Journal of Biomedical Optics

BiomedicalOptics.SPIEDigitalLibrary.org

Effect of thermal denaturation, inhibition, and cleavage of disulfide bonds on the low-frequency Raman and FTIR spectra of chymotrypsin and albumin

Nikolay N. Brandt
Andrey Yu Chikishev
Anna A. Mankova
Inna K. Sakodynskaya

SPIE.

Effect of thermal denaturation, inhibition, and cleavage of disulfide bonds on the low-frequency Raman and FTIR spectra of chymotrypsin and albumin

Nikolay N. Brandt,[a] Andrey Yu Chikishev,[b] Anna A. Mankova,[a,*] and Inna K. Sakodynskaya[c]
[a]Lomonosov Moscow State University, Faculty of Physics, Leninskie gory 1, Moscow, Russia 119991
[b]International Laser Center, Leninskie gory 1/62, Moscow, Russia 119991
[c]Lomonosov Moscow State University, Faculty of Chemistry, Leninskie gory 1, Moscow, Russia 119991

Abstract. The analysis of the structure-function relationship is extremely important in the study of proteins. The importance of function-related motions of large parts or subglobules of protein molecules stimulates the spectroscopic study in the low-frequency (terahertz) domain. However, only tentative assignments are available and the spectroscopic data are insufficiently discussed in terms of structural changes. This work is aimed at the analysis of regularities of changes in the low-frequency (100 to 600 cm^{-1}) FTIR and Raman spectra of proteins related to their structural modifications. We study the spectra of two proteins with substantially different structures (albumin and chymotrypsin) and the spectra of samples in which the structures of protein molecules are modified using inhibition, thermal denaturation, and cleavage of disulfide bonds. The results indicate that the low-frequency spectral interval can be used to characterize protein conformations. Correlated variations in the intensities of several low-frequency bands are revealed in the spectra of the modified proteins. The strongest spectral changes are caused by thermal denaturation of proteins, and the effect of cleavage of disulfide bonds is generally weaker. It is demonstrated that the inhibitor binding in the active site causes spectral changes that can be compared to the changes induced by thermal denaturation.
[DOI: 10.1117/1.JBO.20.5.051015]

Keywords: chymotrypsin; albumin; disulfide bridges; protein structure; FTIR; Raman; low frequencies; denaturation; inhibition.

Paper 140559SSR received Sep. 1, 2014; accepted for publication Oct. 30, 2014; published online Dec. 5, 2014.

1 Introduction

The topicality of the study of protein molecules is related to their decisive role in biochemical processes in living matter. The interpretation of such processes necessitates comprehensive characterization of the functional activity of enzymes, which can be considered as macromolecular machines with specific functions. The enzymatic activity significantly depends on the molecular structure. Even minor structural modifications of an enzyme may lead to substantial changes of conformational dynamics and, hence, functional activity.

Several methods can be used to modify protein structure. The structure of the surface amino acid residues can easily be changed using variations in the native (aqueous) environment of a protein molecule. In particular, the state of surface amino groups of α-chymotrypsin (CT) is modified when the native environment (water) is changed by organic solvents and the functional activity is transformed from the hydrolysis of peptide bonds into transesterification with a significant decrease in the catalytic activity.[1–3] The presence of crown-ether molecules in organic solvents leads to an increase in the activity by several orders of magnitude due to the interaction with surface amino groups.[4]

Thermal denaturation evidently leads to modification of protein structure. The changes of FTIR and Raman spectra of CT upon thermal denaturation have been studied in Ref. 5. The spectral changes show that the denaturation causes changes in the secondary structure, conformation of tyrosine residues, and conformation of disulfide bridges.

