Secondary Structure of Bovine Albumin as Studied by Polarization-Sensitive Multiplex CARS Spectroscopy

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The first application of polarization-sensitive multiplex coherent anti-Stokes Raman spectroscopy (MCARS) in the absence of resonance enhancement to the resolution of the secondary structure of a protein in solution is reported. Polarization MCARS spectra of bovine albumin in D_2O were obtained in the range 1370 to 1730 cm^{-1} with the aid of the background suppression technique. The spectra were fitted simultaneously with a single set of parameters (band positions, bandwidths, amplitudes, and depolarization ratios). Polarized Raman spectra simulated with these parameters revealed a good correspondence with the spontaneous Raman spectra measured. The broad amide I' band was decomposed assuming the three major secondary conformations of protein, of which the contribution of β -sheet structure was found to be negligible. Relative weights of α -helix and random coil conformations agree well with the estimates obtained with Raman and circular dichroism (CD) spectroscopies.

Index Headings: Polarization-sensitive CARS; Multichannel Raman; Conformation of proteins.

INTRODUCTION

Coherent anti-Stokes Raman spectroscopy (CARS) has proven to be a fruitful technique for studies of biophysically important molecules in condensed phase.¹⁻¹⁶ It has become traditional to use an experimental apparatus based on narrow-band laser sources^{1,3,8,17} wherein the dispersion of nonlinear susceptibility is obtained by point-by-point scanning of the laser frequencies through specific Raman resonance. One of the prime concerns¹⁷ has been to obtain high-quality spectra so that the molecular information can be derived by a further CARS line-shape analysis.^{5,4,18,19} However, a more versatile approach involving the combination of narrow-band (ω_{pump}) and broad-band (ω_{Stokes}) semicoherent lasers, with multichannel detection has been proposed.^{20,21} The excitation $\omega_{\text{pump}} - \omega_{\text{Stokes}}$ then simultaneously selects all the vibrational resonances for which the frequency and phase conditions are fulfilled. Such a multiplex CARS (MCARS) technique enables rapid data acquisition and features an improved signal-to-noise ratio²² with respect to scanning methods. A decrease in signal intensity occurs though, owing to a lower spectral density of the light delivered by the broad-band source.

MCARS has commonly been used in combustion diagnostics, plasma studies, and rotational spectroscopy of gases.²²⁻²⁴ It has also been very successful in a number of applications in the condensed phase that included pseudocyanine chloride,² rhodamine 6G,⁷ and bacteriorhodopsin.^{14,15,21} Picosecond MCARS provided a large amount of information on the dynamics of excited states of *trans*-stilbene¹⁰ and the isomerization of all-*trans*- and 9-*cis*-retinal.^{11,12} In all these studies the laser excitation was chosen in the vicinity of an allowed electronic transition,

and hence, the vibrationally resonant susceptibility was electronically resonant enhanced. However, to the authors' knowledge, MCARS has never been applied to non-absorbing samples of biological macromolecules such as proteins. Applications to relatively simple compounds such as pyridine and benzene,² cyclohexane,¹² and neat $(-)\alpha$ -pinene²⁵ have been reported.

Interference of the vibrationally resonant and the non-resonant nonlinear polarizations is a major setback to CARS in the condensed phase. The interference distorts line shapes, thus complicating the resolution of weak and/or overlapping Raman transitions. Discrimination against the nonresonant background can be reached with the use of polarization-sensitive^{1,2,6,18} (PS/CARS) or phase-mismatching^{4,26} techniques. A common feature to the above methods is the ability to control experimentally the portion of nonresonant polarization admixed into the signal. Such a heterodyning method brings an additional "dimension" into the set of spectroscopic observables. The use of heterodyning has been shown^{2,3,8} to heighten the sensitivity to variation of depolarization ratios. It appears to be a feasible method for resolution of broad and composite Raman bands whose substructure is insufficiently resolved with conventional spontaneous Raman (SR) spectroscopy.

