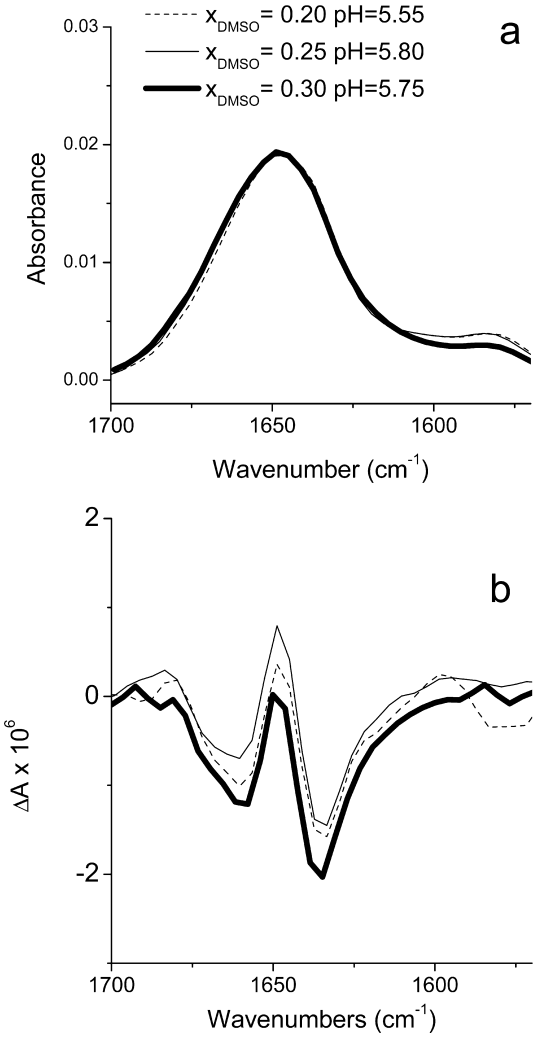


Figure 8. IR (a) and VCD (b) spectra in the amide I band region of lysozyme 120 mg/mL at x_{DMSO} = 0.20 (dotted line), 0.25 (thin line), and 0.30 (thick line).

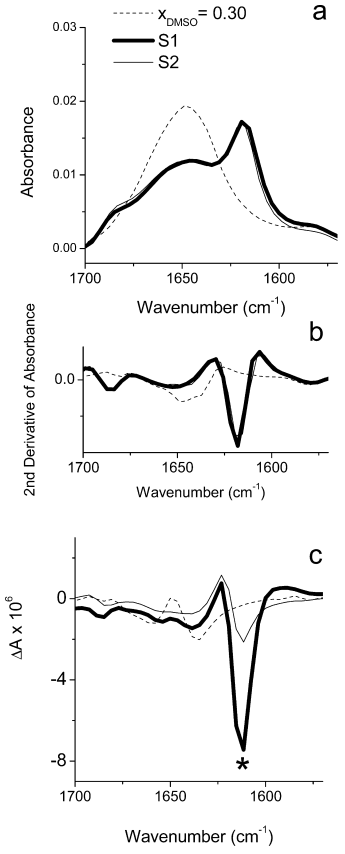


Figure 9. IR (a), second derivative (b), and VCD (c) spectra in the amide I band region of 120 mg/mL lysozyme solutions: native lysozyme at x_{DMSO} = 0.30 (dotted line), thermally treated sample at x_{DMSO} = 0.30 (S1: thick line), and room temperature sample at x_{DMSO} = 0.40 (S2: thin line). *: VCD signal of aggregates.