Several chemical agents specifically interact with the structural elements of a protein globule. In particular, tris(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT) provide the cleavage of disulfide bonds and the corresponding spectral changes have been studied in Refs. 6–8. Enzyme functioning can be terminated using specific agents (inhibitors) that interact with the active site. In particular, phenylmethanesulfonylfluoride (PMSF) may serve as the inhibitor for CT.[9,10]

FTIR and Raman spectroscopic techniques are known to be efficient tools in the study of function-related structural changes of protein molecules. Conformation-sensitive amide I (~1640 cm^{-1}) and amide III (~1230 cm^{-1}) bands can be used to characterize the secondary structure of a protein molecule. The bands assigned to the disulfide bridges are detected in the spectral interval of 500 to 550 cm^{-1}. Relative intensities of the tyrosine doublet (830 and 850 cm^{-1}) are sensitive to H-bonding. These and several additional spectral features from the fingerprint range are normally used in the study of protein molecules. However, there has been considerable recent interest in the spectral measurements in the low-frequency (50 to 500 cm^{-1}) range. (One of the reasons is the significant progress in the terahertz spectroscopy, which can be considered in several applications as a modification of the FTIR spectroscopy.) Subglobular and cooperative oscillations of macromolecules

*Address all correspondence to: Anna A. Mankova, E-mail: mankova@physics.msu.ru

0091-3286/2015/$25.00 © 2015 SPIE

must be manifested in this range[11–13] and, hence, conformational changes of protein molecules can additionally be characterized using the spectroscopic techniques.

Several technical problems of the spectroscopy in the low-frequency range and the problems related to the analysis and interpretation of spectra containing relatively broad overlapped bands impede the application of the potentially informative spectral interval in the analysis of protein conformational changes. Only in a few works are spectral bands assigned to specific vibrational modes of protein molecules (see, for example, Refs. 14 and 15). Note that contradictory interpretations are sometimes proposed in the literature. Thus, the analysis of vibrational spectra of protein molecules in the low-frequency range and application of the corresponding results in the study of the function-related structural changes are topical problems.

In this work, we measure the low-frequency (100 to 600 cm^{-1}) FTIR and Raman spectra of native proteins [CT and bovine serum albumin (BSA)] and samples in which protein molecules are modified due to thermal denaturation, cleavage of disulfide bridges, or inhibition. The similarities and differences of spectral changes are discussed and attempts at interpretation of the spectral data are made.

2 Experimental

In the experiments, we use chymotrypsin from Samson-Med (CAS #9004-07-3) and BSA from MP Biomedicals (CAS #9048-46-8). The molecular masses of the native proteins are $M_{BSA} = 66.5$ kD and $M_{CT} = 25$ kD, and the numbers of disulfide bridges are $N^{SS}_{BSA} = 17$ and $N^{SS}_{CT} = 5$.[16] The dominant element in the secondary structure of BSA is α-helix, whereas CT is almost free of α-helices and the contents of β-sheets and random coils in it are almost equal.[16]

For cleavage of disulfide bridges, we employ DTT from Sigma (CAS #3483-12-3) and TCEP from Thermo Scientific (CAS #51805-45-9). CT is inhibited using PMSF from Helicon (CAS #329-98-6).

Thermally denatured BSA and CT are obtained using 90-min heating of aqueous solutions of proteins (1 mg/ml) at 80°C and subsequent lyophilization.

For inhibition, CT aqueous solution at a concentration of 1 mg/ml is mixed with PMSF solution in acetonitrile (acetonitrile prevents hydrolysis of PMSF prior to mixing). The protein-to-PMSF molar ratio in the solution is 1:3. Then, the mixture is lyophilized.

Proteins are denatured using TCEP and DTT with the aid of the procedures of Refs. 6 and 8, respectively. The protein-to-TCEP and protein-to-DTT molar ratios are 1:15 and 1:10, respectively.

For Raman measurements, lyophilized powders are used. For FTIR measurements, the lyophilized samples are pressed into tablets.

For FTIR measurements, we use a Thermo Scientific Nicolet-6700 spectrometer. Each spectrum results from averaging over 300 scans. The measurement interval is 100 to 600 cm^{-1}, and the resolution is 2 cm^{-1}. Relatively high absorption coefficients of the samples under study necessitate a decrease in the thickness of tablets to several tens of microns. Such thin tablets are mechanically unstable, and we mix lyophilized proteins with paraffin at a protein-to-paraffin ratio of ~1:5. Thus, the samples for FTIR measurements represent protein-paraffin tablets with a mass of 35 to 40 mg, a diameter of 13 mm, and a thickness of ~0.3 mm that are pressed at a pressure of ~20 MPa. The FTIR spectrum of paraffin in the spectral interval under study is free of developed bands, and the paraffin signal represents a relatively low background in the spectra of the protein-paraffin tablets.