Vibrational spectroscopy of proteins, both in solutions and in solid state, has received much attention in recent years,^{3,8,9,27-36} as it complements the data on the structure of proteins obtained with X-ray diffraction, circular dichroism (CD), and infrared spectroscopy. Major protein conformations distinguished in solution^{29,33} (α -helix, β -sheet, and random coil) exhibit Raman bands of similar origin at different frequencies. Raman estimates of the secondary structure distribution are retrieved by resolution of the amide bands which comprise an envelope of vibrations of the polypeptide backbone. The characteristic frequencies are obtained either from model proteins or from proteins with a known structure.²⁷ Decomposition of the prominent and broad amide I band observed between 1630 and 1700 cm^{-1} is generally used to estimate the relative fraction of protein substructures.^{28-30,33-36}

Polarization-sensitive CARS measurements in the off-electronically resonant case of a protein in aqueous solution were first reported for α -chymotrypsin.³⁷ The following application of the PS/CARS technique to a short polypeptide, lysyl-tryptophyl-lysine, in aqueous solution³ indicated that bands have a depolarization ratio notably different from that of the nonresonant background, the latter being $1/3$. A subsequent PS/CARS study of proteins in solutions⁸ (D_2O) focused on the amide I' band and

In the particular polarization scheme with the angles α and β defined as in Fig. 1, the polarization factors in Eq. 4 transform as follows:

$$p^{R,NR} = [(1 - \rho^{R,NR})\cos \alpha \cos \beta + \rho^{R,NR}\cos(\alpha + \beta)]. \quad (6)$$

Equations 3, 5, and 6 form the model for the analytical treatment of susceptibility dispersion profiles obtained experimentally.

The spatial orientation of the nonlinear polarization $\vec{P}^{(3)R}$ for a particular t resonance differs from the one of $\vec{P}^{(3)NR}$ as determined by the magnitudes of the respective depolarization ratios:

$$\begin{aligned} \Theta_t^R &= \arctan(\rho_t^R \cdot \tan \alpha) \\ &\text{and} \\ \Theta^{NR} &= \arctan(\rho^{NR} \cdot \tan \alpha). \end{aligned} \quad (7)$$

Under nonelectron resonant conditions, the polarizations are both linearly polarized, and the nonresonant polarization is much larger than the vibrationally resonant one. The nonresonant background contribution may be suppressed by crossing the analyzer $\vec{e}_A$ (dotted line in Fig. 1) with the direction of $\vec{P}^{(3)NR}$. The contribution of the resonant susceptibility can then be maximized by selecting the α -angle at $\pi/3$.² In such a case the $\vec{P}^{(3)NR}$ vector is expected at $\Theta^{NR} = \pi/6$, while $\vec{P}^{(3)R}$ lies at the angle $\Theta^R = 0$ and $\Theta^R \approx 52.4^\circ$ for respectively polarized ($\rho^R = 0$) and depolarized ($\rho^R = 3/4$) vibrations. Variation in the analyzer position β with respect to direction $\pi/2 - \Theta^{NR}$ admixes a portion of the background into the detected signal. The degree of heterodyning is introduced by the experimentally controlled variation of the ϵ -angle.

Because of the dispersive properties of the sample, the optimal phase-match $\Delta k = [2k_1(\omega_1) - k_2(\omega_2)] - k_{\text{CARS}}(2\omega_1 - \omega_2) = 0$ cannot be achieved simultaneously for all the frequencies within the broad Stokes spectrum $\Delta\omega_2$, though it may be minimized within a chosen spectral range.⁷ The frequency-dependent phase-mismatch narrows the width of an anti-Stokes spectrum in accordance with the expression

$$I_{\text{CARS}}(\omega_2) \propto I_1^2 I_2(\omega_2) \text{sinc}^2(|\Delta k(\omega_2)|L/2)L^2 \quad (8)$$

and limits the principally obtainable spectral range. In the narrow-band CARS experiments, the problem above is usually resolved by scanning the angle of incidence in a mechanical way to compensate for the dispersion of $\Delta k(\omega_2)$. In the MCARS applications, however, the spectral distortions due to the phase-mismatch factor can be significantly reduced by shortening the sample length¹⁵ L and choosing it so that $L \ll L_{\text{coh}}$, where $L_{\text{coh}} = \pi/|\Delta k|$ is the coherence length. For instance, deviations of less than 4% at the edges of a 300-cm⁻¹ broad CARS intensity profile centered at 1550 cm⁻¹ can be expected for a 1-mm-long cuvette with water. Further, the use of tighter focusing into the sample allows one to increase the intensities I_1 and I_2 of incident beams and, therefore, to compensate for the decrease in I_{CARS} caused by the reduction of sample length L . The tighter focusing leads to an even further relaxation of phase-matching requirements¹² as it expands both angular ranges with which $\vec{k}_1$ and $\vec{k}_2$ intersect.