thermally produced (thick line of Figure 9c). As previously described, IR monitors the nucleation of oligomers by the intensity increase of the signal at 1620 cm^{-1}.[14,15] The growth of oligomers to give larger species does not modify the intensity of this absorption;[14] on the contrary, the comparison between the negative VCD peak at 1620 cm^{-1} for S1 and S2 suggests that a different aggregation state is obtained in these two samples. Recently, Nafie and co-workers[19] have revealed a VCD intensity enhancement due to the formation of complex aggregates as fibrils in solution; for the same samples, IR signals only showed minor changes. According to data shown in Figure 9c, we believe that larger species are present in S1, this giving a larger negative intensity at 1620 cm^{-1}. This could be due to the fact that the room temperature aggregation of S2 is limited by the high solvent viscosity; in fact, as soon as a large fraction of protein creates small ordered clusters, the formation of a transparent gel is obtained and the relative increase of viscosity hinders the growing phase of the aggregation process. This effect is reduced for S1 due to the higher aggregation temperature. The thermal treatment applied at 50 °C has a double effect: on one side, it favors the complete unfolding and aggregation of lysozyme; on the other, it reduces the viscosity of solution with respect to room temperature, thus ensuring larger species to be formed before the self-inhibition effect of the polymerization occurs.

All of these observations reveal VCD's high sensitivity to production and developing of aggregated structures. Recently, Kurouski and co-workers[44,45] have also shown the VCD sensitivity to the chirality of fibril in insulin at low pH. Fibrils grown at different pH's showed the same core (β-sheet structure), but different senses of chirality were revealed below pH 2.1 by AFM and, for the first time, by VCD. In fact, the VCD sign pattern of aggregates is directly connected to the chirality sense of fibril growth. For lysozyme in our solvent compositions at the analyzed pH values (Figures 3, 6, 7, and 9), a VCD negative peak at 1620 cm^{-1} confirms that all the aggregates are growing in the normal sense, corresponding to the left-handed supramolecular structures.[46−48] The observation that different environments lead to the same growing mechanism is not obvious. In fact, the nature of the cosolvent dramatically affects the thermal unfolding process of lysozyme.[20,23] A common characteristic is present in our solutions: in the 3.5 < pH < 5.8 range that we probed, the protein has a low positive charge on the surface.[49] According to literature data, the sense of chirality can be influenced by the surface charge and solvent−protein interactions;[44] our results suggest that the sense of growth is mainly dependent on pH.

CONCLUSIONS

VCD shows great sensitivity to the conformational rearrangement of lysozyme dissolved in D_2O, D_2O/EtOD, and D_2O/

dx.doi.org/10.1021/jp311268x | J. Phys. Chem. B 2013, 117, 2645−2652

DMSO medium. The contributions from deuterated α-helices and β-sheets were evidenced and analyzed for the first time for lysozyme dissolved in D_2O and thermally treated in order to achieve the complete deuteration of amidic groups.

Both IR and VCD have a great sensitivity to the aggregate formation, too. Their characteristic band shapes change as intermolecular β-sheet conformations are produced. Moreover, VCD can reveal crucial information about aggregate dimension and chirality. VCD intensity enhancements for aggregate signals reveal the presence of large aggregates, whereas IR spectra are not modified after the nucleation step. It is noteworthy how VCD reveals that aggregates produced under different solvating conditions but similar pH show the same chirality: under acidic conditions ($3.5 < pH < 5.8$), we measured for all our samples a negative peak at 1620 cm^{-1} assigned to left-handed supramolecular structures. Therefore, our findings indicate that the sense of growth of protein assemblies is not affected by the solvent used (water, water/ethanol, or water/DMSO) and aggregates with normal chirality are always produced.

Our experimental results can give an important contribution as a reference VCD study of the effects of solvating conditions on thermal degradation processes. We explored the dependence of the measured VCD profile on isotopic substitution, conformational rearrangement, and assembling of polypeptide chains; a better comprehension of spectral features could come from a theoretical treatment of vibrational optical activity in globular proteins.

AUTHOR INFORMATION

Corresponding Author

*Phone: +39 075 5855585. Fax: +39 075 5855586. E-mail: sassipa@unipg.it.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Esposito, A.; Comez, L.; Cinelli, S.; Scarponi, F.; Onori, G. Influence of Glycerol on the Structure and Thermal Stability of Lysozyme: a Dynamic Light Scattering and Circular Dichroism Study. *J. Phys. Chem. B* **2009**, *113*, 16420−16424.

(2) Prabhu, N.; Sharp, K. Protein-Solvent Interactions. *Chem. Rev.* **2006**, *106*, 1616−1623.

(3) Csermely, P. Water and Cellular Folding Processes. *Cell. Mol. Biol.* **2001**, *47*, 791−800.

