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CARS and Raman spectroscopy of function-related conformational changes of chymotrypsin

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We report on the comparative analysis of the conformation-sensitive bands of free enzyme (chymotrypsin), liganded enzyme (chymotrypsin anthranilate) and enzyme complex with 18-crown-6. The studies were carried out by Raman scattering spectroscopy and polarization-sensitive coherent anti-Stokes Raman scattering spectroscopy (CARS). The bands of tyrosine and tryptophan residues, disulfide bridges and amide I and amide III were analyzed. It is concluded that the conformation of the enzyme changes much more in the case of liganding than in the case of the formation of a complex with the crown ether.

INTRODUCTION

It is well known that any biological molecule, in particular an enzyme molecule, is designated for the performance of one or several functions. Normally the active site (working part) of an enzyme molecule is not large and the rest of the molecule provides its environment. Enzyme function depends not only on the primary structure (the number and sequence of amino acid residues) but also on spatial organization of the polypeptide chain (secondary and tertiary structure), i.e. 3D protein conformation.

The conformation of the enzyme molecule depends on interactions with the solvent molecules and also on temperature. It is also believed that the interaction with ligands changes the conformation of enzyme molecules.

There are numerous methods for determining the conformation of enzyme molecules, including Raman scattering spectroscopy. This method is widely used for the study of enzyme–substrate interactions.[1] Most experimental studies in this field are carried out by resonance Raman scattering spectroscopy. In this case the spectral changes of a chromophore which forms part of the protein molecule (e.g. in hemoglobin[2]) or a ligand (e.g. in serine proteases[3–7]) are studied.

There are several bands in the Raman spectra of proteins that are sensitive to changes of the conformation of a molecule, namely amide I (1640–1660 cm^{-1}) and amide III (1200–1240 cm^{-1}) bands, tyrosine doublet (830 and 850 cm^{-1}), bands of disulfide bridges (510, 525 and 540 cm^{-1}) and some of the bands corresponding to tryptophan amino acid residues (1337 and 1361 cm^{-1}).

Polarization-sensitive coherent anti-Stokes Raman scattering spectroscopy (PSCARS) is another method for studying biological molecules. Earlier we demonstrated that PSCARS may give new, direct spectroscopic information on the conformation-sensitive bands of proteins and amino acids.[8] Although difficult to implement, the method provides several important advantages over spontaneous Raman scattering spectroscopy. Among the most important are the absence of a luminescent background (scattering in the anti-Stokes part of spectrum), high spectral resolution (limited only by the bandwidths of the lasers being used and not related to the detected signal level) and high collection efficiency (laser-like character of the scattered radiation). Changes in the polarization conditions often allow one to increase further the spectral resolution due to the interference character of the CARS signal.

Chymotrypsin is one of the best studied proteins.[9] In native aqueous solution chymotrypsin performs the hydrolysis of the peptide chains of proteins. It has been demonstrated[10,11] that in organic solvents this enzyme can perform synthesis instead of hydrolysis. It was found also that in organic solvents the activity of chymotrypsin increases substantially in the presence of crown ethers.[3–7] The mechanism of this effect is related to the formation of a complex of the surface amino groups of the protein with the molecules of the crown ether.

It is hardly possible to study the changes in the surface amino groups of the protein by means of Raman spectroscopy because of the overlap of the spectral bands of amino groups with other protein bands and especially with amides. However, it is possible to study the interaction of amino groups with crown ethers in model systems under conditions similar to those in the protein molecule. In our experiments we used tris(hydroxymethyl)aminomethane, $(HOCH_2)_3CNH_2$ (Tris), as a model compound.[12] The study of more closely related structural analogs (such as amino acids or their derivatives) is also difficult because of the spectral overlap with bands of the carboxylic group.[12]

The interaction of crown ethers with amines results in the formation of complexes[13,14] in which the amino group is located inside the macrocycle as in the case

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of the complexes with alkali metals. It is assumed that complex formation is determined by hydrogen bonding of the amino group with the oxygen atoms of 18-crown-6.[14]