MATERIALS AND METHODS

Bovine albumin (No A-7906) was purchased from Sigma and used without further purification. The protein was dissolved in D₂O to a concentration of 180 mg/mL and stored for a few hours at +4°C to ensure the completion of the exchange of deuterium with amide protons.²⁹ Heavy water instead of H₂O was chosen as the solvent to avoid the interference of the protein vibrations in the 1400–1700 cm⁻¹ Raman range with the pronounced and broad water resonance at ~1640 cm⁻¹, which shifts to ~1200 cm⁻¹ in the D₂O spectrum. The stock solution was used in CARS experiments. For spontaneous Raman experiments, the solution was diluted to a concentration of 40 mg/mL.

The layout of our picosecond multiplex polarization CARS spectrometer¹³ is based on two cavity-dumped dye lasers (3.8 MHz, Coherent 700) synchronously pumped by a mode-locked Nd:YLF laser (Antares 76-YLF, Coherent). Broad-band emission was obtained from the DCM-filled dye laser by removing the standard birefringent plate from the cavity. This semi-coherent output (627.5–645 nm) with 8-ps auto-correlation function (obtained with Femtochrome, FR 103 XL) was used as the Stokes beam. The narrow-band dye laser (Rh6G filled, 5 ps), delivering the pump beam, was tuned to 578.3 nm so that the Raman range 1370 to 1730 cm⁻¹ could be covered with the excitation $\bar{\nu}_{\text{pump}} - \Delta\bar{\nu}_{\text{Stokes}}$ (see Fig. 2). Separate optical channels allowed for the adjustment of temporal overlap between pump and Stokes pulses (cross-correlation 9.6 ps) and their polarization (Glan–Taylor polarizers, $\epsilon < 10^{-6}$, Melles Griot). A focusing lens ($L = 7$ cm) combined the parallel beams (pulse energy ~9 nJ each) in the sample, which was kept in a 1-mm-long cuvette. Thin (150- μ m) anti-reflection coated glass windows were applied to minimize stress-induced birefringence and to avoid multiple interference at the air boundaries.

The CARS continuum was collimated by a lens ($L = 10$ cm), spatially separated at the appropriate phase-match angle, analyzed with a Glan–Taylor polarizer, further attenuated, and led into a single $f/12$ home-made polychromator. Residues of the pump and Stokes beams present in the signal beam were suppressed with holographic notch (Kaiser Optics, HNF 2090) and interference filters, respectively. To maximize dispersion on the detector, we magnified the spectral images created by the polychromator by a factor of 3 with a lens ($L = 5$ cm) attached to its exit port. The spectra were detected by an image-intensified photodiode array (EG&G 1421) and acquired by a multichannel analyzer (EG&G 1460). The MCARS spectra were accumulated at room temperature with an exposure time of 300 s and read out every 0.5 s. All spectra measured were corrected for the residual contribution of the pump beam and detector dark current. These were acquired while the Stokes beam was being blocked. Wavelength calibration was carried out with Ne and Ar emission lamps.

The MCARS dispersion profiles are affected by (1) the spectral distribution $I_2(\omega_2)$ of the Stokes laser; (2) the extent of the phase-mismatch; (3) the wavelength-dependent response of the detection system; and (4) the individual pixel sensitivity of the multichannel detector. The

FIG. 2. Excitation scheme in the multiplex CARS experiments. Emission profiles of the broad-band Stokes laser (right) and narrow-band pump laser (center) are plotted along with the dispersion profile of the nonresonant background of D₂O (left). The intensity scales differ.

MCARS spectrum of the sample and the dispersion of the nonresonant background of the solvent are obtained under the same experimental conditions. These spectra are equally influenced by the factors above. The nonresonant susceptibility of D₂O shows no vibrational features within the Raman range used and is independent of the wavelength. The spectrum of the nonresonant background thus provides a convenient reference for the intensity calibration.