For Raman measurements, we use a Thermo Scientific DXR Raman confocal microscope. The excitation wavelength is 532 nm, mean power at the sample is no greater than 10 mW, and the spectral resolution is 5 cm^{-1} (the dispersion is ~1 cm^{-1} per pixel). In the experiments, we use lyophilized powders.

To analyze the FTIR data, we follow the approach of Ref. 14 and fit the spectra using Lorentzian curves to determine the peak positions. Such a procedure is needed since the FTIR spectra of the samples under study contain relatively broad overlapped spectral bands. We use the initial set of the Lorentzian components of Ref. 14, where the FTIR spectra of several proteins (trypsin, lysozyme, and BSA) have been fitted. For several samples, a few bands were added to appropriately fit the measured spectra. Below, we use the positions of the fitting Lorentzian components in the analysis of the FTIR spectra.

The measured Raman spectra are $R(\nu)$-converted.[17] Then, the background is subtracted,[18] and the spectra are smoothed using 20-points Savitzky-Golay filter.

To determine the positions of the spectral bands in both Raman and FTIR spectra, we additionally use the second derivatives of the spectral curves.

3 Results

Figure 1(a) presents the FTIR spectra of BSA in the spectral interval of 100 to 370 cm^{-1}. For comparison, the spectra are arbitrarily shifted along the vertical axis. It is seen that the samples exhibit broad asymmetric spectral bands in the interval of 100 to 200 cm^{-1} that cannot be fitted using a single Lorentzian

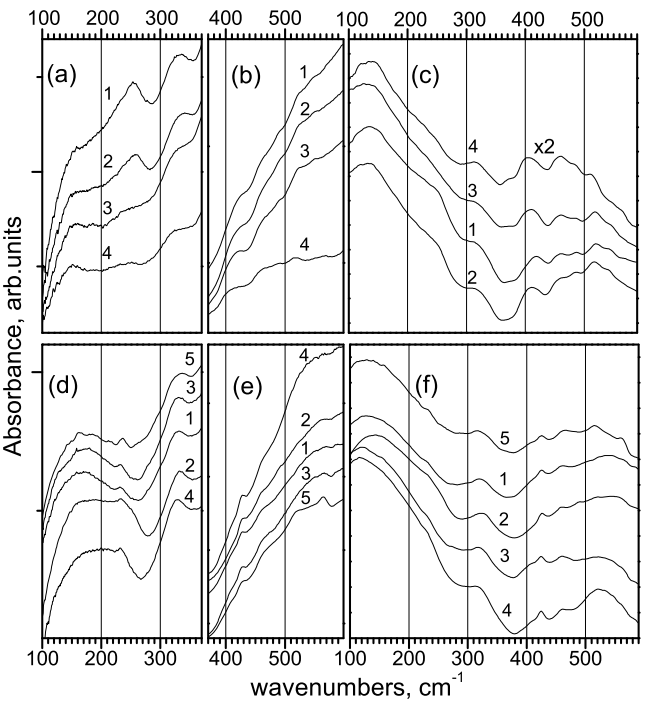

Fig. 1 FTIR spectra of [(a) to (c)] bovine serum albumin (BSA) and [(d) to (f)] chymotrypsin (CT) samples: (1) tris(2-carboxyethyl)phosphine (TCEP)-protein complexes, (2) thermally denatured proteins, (3) dithiothreitol (DTT)-protein complexes, (4) native proteins, and (5) inhibited CT.

component. At least two Lorentzian components in this interval are needed for the approximation. In particular, the peak positions for the native BSA are 118 and 168 cm^{-1}. The position of the first peak remains unchanged for the remaining samples, whereas the position of the second component ranges from 159 to 167 cm^{-1}. Note also additional variations in the widths and relative amplitudes. The bands in this interval can be assigned to the NH out-of-plane bending, CN torsion, or C═O out-of-plane bending.[14]

Similar results are obtained for CT [Fig. 1(d)]. The positions of the fitting components for the native protein are 115 and 148 cm^{-1}. Almost the same positions are obtained for the sample with TCEP and denatured protein although the relative amplitudes are different. For the sample with DTT, the fitting components are shifted to 109 and 141 cm^{-1}, respectively. For the inhibited protein, the peak positions are 121 and 155 cm^{-1}. For both proteins, the widths of the fitting Lorentzian components are ~100 cm^{-1}.