(4) O'Connor, T. F.; Debenedetti, P. G.; Carbeck, J. D. Simultaneous Determination of Structural and Thermodynamic Effects of Carbohydrate Solutes on the Thermal Stability of Ribonuclease A. *J. Am. Chem. Soc.* **2004**, *126*, 11794−11795.

(5) Dobson, C. M. Principles of Protein Folding, Misfolding and Aggregation. *Semin. Cell Dev. Biol.* **2004**, *15*, 3−16.

(6) Gremlich, H. U., Yan, B. *Infrared and Raman Spectroscopy of Biological Materials*; Marcel Dekker Inc.: New York, 2001.

(7) Mantsch, H. H., Chapman, D., Eds. *Infrared Spectroscopy of Biomolecules*; Wiley-Liss Inc.: New York, 1996.

(8) Keiderling, T. A.; Silva, R. A.; Yoder, G.; Dukor, R. K. Vibrational Circular Dichroism Spectroscopy of Selected Oligopeptide Conformations. *Bioorg. Med. Chem.* **1999**, *7*, 133−141.

(9) Keiderling, T. A.; Wang, B.; Urbanova, M.; Pancoska, P.; Dukor, R. K. Empirical Studies of Protein Secondary Structure by Vibrational Circular Dichroism and Related Techniques. *A*-Lactalbumin and Lysozyme as Examples. *Faraday Discuss.* **1994**, 263−285.

(10) Ma, S.; Freedman, T. B.; Dukor, R. K.; Nafie, L. A. Near-Infrared and Mid-Infrared Fourier Transform Vibrational Circular Dichroism of Proteins in Aqueous Solution. *Appl. Spectrosc.* **2010**, *64*, 615−626.

(11) Paterlini, M. G.; Freedman, T. B.; Nafie, L. A. Vibrational Circular Dichroism Spectra of Three Conformationally Distinct States and an Unordered State of Poly(L-Lysine) in Deuterated Aqueous Solution. *Biopolymers* **1986**, *25*, 1751−1765.

(12) Chiti, F.; Dobson, C. M. Protein Misfolding, Functional Amyloid, and Human Disease. *Annu. Rev. Biochem.* **2006**, *75*, 333−366.

(13) Ross, C. A.; Poirier, M. A. Protein Aggregation and Neurodegenerative Disease. *Nat. Med.* **2004**, *10* (Suppl), S10-7.

(14) Benseny-Cases, N.; Cocera, M.; Cladera, J. Conversion of Non-Fibrillar β-sheet Oligomers into Amyloid Fibrils in Alzheimer's Disease Amyloid Peptide Aggregation. *Biochem. Biophys. Res. Commun.* **2007**, *361*, 916−921.

(15) Sassi, P.; Giugliarelli, A.; Paolantoni, M.; Morresi, A.; Onori, G. Unfolding and Aggregation of Lysozyme: a Thermodynamic and Kinetic Study by FTIR Spectroscopy. *Biophys. Chem.* **2011**, *158*, 46−53.

(16) Morris, A. M.; Watzky, M. A.; Finke, R. G. Protein Aggregation Kinetics, Mechanism, and Curve-Fitting: a Review of the Literature. *Biochim. Biophys. Acta* **2009**, *1794*, 375−397.

(17) Blanch, E. W.; Morozova-Roche, L. A.; Cochran, D. A.; Doig, A. J.; Hecht, L.; Barron, L. D. Is Polyproline II Helix the Killer Conformation? A Raman Optical Activity Study of the Amyloidogenic Prefibrillar Intermediate of Human Lysozyme. *J. Mol. Biol.* **2000**, *301*, 553−563.

(18) Measey, T. J.; Schweitzer-Stenner, R. Vibrational Circular Dichroism as a Probe of Fibrillogenesis: The Origin of the Anomalous Intensity Enhancement of Amyloid-like Fibrils. *J. Am. Chem. Soc.* **2010**, *133*, 1066−1076.

(19) Ma, S.; Cao, X.; Mak, M.; Sadik, A.; Walkner, C.; Freedman, T. B.; Lednev, I. K.; Dukor, R. K.; Nafie, L. A. Vibrational Circular Dichroism Shows Unusual Sensitivity to Protein Fibril Formation and Development in Solution. *J. Am. Chem. Soc.* **2007**, *129*, 12364−12365.