Raman spectroscopy seems to be a promising method for the study of the processes of complex formation and of the conformation of such complexes because amines feature vibrational bands in the spectral range 1500–1600 cm^{-1}. The positions and relative intensities of the bands depend on the protonation of the amino group and its hydrogen bonding.[15–18]

In this paper we report a comparative analysis of the conformation-sensitive bands of free enzyme (chymotrypsin), liganded enzyme (chymotrypsin anthranilate) and enzyme complex with 18-crown-6. Conformational changes of the enzyme molecule caused by ligand binding were studied by means of PSCARS. The conformational changes of chymotrypsin and Tris induced by complex formation with 18-crown-6 were studied with the use of Raman spectroscopy. All the vibrational bands studied were not directly related to functional groups of the active site. Hence the spectral changes observed were related to the conformational changes of the protein molecule as a whole.

EXPERIMENTAL

Raman spectrometer

We used a Spectra-Physics Model 170 argon ion laser ($\lambda = 488$ nm) for excitation of Raman spectra. Stray light rejection was performed with the help of a holographic SuperNotch filter (Kaiser Optical Systems). The signal was detected with a Princeton Instruments LN/CCD-512TK/S detector. The spectral resolution was 2 cm^{-1}. Raman spectra were measured in the range 490–1720 cm^{-1}. The laser power at the sample was about 100 mW.

PSCARS spectrometer

The experimental setup has been described in detail elsewhere.[8,19] Here we present only the main parameters of the spectrometer. The degenerate variant of CARS was used and the signal was detected at the anti-Stokes wavenumber $\omega_a = 2\omega_1 - \omega_2$. The radiation of the second harmonic of a Quanta Ray DCR 2 Nd:YAG laser (ω_1) and of a PDL2 dye laser, containing either rhodamine 590 or kiton red (ω_2), was used in the CARS process. The dye laser was operating within the wavelength range 555–606 nm, providing CARS wavenumber detunings $(\omega_1 - \omega_2)/2\pi c = 800–2200$ cm^{-1}. The energy at the sample in the pumping beams was 400 mJ per pulse. Polarization suppression of the coherent non-resonant background was used. The polarizations of the beams were set by high-quality Glan–Taylor prisms (extinction ratio 10^{-7} at crossed polarization). The angle between polarizations of the pumping beams $\mathbf{P}_1$ and $\mathbf{P}_2$ (Φ) was 60°. The CARS signal was analyzed using a Glan–Taylor polarizing prism. This polarization analyzer was set at an angle Ψ relative to the polarization $\mathbf{P}_1$. The values of Ψ were chosen close to that providing polarization suppression of the non-resonant background.

The PSCARS spectra measured under different polarization conditions (usually 4–5 positions of the polarization analyzer for each sample) were fitted with an original fit program.[8,19] The simultaneous fit of several PSCARS spectra increases the accuracy of the procedure significantly. Thus we could determine the main parameters of the vibrational bands, i.e. wavenumbers, amplitudes and bandwidths. The interference character of PSCARS spectra makes it difficult to extract the information directly from the spectroscopic data.

Samples

Chymotrypsin (CT) and anthranilic acid were obtained from Sigma, Tris from Reakhim and 18-crown-6 from Fluka.

Chymotrypsin and its complexes with crown ether were lyophilized from solution at pH 7.8. The ratios of the molar concentrations of crown ether and chymotrypsin were 10, 50 and 100.

The liganded enzyme [chymotrypsin complex with the anthranilic acid (CT–AA)] was prepared according to the literature.[20,21] Both the pure and liganded enzyme were dissolved in water or heavy water to obtain a protein concentration of 100–150 mg ml^{-1}. Heavy water was used to avoid the overlap of the vibrations of normal water and the amide I band in the 1600–1700 cm^{-1} region. According to Blout *et al.*,[22] 85–90% deuterium exchange of the amide protons takes place in 3 h. The PSCARS spectra were recorded not earlier than 3 h after sample preparation. Until that time the solutions were kept in a refrigerator under sterile conditions at 4 °C. The front and back windows of the sample cells (1.0–2.0 mm thick) were made from microscope cover-glass slides, which were antireflection coated at the glass–air interface to minimize multiple interference effects.