Significant changes are observed in the BSA spectra in the interval of 200 to 280 cm^{-1}. The spectrum of the native protein exhibits a very weak band. The interaction with DTT leads to an increase in the band at 255 cm^{-1}. The band further increases in the thermally denatured sample and the sample with TCEP. The band at 250 to 260 cm^{-1} has been assigned to the C─C─C bending in Ref. 15.

Each CT sample exhibits a band at ~235 cm^{-1}. In comparison with the spectrum of the native CT [curve 4 in Fig. 1(d)], all of the remaining samples exhibit an increase in the intensity at ~250 cm^{-1} (similar to the BSA samples). The band at 235 cm^{-1} has been assigned to the methyl torsion in Ref. 2. A weak band at 290 cm^{-1} in the spectrum of the inhibited CT can be assigned to the C─CN deformation or N─C torsion.[2]

The band at ~325 cm^{-1} in the spectra of all samples can be assigned to the CNC deformation, C═O in-plane bending, and CCN deformation.[1]

Figures 1(b) and 1(e) show the FTIR spectra in the interval of 370 to 600 cm^{-1}. The presence of significant almost linear backgrounds impedes the analysis of spectral changes. Therefore, we additionally process the experimental data using double differentiation and double integration of the curves with subtracting mean levels prior to integration. Such a procedure makes it possible to delete constant and linear background components. The processed spectra are presented in Figs. 1(c) and 1(f). For comparison, curve 4 in Fig. 1(c) (native BSA) is scaled up by a factor of 2.

An asymmetric spectral feature is observed in the BSA spectra in the interval of 380 to 440 cm^{-1}. The modifications of the protein molecule lead to the shift of the spectral maximum from 403 to 420 cm^{-1} if the samples are ordered in the following way: native protein, sample with DTT, thermally denatured sample, and sample with TCEP. The bands in this interval are assigned to side-chain vibrations.[2]

A developed peak at 425 cm^{-1} and a shoulder at 403 cm^{-1} are observed in the CT spectra. Based on this result, we may assume that the same doublet is observed in BSA spectra and the visible shift of the spectral maximum is due to a decrease in the relative intensity of the low-frequency component. Such a decrease is also observed in Fig. 1(f) for almost the same order of the samples. The spectrum of the inhibited CT is most similar to the spectrum of the sample with TCEP.

The redistribution of the relative intensities is also observed for the bands at 460 and 490 cm^{-1} in the BSA spectra. These bands are assigned to the C─C─C bending and side-chain vibrations, respectively.[2] Both bands are clearly visible only in the spectrum of the thermally denatured CT. The remaining CT samples exhibit only the band at 460 cm^{-1}.

The BSA spectra exhibit the band at 515 cm^{-1}. For the above order of the samples, we observe an increase in the relative intensity of the band at 545 cm^{-1}. The spectrum of the native BSA exhibits the band at 565 cm^{-1}, which is almost undetectable in the spectra of the remaining samples.

In the spectral interval of 500 to 600 cm^{-1}, the spectrum of the native CT substantially differs from the spectra of the remaining samples. The original spectra in Fig. 1(e) show an increase in the relative intensity of the band at 565 cm^{-1} in the modified samples, and the most developed band corresponds to the inhibited CT.

To additionally characterize protein changes related to the interaction with reagents, we employ Raman spectroscopy. Figure 2 shows the processed Raman spectra that are arbitrarily shifted along the vertical axis.

Figures 2(a) and 2(b) present the spectra of native and modified BSA. An asymmetric spectral feature at 150 cm^{-1} that presumably consists of several spectral components is observed in all spectra. The interactions with reagents cause redistribution of intensities and, hence, variations in the band shape. Note an increase in the intensity at 170 cm^{-1}, which is most developed in the spectrum of the thermally denatured sample. This sample also exhibits a small band at 190 cm^{-1}, which is not observed in the spectra of the remaining samples. The Raman bands at frequencies of <200 cm^{-1} in the CT spectra are also sensitive to interactions. In particular, the band of native protein at 110 cm^{-1} vanishes in the spectrum of the denatured sample and the intensity of the broad band at 155 cm^{-1} decreases. In the remaining modified samples, we observe an increase in the intensity at 175 cm^{-1}.