The MCARS spectrum shown in Fig. 2 (left) was obtained from the protein solution in D₂O by setting the transmission plane of the analyzer $\beta = -\theta^{\text{NR}} = -30^\circ$, that is, along the theoretically predicted direction of the polarization $\hat{P}^{(3)\text{NR}}$. The spectrum exhibits a width of $\sim 190 \text{ cm}^{-1}$ full weight at half-maximum (FWHM), which enables MCARS coverage of an approximately 400-cm^{-1} broad Raman range. Because of large differences in the

relative magnitudes of nonresonant and resonant polarizations ($\chi^{(3)\text{R}}/\chi^{(3)\text{NR}} < 10^{-2}$), the spectrum in Fig. 2 shows solely the contribution of the nonresonant background. This profile was used for intensity calibration. Regular modulations in the resultant spectra, caused by unequal offset of even and odd pixels over the dynamic range of the detector, were eliminated with the use of the routine of fast Fourier transform filtering.

Spontaneous Raman spectra were obtained with a Jobin-Yvon Ramanor HG 2S spectrometer (excitation at 514.5 nm, Spectra-Physics 2025-03) in a 90° geometry. Polarized measurements were performed with the use of a sheet analyzer (Spindler & Hoyer, $\epsilon < 10^{-4}$) in the detection path and a $\lambda/2$ plate in the excitation path. The VV and VH spectra of albumin solution were obtained after subtraction of the respective spectra of neat D₂O. Spectra were accumulated for 10 s/cm^{-1} at $+5^\circ\text{C}$ with

slitwidths of 300 μm , corresponding to a spectral resolution of 2.5 cm^{-1} .

RESULTS AND DISCUSSION

The resolution in the MCARS experiments is dependent on both the spectral width of the pump laser λ_1 and the instrumental response of spectrometer. The width of 2.1 cm^{-1} (FWHM) of the laser line λ_1 was measured independently with the Ramanor spectrometer (slits 50 μm , resolution $\sim 0.3 \text{ cm}^{-1}$). The instrumental function is mainly determined by the spectral slitwidth of the polychromator (1.35 cm^{-1} , as obtained from deconvolution) and the image distortions²⁴ in the image intensifier of the detector. Since the relay lens at the exit port of the polychromator magnified the linear dispersion, the decrease in spectral resolution due to distortions in the image intensifier was minimized. The threefold expansion of the spectrum enabled us to improve the response of the image-intensified photodiode array (IPDA) from 2.2 to 0.7 cm^{-1} . Additionally, the use of the relay lens reduced the risk that the IPDA would be saturated by the incoming photons. The laser line λ_1 detected in this configuration displayed an FWHM of 3.4 cm^{-1} , which should be recognized as the actual resolution in MCARS experiments. Since the expected Raman bandwidths for the protein are typically in a range of 12 to 20 cm^{-1} (FWHM),^{3,32} the line broadening due to the finite system resolution was not further considered.

MCARS spectra of bovine albumin in D_2O were resolved in the 1370–1730 cm^{-1} range by varying the ϵ -angle over $\pm 1.5^\circ$ with a step of 0.5° around the position of maximal background suppression. Four of the six MCARS profiles are represented in Fig. 3a–3d. Two broad bands found at 1450 and 1650 cm^{-1} dominate the spectra. They can, respectively, be assigned to the envelopes of CH_2 and CH_3 deformations and the vibrations of the deuterated polypeptide backbone (amide I'). The substructure of the amide I' band is best seen in Fig. 3b and 3c obtained with the least fraction of the nonresonant background. Resolution appeared possible because of a small variation in the vibrational depolarization ratio within the broad bands.

In a particular polarization scheme (Fig. 1) the polarization vectors $\vec{P}^{(3)\text{R}}$ appear between $\vec{z}_1$ and $\vec{P}^{(3)\text{NR}}$ for polarized bands ($\rho^{\text{R}} < \rho^{\text{NR}}$) and between $\vec{P}^{(3)\text{NR}}$ and $\Theta^{\text{R}} \approx 52.4^\circ$ for depolarized ($\rho^{\text{R}} > \rho^{\text{NR}}$) bands. An analyzer used for suppression of $\vec{P}^{(3)\text{NR}}$ introduces a sign with which the resonant polarization $\vec{P}^{(3)\text{R}}$ contributes to the overall polarization. Since the heterodyned spectra represent the dispersion of χ^{R} in the presence of a controlled amount of the nonresonant background, the sign of the dispersive contribution at the particular ϵ -angle indicates the depolarization ratio of a band as compared to ρ^{NR} . One can see that the constituents of the band at around 1450 cm^{-1} all have $\rho^{\text{R}} > \rho^{\text{NR}}$, while those of the amide I' band show $\rho^{\text{R}} < \rho^{\text{NR}}$.