(20) Sassi, P.; Onori, G.; Giugliarelli, A.; Paolantoni, M.; Cinelli, S.; Morresi, A. Conformational Changes in the Unfolding Process of Lysozyme in Water and Ethanol/Water Solutions. *J. Mol. Liq.* **2011**, *159*, 112−116.

(21) Giugliarelli, A.; Sassi, P.; Paolantoni, M.; Onori, G.; Cametti, C. Heat-Denatured Lysozyme Aggregation and Gelation As Revealed by Combined Dielectric Relaxation Spectroscopy and Light Scattering Measurements. *J. Phys. Chem. B* **2012**, *116*, 10779−10785.

(22) Kamiyama, T.; Liu, H.; Kimura, T. Preferential Solvation of Lysozyme by Dimethyl Sulfoxide in Binary Solutions of Water and Dimethyl Sulfoxide. *J. Therm. Anal. Calorim.* **2009**, *95*, 353−359.

(23) Torreggiani, A.; Di Foggia, M.; Manco, I.; De Maio, A.; Markarian, S. A.; Bonora, S. Effect of Sulfoxides on the Thermal Denaturation of Hen Lysozyme: A Calorimetric and Raman Study. *J. Mol. Struct.* **2008**, *891*, 115−122.

(24) Arakawa, T.; Kita, Y.; Timasheff, S. N. Protein Precipitation and Denaturation by Dimethyl Sulfoxide. *Biophys. Chem.* **2007**, *131*, 62−70.

(25) Giugliarelli, A.; Paolantoni, M.; Morresi, A.; Sassi, P. Denaturation and Preservation of Globular Proteins: the Role of DMSO. *J. Phys. Chem. B* **2012**, *116*, 13361−13367.

(26) Goda, S.; Takano, K.; Yamagata, Y.; Nagata, R.; Akutsu, H.; Maki, S.; Namba, K.; Yutani, K. Amyloid Protofilament Formation of Hen Egg Lysozyme in Highly Concentrated Ethanol Solution. *Protein Sci.* **2000**, *9*, 369−375.

(27) Meersman, F.; Heremans, K. Temperature-Induced Dissociation of Protein Aggregates: Accessing the Denatured State. *Biochemistry* **2003**, *42*, 14234−14241.

(28) Vrettos, J. S.; Meuse, C. W. Infrared Techniques for Quantifying Protein Structural Stability. *Anal. Biochem.* **2009**, *390*, 14−20.

(29) Urbanova, M.; Dukor, R. K.; Pancoska, P.; Gupta, V. P.; Keiderling, T. A. Comparison of Alpha.-Lactalbumin ond Lysozyme Using Vibrational Circular Dichroism. Evidence for a Difference in Crystal and Solution Structures. *Biochemistry* **1991**, *30*, 10479−10485.

(30) Sen, A. C.; Keiderling, T. A. Vibrational Circular Dichroism of Polypeptides. III. Film Studies of Several α-Helical and β-Sheet Polypeptides. *Biopolymers* **1984**, *23*, 1533−1545.