The thermal denaturation of BSA also leads to noticeable changes of the broad band at 220 cm^{-1}: the intensity of the

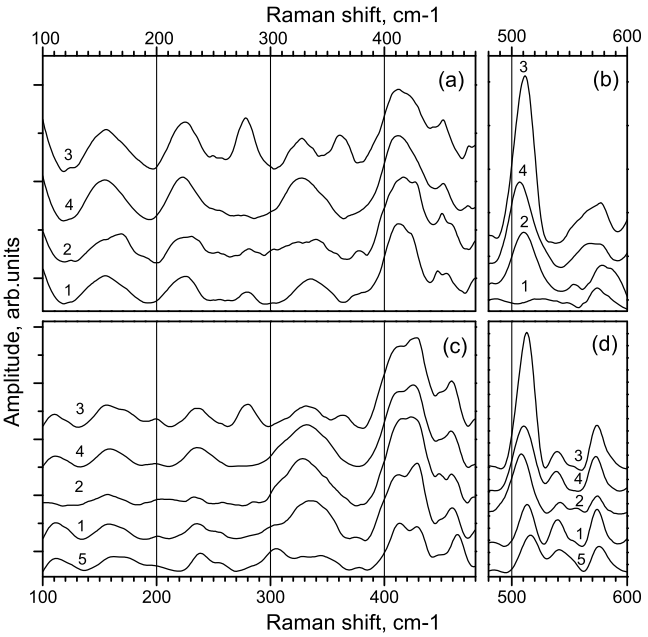

Fig. 2 Raman spectra of [(a) and (b)] BSA and [(c) and (d)] CT samples: (1) TCEP-protein complexes, (2) thermally denatured proteins, (3) DTT-protein complexes, (4) native proteins, and (5) inhibited CT.

low-frequency (high-frequency) component decreases (increases). This band is slightly shifted in the TCEP-protein sample and remains almost unchanged in the DTT-protein sample. The broad band at 235 cm^{-1} in the spectrum of the native CT is transformed into a doublet in the spectra of the inhibited protein and TCEP-protein complex. As in the spectra of albumin, the thermal denaturation causes a significant decrease in the intensity of the band and the interaction with DTT does not cause significant changes.

The thermal denaturation causes significant changes of the BSA band at 325 cm^{-1}. The interaction with TCEP leads to the shift of this band to 335 cm^{-1}, and the interaction with DTT results in the changes of the band shape. The broad band at 335 cm^{-1} in the CT spectrum is similar to the corresponding band in the BSA spectrum, but a developed shoulder appears at 305 cm^{-1} in the former spectrum. The spectral component at 305 cm^{-1} becomes dominant in the spectrum of inhibited CT. The redistribution of the relative intensities of spectral components in the frequency interval of 300 to 370 cm^{-1} is observed in the spectra of the denatured sample and the sample with TCEP. In the spectra of both proteins, the interaction with DTT predominantly causes a decrease in the total intensity of the band at 330 to 335 cm^{-1}.

Note that the bands at 280 and 360 to 375 cm^{-1} appear in the spectra of modified proteins. The most intense bands at 280 and 360 cm^{-1} are observed in the spectra of the DTT-protein samples. The intensity of the band at 280 cm^{-1} is also significant in the spectra of the TCEP-BSA and denatured BSA samples. The spectra of the modified proteins (except for the DTT-protein samples) exhibit the spectral bands shifted to 375 cm^{-1}. The bands at 280 and 360 to 375 cm^{-1} cannot be assigned to the reagents, since the characteristic bands of reagents are not observed in the spectra at the experimental concentrations. The corresponding measurements in the fingerprint range have also proven the absence of the characteristic bands of reagents.

A doublet at 410 and 425 cm^{-1} is observed in the spectra of all samples. As for several bands above, the interaction with DTT does not lead to significant spectral changes. However, the spectra of the remaining modified samples exhibit a decrease in the low-frequency component of the doublet and an increase in the high-frequency component.