Note that the spectra of chymotrypsin and its complexes with crown ether were measured in tablets (pressed powders), whereas the comparative studies of chymotrypsin and liganded chymotrypsin were carried out in aqueous solutions.

The protonation of the amino group of Tris is determined by the pH of the solution. The pH of the aqueous solution of Tris was adjusted by addition of hydrochloric acid. We studied complexes of Tris with 18-crown-6 at equimolar concentrations of the components at pH 10, 3 and 7.8. The first two pH values correspond to the deprotonated and the protonated forms of Tris, respectively. pH 7.8 is an intermediate value that corresponds to the pH optimum of the enzyme.

The samples (pure Tris and complexes with crown ether) at different pH were lyophilized from aqueous solution and the Raman spectra of powders were measured. Hereafter we refer to the sample lyophilized from the solutions at a certain pH as simply by 'the sample at this pH value.'

RESULTS

We measured Raman spectra of 18-crown-6, Tris, chymotrypsin and its complexes with 18-crown-6 in the spectral range 450–1750 cm^{-1}. We measured also Raman spectra of the liganded chymotrypsin (complex with anthranilic acid) in the spectral range 450–700 cm^{-1}. All

spectra were 13-point smoothed with the help of Origin 5.0. The positions of the bands were determined after fitting of the spectra.

PSCARS spectra of chymotrypsin and its complex with anthranilic acid were measured within the wavenumber ranges 800–900, 1180–1340 and 1580–1700 cm^{-1} under different polarization conditions. Within all spectral ranges the input of the ligand signal (measured separately) was negligibly low and therefore all the spectral differences were assigned to the protein itself.

First we checked the reproducibility of the data obtained with the help of PSCARS and the possible error margins. In Fig. 1 we present two PSCARS spectra of chymotrypsin heavy water solutions measured under the same polarization conditions several days after one another and after several realignments of the setup. It follows from the comparison that spectral differences exceeding ~10% are significant.

The reproducibility of the Raman spectra was higher and spectral differences exceeding 2% are significant.

All the excitation wavelengths in Raman and CARS experiments were outside the absorption bands of the protein, ligand and crown ether. The coincidence of the Raman and CARS spectra measured at the beginning and in the end of the experimental session was considered as proof of the integrity of the sample (the absence of influence of the laser radiation on the sample).

CARS is a non-linear spectroscopic technique and the signal amplitude at a given detuning is not a result of just the summation of the amplitudes of different vibrational bands and background. The signal intensity is proportional to the square of the third-order susceptibility and thus contains the cross-terms or interference of different vibrational bands and the non-resonant background. This interference and hence the spectrum detected depend strongly on the polarization conditions of the experiment. Variations of e.g. the polarization analyzer position may lead to dramatic changes in the spectral bandshapes.[8] Hence it is necessary to fit the PSCARS spectra to obtain the parameters of the vibrational bands. To increase the accuracy of the procedure, for each sample we carried out the fit with one set of parameters for a series of spectra measured under different polarization conditions.

Amide I band

Figure 2 shows the amide I Raman band of chymotrypsin and its complex with crown ether (1 : 10). It is seen

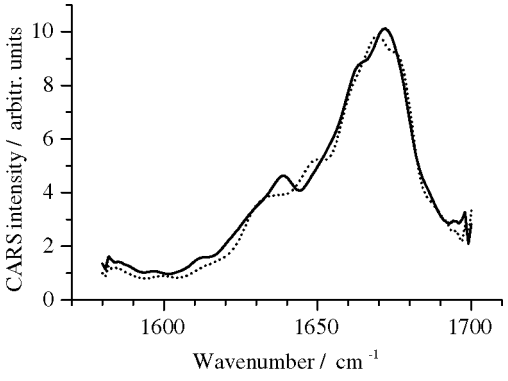

Figure 1. Reproducibility of PSCARS spectra of chymotrypsin in aqueous solution; $\Psi = 62.63°$.