(31) Kubelka, J.; Keiderling, T. A. Differentiation of β-Sheet-Forming Structures: Ab Initio-Based Simulations of IR Absorption and Vibrational CD for Model Peptide and Protein β-Sheets. *J. Am. Chem. Soc.* **2001**, *123*, 12048−12058.
(32) Hilario, J.; Kubelka, J.; Keiderling, T. A. Optical Spectroscopic Investigations of Model β-Sheet Hairpins in Aqueous Solution. *J. Am. Chem. Soc.* **2003**, *125*, 7562−7574.
(33) Yasui, S. C.; Keiderling, T. A. Vibrational Circular Dichroism of Polypeptides. 8. Poly(Lysine) Conformations as a Function of pH in Aqueous Solution. *J. Am. Chem. Soc.* **1986**, *108*, 5576−5581.
(34) Yasui, S. C.; Keiderling, T. A. Vibrational Circular Dichroism of Polypeptides. VI. Polytyrosine α-Helical and Random-Coil Results. *Biopolymers* **1986**, *25*, 5−15.
(35) Yasui, S. C.; Keiderling, T. A.; Bonora, G. M.; Toniolo, C. Vibrational Circular Dichroism of Polypeptides, V. A Study of 3_{10}-Helical-Octapeptides. *Biopolymers* **1986**, *25*, 79−89.
(36) Kubelka, J.; Huang, R.; Keiderling, T. A. Solvent Effects on IR and VCD Spectra of Helical Peptides: DFT-Based Static Spectral Simulations with Explicit Water. *J. Phys. Chem. B* **2005**, *109*, 8231−8243.
(37) Lipp, E. D.; Nafie, L. A. Vibrational Circular Dichroism in Amino Acids and Peptides. 10. Fourier Transform VCD and Fourier Self-Deconvolution of the Amide I Region of Poly(γ-Benzyl-L-Glutamate). *Biopolymers* **1985**, *24*, 799−812.
(38) Aviles-Moreno, J. R.; Urena Horno, E.; Partal Urena, F.; Lopez Gonzalez, J. IR−Raman−VCD Study of R-(+)-Pulegone: Influence of the Solvent. *Spectrochim. Acta, Part A* **2010**, *79*, 767−776.
(39) Arnaudov, L. N.; de Vries, R. Thermally Induced Fibrillar Aggregation of Hen Egg White Lysozyme. *Biophys. J.* **2005**, *88*, 515−526.
(40) Bonincontro, A.; De Francesco, A.; Onori, G. Influence of pH on Lysozyme Conformation Revealed by Dielectric Spectroscopy. *Colloids Surf., B* **1998**, *12*, 1−5.
(41) Yan, H.; Saiani, A.; Miller, A. F. Gelation of a Model Globular Protein. *Macromol. Symp.* **2007**, *251*, 112−117.
(42) Jackson, M.; Mantsch, H. H. Beware of Proteins in DMSO. *Biochim. Biophys. Acta* **1991**, *1078*, 231−235.
(43) Kamiyama, T.; Morita, M.; Kimura, T. Rheological Study of Lysozyme in Dimethyl Sulfoxide + Water Solution at 298.15 K. *J. Chem. Eng. Data* **2004**, *49*, 1350−1353.
(44) Kurouski, D.; Lombardi, R. A.; Dukor, R. K.; Lednev, I. K.; Nafie, L. A. Direct Observation and pH Control of Reversed Supramolecular Chirality in Insulin Fibrils by Vibrational Circular Dichroism. *Chem. Commun.* **2010**, *46*, 7154−7156.
(45) Kurouski, D.; Dukor, R. K.; Lu, X.; Nafie, L. A.; Lednev, I. K. Spontaneous Inter-Conversion of Insulin Fibril Chirality. *Chem. Commun.* **2012**, *48*, 2837−2839.
(46) Khurana, R.; Ionescu-Zanetti, C.; Pope, M.; Li, J.; Nielson, L.; Ramirez-Alvarado, M.; Regan, L.; Fink, A. L.; Carter, S. A. A General Model for Amyloid Fibril Assembly Based on Morphological Studies Using Atomic Force Microscopy. *Biophys. J* **2003**, *85*, 1135−1144.
(47) Serpell, L. C.; Smith, J. M. Direct Visualisation of the β-sheet Structure of Synthetic Alzheimer's Amyloid. *J. Mol. Biol.* **2000**, *299*, 225−231.
(48) Fulara, A.; Lakhani, A.; Wójcik, S.; Nieznańska, H.; Keiderling, T. A.; Dzwolak, W. Spiral Superstructures of Amyloid-Like Fibrils of Polyglutamic Acid: An Infrared Absorption and Vibrational Circular Dichroism Study. *J. Phys. Chem. B* **2011**, *115*, 11010−11016.
(49) Kuehner, D. E.; Engmann, J.; Fergg, F.; Wernick, M.; Blanch, H. W.; Prausnitz, J. M. Lysozyme Net Charge and Ion Binding in Concentrated Aqueous Electrolyte Solutions. *J. Phys. Chem. B* **1999**, *103*, 1368−1374.