A single peak at 450 cm^{-1} in the spectra of the native BSA and DTT-BSA sample is transformed into a doublet in the spectra of the remaining modified samples with the second component at 460 cm^{-1}. Almost the same doublet is observed in the spectra of the CT samples but the high-frequency component dominates. Only in the spectrum of the native CT is the doublet not developed and the high-frequency component is observed at 460 cm^{-1}. In the modified samples, the intensity of the low-frequency component increases.

In general, the thermal denaturation causes the most significant changes in the Raman spectra in the interval of 100 to 480 cm^{-1}.

The bands assigned to disulfide bridges are observed in the spectral interval of 500 to 550 cm^{-1} [Figs. 2(b) and 2(d)]. It is commonly accepted that the bands at 510, 525, and 540 cm^{-1} are assigned to the gauche-gauche-gauche, gauche-gauche-trans, and trans-gauche-trans conformations of S-S bridges.[19]

The spectrum of the native BSA exhibits a single band at 506 cm^{-1}, which indicates the dominant gauche-gauche-gauche conformation of the disulfide bridges. The thermal denaturation causes a minor blue shift of the band and, hence, minor modification of the conformation of bridges. The effect of DTT involves the cleavage of the disulfide bonds in the protein molecule and the formation of such bridges in the oxidized DTT (trans-4,5-dihydroxy-1,2-dithiane). Thus, we observe an increase in the intensity of the blue-shifted band, which must be assigned to the oxidized DTT rather than the protein. TCEP also provides the cleavage of disulfide bonds, but, in this case, new bridges are not formed and we observe a dramatic decrease in the intensity in the spectral interval of 500 to 550 cm^{-1}, which indicates almost complete absence of disulfide bridges in the TCEP-BSA sample.

The Raman spectra of CT samples show that the denaturation weakly affects the conformations of the disulfide bridges. The inhibition and the interaction with TCEP lead to the shift of the most intense band from 510 to 515 cm^{-1} and an increase in the relative intensity of the band at 540 cm^{-1}. The intensity of the band at 515 cm^{-1} in the Raman spectrum of the DTT-CT sample increases due to the S-S bridges in the oxidized DTT, but the intensity of the band at 540 cm^{-1} remains almost unchanged. Thus, the results for DTT-CT and TCEP-CT samples indicate that CT is more stable than BSA against the agents that provide the cleavage of disulfide bonds.

4 Discussion

The low-frequency FTIR and Raman spectra of BSA and CT are generally similar. However, developed spectral differences can be due to significantly different structures of the two proteins.

The analysis of the FTIR and Raman spectra shows that the most significant structural changes of the proteins result from thermal denaturation. The changes of several bands in the spectra of modified proteins are similar. In particular, the FTIR data for CT show that the amplitude of the band at 255 to 260 cm^{-1} increases if the samples are ordered in the following way: native protein, sample with DTT, sample with TCEP, and thermally denatured protein. The intensity of this band in the BSA spectra also increases for almost the same order: native protein, sample with DTT, thermally denatured protein, and sample with TCEP. Note that the bands in the spectra of the last two samples are almost identical so that the orders for the two proteins are virtually identical. When the spectra are ordered in the same way, the intensity of the band at 403 cm^{-1} decreases for both proteins.

For the above order of the CT samples, we observe an increase in the intensity of the Raman band at 255 cm^{-1} relative to the intensity of the band at 235 cm^{-1}. The bands at 225 and 230 cm^{-1} in the Raman spectra of BSA exhibit the same transformations.

The bands at 410 and 425 cm^{-1} are observed in the Raman spectra of both proteins. For BSA (CT) samples, the intensity of the low-frequency (high-frequency) component is higher. The same relationship of the intensities is valid for the bands at 450 and 460 cm^{-1}. For the above order of the samples, the intensity of the band at 450 (460) cm^{-1} increases in the Raman spectra of CT (BSA).

A relatively small PMSF (inhibitor) molecule ($M_{CT}/MP_{MSF} \approx 150$) interacts with the CT active site and binding pocket. Such interaction can be classified as local with respect to the protein molecule as a whole and similarity of the spectra of native and inhibited CT can be expected. However,

the spectra of inhibited CT noticeably differ from the spectra of the native protein, which indicates that the ligand binding induces significant conformational changes of the protein molecule in agreement with the general concept of the structurefunction relationship.