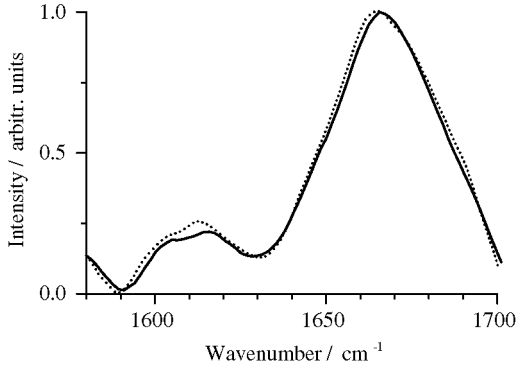

Figure 2. Raman spectra of chymotrypsin (solid line) and its complex with 18-crown-6 (1 : 10) (dashed line).

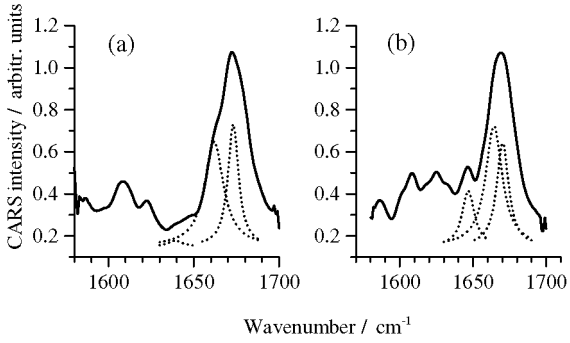

Figure 3. PSCARS spectra of (a) CT and (b) CT–AA heavy water solutions (solid lines) and the Lorentzian components used for an approximation of these spectra (dashed lines); $\Psi = 62.88°$.

that complex formation does not influence the parameters of this band. In Fig. 3 we present PSCARS spectra of the free and liganded enzyme. Each spectrum was approximated with three Lorentzian components.[8] The positions, widths and amplitudes of the bands were varied. In Fig. 3 we also show the Lorentzian components with the parameters obtained after the fitting procedure. The amplitudes of the bands are normalized to the sum of three amplitudes.

Amide III band

Figure 4 shows spectra of chymotrypsin and its complex with crown ether (1 : 10). A shoulder in the high-wavenumber part of the spectrum of the complex is assigned to the crown ether (1271 cm^{-1}). The amide III band of the free enzyme can be approximated with two Lorentzian bands at the wavenumbers 1241 and 1265 cm^{-1}. The spectrum of the complex is approximated with three bands at 1241, 1265 and 1271 cm^{-1}. The relative amplitudes and widths of the first two are the same as those of the free enzyme. Hence the amide III bands of the free enzyme and its complex with crown ether are virtually identical.

Figure 5(a) shows PSCARS spectra of the free and liganded enzyme. To underline the differences in the vibrational spectra of two samples under study, we present in Fig. 5(b) the difference in spontaneous Raman spectra calculated as sums of Lorentzians with the parameters (amplitudes, wavenumbers and bandwidths), obtained after PSCARS spectra fitted according to the literature.[8,19]

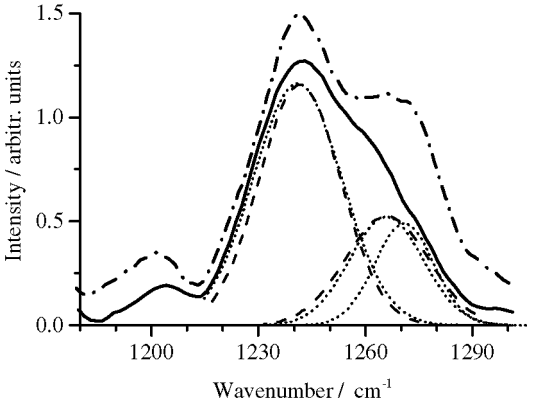

Figure 4. Raman spectra of chymotrypsin (solid line) and its complex with 18-crown-6 (1 : 10) (dashed dotted line) and the Lorentzian components used for approximation of these spectra (dashed and dotted lines, respectively).