The spectral changes resulting from CT inhibition are similar to the spectral changes due to CT inhibition with an alternative ligand (anthranilic acid).[20] In both cases, the redistribution of relative intensities at 510 and 540 cm^{-1} can be due to transformation from the gauche-gauche-gauche to trans-gauche-trans conformation. Such a similarity is an expected result, since the conformations of the S-S bridges, which are not in direct contact with the CT active site, must not be sensitive to a specific ligand.

Raman spectra of DTT-protein and TCEP-protein samples significantly differ from Raman spectra of native proteins. Raman data show that (1) TCEP provides almost complete cleavage of disulfide bridges in BSA and (2) the cleavage of S-S bridges in proteins due to the effect of DTT is accompanied by the formation of the bridges in the oxidized DTT. Taking into account the absence of significant changes in FTIR spectra of the same samples, we may conclude that the bands of disulfide bridges are not observed in FTIR spectra.

In accordance with the published experimental data and model calculations, amide VI and amide VII bands must be observed in spectral intervals of 500 to 610 and 160 to 250 cm^{-1}, respectively.[21–25] Both Raman and FTIR spectra of BSA differ from the corresponding spectra of CT in the above intervals (recall that the secondary structures of the two proteins are significantly different). Interaction with chemical agents and thermal denaturation also lead to spectral changes in the above intervals. However, only tentative assignments can be found in the literature and the results on the relation of the corresponding band shapes and contents of secondary-structure elements are missing. Thus, the existing data are insufficient for analysis in terms of amide bands.

5 Conclusions

Based on the differences between the low-frequency (100 to 600 cm^{-1}) vibrational spectra of proteins with significantly different structures, we assume that the spectral interval can be used to characterize protein conformations.

The experimentally observed correlated variations in the intensities of several low-frequency bands in the series natural protein, DTT-protein sample, TCEP-protein sample, and denatured protein may indicate increasing conformational changes in such a series.

The strongest spectral changes are caused by thermal denaturation of the protein samples. However, comparable changes result from the cleavage of disulfide bonds.

In spite of the local character of the PMSF interaction with CT, the ligand binding induces significant spectral changes. Presumably, the corresponding conformational changes of the protein molecule can be compared to the changes induced by thermal denaturation.

Acknowledgments

This work was supported by the Russian Foundation for Basic Research (Grant No. 13-02-01177) and Lomonosov Moscow State University Program of Development.