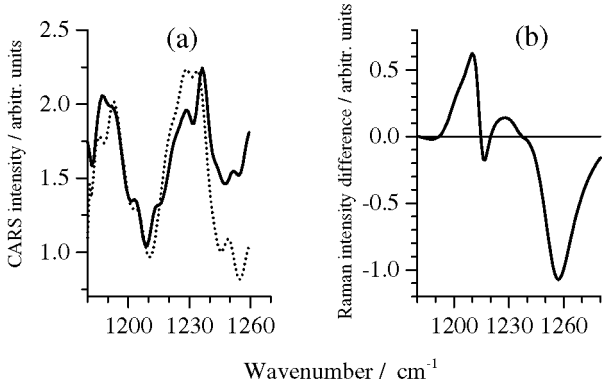

Figure 5. (a) PSCARS spectra of CT (solid line) and CT–AA (dashed line) aqueous solutions and (b) the difference of the calculated Raman spectra of CT and CT–AA aqueous solutions.

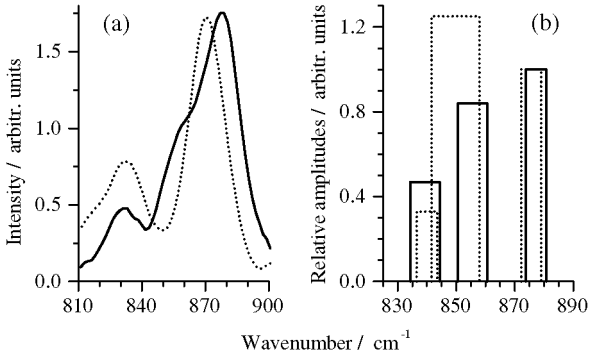

Figure 6. (a) Raman spectra of chymotrypsin (solid line) and its complex with 18-crown-6 (1 : 100) (dashed line) and (b) fit parameters for PSCARS spectra of CT (solid line) and CT–AA (dashed line) aqueous solutions.

Tyrosine bands

In Fig. 6(a) we show Raman spectra of chymotrypsin and its complex with crown ether (1 : 100). The spectra can be approximated with three (four) bands: 877 cm^{-1} (tryptophan), 856 and 830 cm^{-1} (tyrosine doublet) and 868 cm^{-1} (crown ether). The intensities of the bands were normalized to the intensity of the tryptophan band at 877 cm^{-1}. The ratio of the intensities of the tyrosine bands ($R_{Tyr} = I_{856}/I_{830}$) for the free enzyme and for its complexes with crown ether (1 : 10, 1 : 50, 1 : 100) is 2.3, 1.2, 0.25 and 0.02, respectively.

PSCARS spectra of the free and liganded enzyme were also measured. The results of the approximation of these spectra with three bands are presented schematically in Fig. 6(b) as a histogram. The positions, widths and amplitudes of the columns correspond respectively to the wavenumbers, bandwidths and amplitudes of the bands used for fitting of PSCARS spectra. The ratio R_{Tyr} was 1.8 for CT and 3.8 for CT–AA.

Tryptophan bands

Spectral changes resulting from complex formation with crown ether are observed in the range 1300–1380 cm^{-1}. The spectrum here can be fitted with two bands (1337 and 1354 cm^{-1}) that can be assigned to the vibrations of tryptophan. No spectral features of crown ether are observed in this range. After normalizing the spectra to the intensity of the band at 1337 cm^{-1} one can see that the relative intensity of the band at 1354 cm^{-1} increases with increase in the relative concentration of the crown ether in the complex (four times if the free enzyme is compared with the 1 : 100 complex).

Disulfide bridges

In Fig. 7(a) and (b) we present the Raman spectra of solutions of the free and liganded chymotrypsin in normal and heavy water. All the spectra in the range 500–550 cm^{-1} can be fitted with three bands (510, 519 and 537 cm^{-1}). It is seen that the spectra of the free enzyme in both solvents are similar and that the ligandation results in a relative increase in the intensity of the high-wavenumber feature accompanied by a corresponding decrease in the amplitude of the low-wavenumber one.

In Fig. 7(c) we show spectra of chymotrypsin and its complex with crown ether (1 : 100). The spectra were fitted with three bands (510, 520 and 547 cm^{-1}). The formation of the complex influences mainly the high-wavenumber spectral component: its width decreases and the amplitude increases approximately twofold.

Bands of amino groups

As mentioned above, Raman studies of model systems can give some idea about changes in the surface amino groups

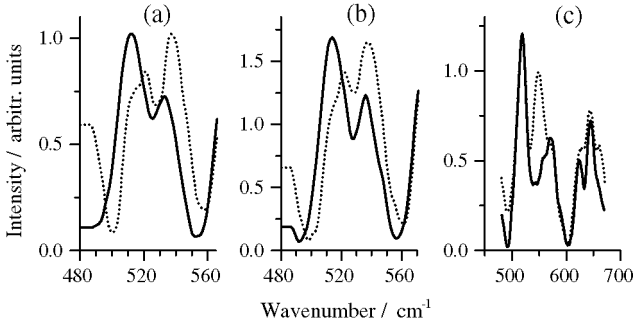

Figure 7. Raman spectra of CT (solid line) and CT–AA (dashed line) solutions in (a) normal and (b) heavy water. (c) Raman spectra of CT (solid line) and its complex with crown ether (1 : 100) (dashed line).

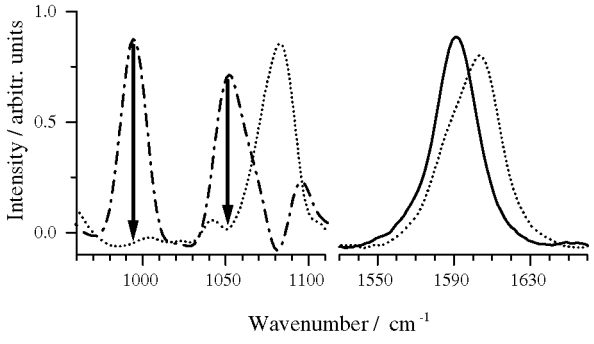

Figure 8. Raman spectra of Tris (solid line), crown ether (dashed dotted line) and their complex at pH 10 (dashed line).

of chymotrypsin induced by the formation of the complex with crown ether.

Figure 8 shows Raman spectra of Tris, crown ether and their complex at pH 10. In particular, it is seen that the band of the crown ether at 995 cm^{-1} and the band of Tris at 1100 cm^{-1} are absent in the spectrum of the complex, but some new bands, that are not seen in the spectra of Tris and the crown ether, are present in the spectrum of the complex (e.g. 1083 cm^{-1}). Note also the appearance of a new spectral component at 1606 cm^{-1} which results in broadening of the band at ca 1600 cm^{-1}. The ratio of the amplitudes of the components at 1606 and 1591 cm^{-1} is 1.4.

The spectrum of complex at pH 3 is similar to that at pH 10, but the ratio of the amplitudes of the bands at 1606 and 1591 cm^{-1} is 0.8.

Figure 9 shows Raman spectra of Tris and its complexes with the crown ether at pH 7.8. Note that the spectrum of Tris contains features seen in its spectrum at pH 3 (1548 and 1646 cm^{-1}) and pH 10 (1591 cm^{-1}). The bands of the crown ether at 995 cm^{-1} can be seen in the spectrum of the complex. The bands at 1548 and 1646 cm^{-1} are absent in the spectra of complexes. The ratio of amplitudes of the bands at 1606 and 1591 cm^{-1} is 0.35. Note also that the band of the complex at pH 10 (1341 cm^{-1}) is not seen in the spectrum of the complex at pH 7.8.

DISCUSSION

Consider first the results obtained for chymotrypsin and its complex with the crown ether. Note that at all concentrations the crown ether molecules are bound in a complex with the protein. This is proved by the absence of the bands of the free crown ether (995 and 1483 cm^{-1}) in the spectra.

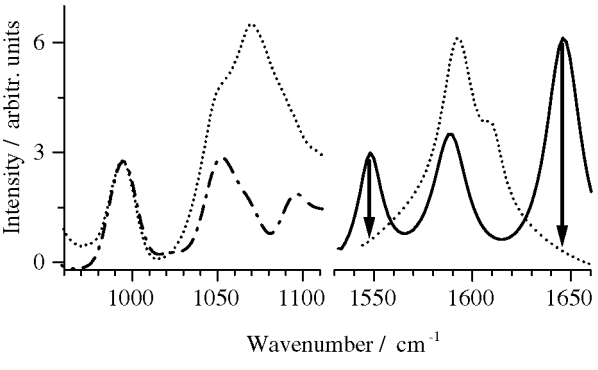

Figure 9. Raman spectra of Tris (solid line), crown ether (dashed dotted line) and their complex at pH 7.8 (dashed line).