References

1. N. N. Brandt, A. Y. Chikishev, and I. K. Sakodynskaya, "Raman spectroscopy of tris-(hydroxymethyl)aminomethane as a model system for the studies of α-chymotrypsin activation by crown ether in organic solvents," *J. Mol. Struct.* **648**(3), 177–182 (2003).
2. A. A. Mankova et al., "Terahertz time-domain and FTIR spectroscopy of tris-crown interaction," *Chem. Phys. Lett.* **554**, 201–207 (2012).
3. A. A. Mankova et al., "Terahertz time-domain and FTIR spectroscopic study of interaction of a-chymotrypsin and protonated tris with 18-crown-6," *Chem. Phys. Lett.* **560**, 55–59 (2013).
4. J. Broos et al., "Large activation of serine proteases by pretreatment with crown ethers," *J. Chem. Soc., Chem. Commun.* **2**, 255–256 (1995).
5. N. N. Brandt et al., "FTIR and THz spectroscopy of chymotrypsin upon interaction with crown ether, denaturation, and inhibition," *Biomed. Spectrosc. Imaging* **3**, 219–224 (2014).
6. C. David, S. Foley, and M. Enescu, "Protein S-S bridge reduction: a Raman and computational study of lysozyme interaction with TCEP," *Phys. Chem. Chem. Phys.* **11**, 2532–2542 (2009).
7. E. B. Getz et al., "A comparison between the sulfhydryl reductants tris (2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry," *Anal. Biochem.* **273**, 73–80 (1999).
8. C. David et al., "Reductive unfolding of serum albumins uncovered by Raman spectroscopy," *Biopolymers* **89**(7), 623–634 (2008).
9. G. T. James, "Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers," *Anal. Biochem.* **86**, 574–579 (1978).
10. Y. I. Khurgin and E. Y. Maksareva, "Study of the solid-state enzyme reactions. 3. Irreversable inactivation of α-chymotrypsin by benzylsulfonyl fluoride (in Russian)," *Boioorganicheskaya khimiya* **17**(1), 76–80 (1991).
11. A. I. Kitaigorodsky, *Molecular Crystals*, Nauka, Moscow (1971). (in Russian)
12. M. C. Beard, G. M. Turner, and C. A. Schmuttenmaer, "Measuring intramolecular charge transfer via coherent generation of THz radiation," *J. Chem. Phys. A* **106**, 878 (2002).
13. R. J. Falconer and A. G. Markelz, "Terahertz spectroscopic analysis of peptides and proteins," *J. Infrared Milli Terahertz Waves* **33**, 973 (2012).
14. Ch. U. Stehle et al., "Far-infrared spectroscopy on free-standing protein films under defined temperature and hydration control," *J. Chem. Phys.* **136**, 075102 (2012).
15. J. Twardowski, "Far infrared spectra of acid phosphatase from rat liver. Spectra of the native and the heat- and acid-denatured isoenzymes," *Acta biochimica polonica* **27**(1), 1–7 (1980).
16. http://www.rcsb.org/pdb/home/home.do.
17. O. F. Nielsen, "Low frequency spectroscopic studies of interactions in liquids," *Annu. Rep. Prog. Chem. C Phys. Chem.* **90**, 3–44 (1993).
18. Ch. A. Lieber and A. Mahadevan-Jansen, "Automated method for subtraction of fluorescence from biological Raman spectra," *Appl. Spectrosc.* **57**(11), 1363–1367 (2003).
19. F. S. Parker, *Applications of Infrared, Raman, and Resonance Raman Spectroscopy in Biochemistry*, Plenum Press, New York (1983).
20. N. N. Brandt et al., "CARS and Raman spectroscopy of function-related conformational changes of chymotrypsin," *J. Raman Spectrosc.* **31**, 731–737 (2000).
21. H. Susi, "The strength of hydrogen bonding: infrared spectroscopy," *Methods Enzymol.* **26**, 381–391 (1972).
22. Y. El Khoury et al., "On the specificity of the amide VI band for the secondary structure of proteins," *Vibr. Spectrosc.* **55**(2), 258–266 (2011).
23. A. Elliott and E. J. Ambrose, "Structure of synthetic polypeptides," *Nature* **165**, 921–922 (1950).
24. S. Krimm and J. Bandekar, "Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins," *Adv. Protein Chem.* **38**, 181–364 (1986).
25. G. G. Suchkova and L. I. Maklakov, "Amide bands in the IR spectra of urethanes," *Vibr. Spectrosc.* **51**, 333–339 (2009).

Nikolay N. Brandt graduated and received his PhD degree from the Faculty of Physics, Moscow State University in 1998 and 2001, respectively. He is a participant of International Research Projects in Tokyo and Berlin. He is an associate professor at the Faculty of Physics, Moscow State University. His areas of expertise are

Raman, IR, and fluorescence spectroscopy of biological macromolecules aimed at the analysis of the relationship of conformational dynamics and functional activity.

Andrey Yu Chikishev graduated and received his PhD and DrSci degrees from the Faculty of Physics, Moscow State University, in 1982, 1986, and 2002, respectively. He is a leader and participant of International Research Projects on laser spectroscopy of biomolecules and organic molecules in the Netherlands, Germany, Japan, and Italy. He is a head of the Laboratory of Laser Diagnostics of Biomolecules and Methods of Photonics in the study of objects of cultural heritage, International Laser Center, Moscow State University.

Anna A. Mankova graduated from the Faculty of Physics, Moscow State University in 2012. Since 2012 she has been a PhD student at the Faculty of Physics, Moscow State University. Her area of interest is low-frequency spectroscopy of biological molecules.

Inna K. Sakodynskaya graduated and received his PhD degree from the Chemistry Department at Moscow State University in 1981 and 1985, respectively. He is a participant of International Research Projects in the Netherlands, United States, and Italy. He is a leading researcher in the Chemistry Department, Moscow State University. His areas of expertise are kinetics and catalysis, chemical enzymology.