The absence of changes in the amide I and amide III bands allows one to conclude that the complex formation does not influence the secondary structure of the protein. The liganding of the enzyme, in contrast, results in substantial changes in the amide I and amide III bands. Earlier we demonstrated that PSCARS allows one to detect three bands that are not resolved in spontaneous Raman spectroscopy and that form the contour of amide I; the relative amplitudes of these bands displayed good correlation with the x-ray data on the content of the elements of the secondary structure.[8] Hence the results obtained for the pure and liganded enzyme (CT and CT–AA) in the amide I range can be considered as indicating the differences in the secondary structure. Under the assumption that different bands correspond to different elements of the secondary structure, we may say that the contents of α-helix, β-sheet and random coil in the free enzyme are 3, 52 and 45%, respectively. In the liganded chymotrypsin (CT–AA) they are 20, 37 and 43%, respectively (Fig. 3). This straightforward interpretation results in the conclusion that the increase in α-helix is due to a decrease in β-sheet. Similar results have been reported recently.[23] An alternative interpretation was discussed in our previous paper.[19] It may be possible that the bands detected correspond to some conformations of the peptide backbone which are not related directly to the generally accepted elements of the secondary structure.

The spectral changes observed for the amide III correlate fairly well with those observed for the amide I. Although the amide III band is certainly sensitive to the conformations of the peptide bonds, its deconvolution is not used as widely as the deconvolution of amide I for the determination of the secondary structure. The main reason is that some uncertainty exists in the assignment of the vibrational bands within the 1220–1320 cm^{-1} range. The presence of β-conformation results in a significant Raman signal at 1235–1240 cm^{-1}, although this spectral range may be overlapped by the vibrations of random coil conformation. According to Lippert *et al.*,[24] '... a protein band at 1300–1320 cm^{-1} may not be used as a unique identification of α-helical content. However, the absence of spectral intensity at 1235–1240 cm^{-1} does seem to be diagnostic of helix.' The formal analysis of the amide I band in CT and CT–AA showed the relative increase in α-helical content upon ligand binding. Hence we could expect a corresponding decrease in the Raman intensity at 1235–1240 cm^{-1} and, possibly, some increase at 1300–1320 cm^{-1}. This is just what we see in Fig. 5(b) where the difference in the calculated Raman spectra is presented.

Consider the changes in the tyrosine doublet. The value of the parameter R_{Tyr} is determined by the strength of the H-bond formed by tyrosine with the adjacent groups.[25] Specifically, the values 2.3 and 1.8 for the free protein in powder and in aqueous solution, respectively, suggest that in the former case the OH groups of tyrosines are stronger proton acceptors. NH_3^+ or CO_2H groups of the protein can be proton donors. In the presence of 18-crown-6 the ratio decreases, showing that tyrosines become strong proton donors, most likely forming H-bonds with oxygen atoms of 18-crown-6. In the case of the liganded chymotrypsin (CT–AA), tyrosines are stronger proton

acceptors compared with the free enzyme. Hence, the state of the H-bonds formed by tyrosines in the liganded chymotrypsin is substantially different from that in its complex with the crown ether and in the free form.

It is known that the strong band at 1354 cm^{-1} corresponds to 'hidden' tryptophan residues.[1] Therefore, the increase in the relative intensity of the band at 1354 cm^{-1} observed in case of complex formation is a consequence of the transformation of tryptophans from the 'open' to the 'hidden' configuration. The number of 'open' tryptophans in the lyophilized enzyme powder is higher than that in a buffer solution owing to partial denaturation of the protein in course of lyophilization. In the presence of 18-crown-6 at a 1 : 50 molar ratio the relationship between 'open' and 'hidden' tryptophans is similar to that in the aqueous solution. Hence 18-crown-6 may prevent the enzyme from being denatured during lyophilization.

It is assumed that the vibrational bands at ca 510, 525 and 540 cm^{-1} in the vibrational spectra of proteins correspond to *gauche–gauche–gauche* ($g-g-g$), *gauche–gauche–trans* ($g-g-t$) and *trans–gauche–trans* ($t-g-t$) conformations, respectively, of the disulfide bridges. Each of the chymotrypsin spectra in the range 500–550 cm^{-1} (Fig. 7) was fitted with three bands, the positions of which are close to those assigned to the conformers of the disulfide bridges.[26,27] Hence the transformations in the corresponding spectral interval resulting from either liganding or complex formation can be interpreted as follows. Liganding of chymotrypsin (in both normal and heavy water) leads to a decrease in the $g-g-g$ content and a corresponding increase in the $t-g-t$ content. Formation of the complex with crown ether does not cause substantial changes of the relative content of the conformers. The content of the $g-g-g$ form decreases slightly. A substantial decrease in the width of the band corresponding to the $t-g-t$ form accompanied by an increase in its intensity can be interpreted as the decrease of the spread of the corresponding angles in the bridges.

The results obtained for Tris (the model system for protein amino groups) are in good agreement with the IR data, according to which the band at 1591 cm^{-1} is assigned to vibrations of the deprotonated amino group and the bands at 1548 and 1646 cm^{-1} are assigned to vibrations of the protonated amino group.[16]

The absence of bands of free crown ether in the spectra of the Tris complex with crown ether at pH 3 and 10 can be considered as an indication of the complete complexation. It is likely that 1 : 1 complex formation takes place. The absence of the bands of the protonated Tris in the spectrum of complex at pH 7.8 indicates that all the protonated Tris molecules are bound in complexes. However, the presence of the bands of the free crown ether in this spectrum allows one to assume that there are free deprotonated Tris molecules in the sample. This assumption is supported by consideration of the relationship between the intensities of the bands at 1606 and 1591 cm^{-1}. In the case when all Tris molecules are bound in complexes, the ratio of the intensities at 1606 and 1591 cm^{-1} for pH 7.8 must be between 0.8 and 1.4. However, this ratio is 0.35, which is possible only if the band at 1591 cm^{-1} is additionally 'enhanced.' As the band at 1591 cm^{-1} is seen in the spectrum of the free deprotonated Tris, we again arrive at the conclusion that there are free deprotonated Tris molecules in the complexes at intermediate pH. Hence the presence of the protonated molecules 'prevents' the deprotonated molecules from forming complexes with the crown ether.

Based on the results obtained for the complex of Tris with the crown ether, we can assume that each molecule of crown ether forms a complex with one amino group of the protein and that the complexes are formed predominantly with the protonated amino groups of the protein molecule.

CONCLUSION

The formation of the complex of chymotrypsin with 18-crown-6 does not influence the secondary structure of the protein, whereas liganding of the enzyme by anthranilic acid results in substantial changes in the secondary structure. Liganding results in a decrease in the α-helix content and an increase in the content of β-sheet. The relative amount of random coil remains virtually unchanged.

In pure chymotrypsin, OH groups of tyrosine residues are proton acceptors in H-bonds formed with the neighboring groups of the protein globule. In the presence of 18-crown-6 tyrosines become strong proton donors and presumably form H-bonds with the crown ether molecules. In the case of anthranilated chymotrypsin, tyrosines are stronger proton acceptors than in the case of the free enzyme.

In free chymotrypsin tryptophan residues are located in the vicinity of the protein surface. Formation of the complex with crown ether leads to 'hiding' of tryptophans inside the protein globule, which makes the conformation of the latter more close to the native one.

The conformations of the disulfide bridges remain virtually unchanged upon formation of a complex with the crown ether. Only a decrease in the spread of angles corresponding to the $t-g-t$ conformation is observed. Liganding of the enzyme results in a decrease in the content of $g-g-g$ isomers and a corresponding increase of the content of $t-g-t$ isomers.

It is likely that surface amino groups of proteins form 1 : 1 complexes with 18-crown-6 under lyophilization. These are mainly protonated amino groups that form complexes with the crown ether.

It can be concluded that the conformation of the enzyme changes much more in the case of liganding than in the case of the formation of a complex with the crown ether.

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