As can be seen from Fig. 3a–3d, a slight (0.5°) variation in the analyzer setting results in a drastic change of the band shapes. The heterodyning effect allows one to resolve weak vibrations at 1580 and 1603 cm^{-1} as well. These bands, assignable, respectively, to tryptophane and/or phenylalanine and to tyrosine modes,^{28,31} both display

TABLE I. The best parameter sets as obtained from a simultaneous fit on the six polarization multiplex CARS spectra of bovine albumin in D_2O . The parameters are the Raman band positions, bandwidths, relative amplitudes, and depolarization ratios. Fitting parameters for the nonresonant background are $\chi^{(3)\text{NR}} = 1320 \text{ a.u.}$ and $\rho^{\text{NR}} = 0.304$. Heavy water bands are incorporated into the fit at 1202, 1558, 2385, and 2490 cm^{-1} , with relative amplitudes and depolarization ratios taken from polarized spontaneous Raman measurements. A number of protein modes, such as NO, stretching and contributions from Trp and *N*-deuterated Trp, are introduced into the fit as a broad (40- cm^{-1}) band at 1329 cm^{-1} , $\rho^{\text{R}} = 0.3$. Abbreviations used are His (Histidine), Phe (Phenylalanine), Trp (Tryptophan), and Tyr (Tyrosine).

Ω_i $\frac{2\pi c}{\text{cm}^{-1}}$	Γ_i $\frac{2\pi c}{\text{cm}^{-1}}$	A_i^{R} (a.u.)	ρ_i^{R}	Assignment
1405	13	5.1	0.1	His: $\text{N}_1\text{--C}_2\text{--N}_3$ sym. stretching N–D bending
1421	16	10.2	0.08	COO^- sym. stretching.
1446	16	38.7	0.63	C–H def. overtone CH_2 bending Phe (?)
1457	16	32.0	0.64	CH_2 sym. def.
1469	14	15.3	0.67	CH_2 asym def. 1477 amide II' (?)
1584	11	9.4	0.73	Phe, Trp, <i>N</i> -deuterated Trp
1605	12	14.3	0.74	Tyr, ring stretching Phe, ring
1615	13	7.4	0.7	Phe, ring stretching Tyr ring stretching Trp (indole ring)
1634	24	56.5	0.09	α -Helix
1651	24	44.3	0.05	Random coil
1661	24	1	0.0	β -Sheet

$\rho^{\text{R}} > \rho^{\text{NR}}$. The best discrimination against the background is achieved at $\beta \approx 62.5^\circ$. The efficiency of the suppression was judged by observing the minimal background contribution in the range of 1500 to 1550 cm^{-1} and at the spectral edges where no vibrations were expected. The exact value of the angle Θ^{NR} and, thus, the depolarization ratio ρ^{NR} could only be obtained by fitting the MCARS spectra.

In accordance with the above model, the six PS/MCARS spectra were fitted simultaneously with a single set of vibrational parameters including band positions, bandwidths, amplitudes, and depolarization ratios. In the fit, the vibrational phases were all assumed to be zero. It appeared necessary to incorporate in the fitting twelve protein modes with known frequencies and assignments.^{27,28,31} The fit quality was judged by the minimal achievable value of the chi-square function weighted with reciprocal experimental data. The best parameter set obtained is given in Table I along with the band assignments. The corresponding curves are plotted in Fig. 3a–3d in bold. Four solvent modes were also introduced with vibrational parameters as obtained from a fit of the polarized SR spectra of D_2O . In fitting the amide I' region only α -helix, β -sheet, and undefined (random coil) conformations^{29,33} were considered. The amount of respective fractions can be estimated from the amide spectral intensities (band areas³³). Representing the amide bands as linear combinations is allowed under the assumption that the Raman polarizability tensor is nearly constant for different structure types. The amide I band arises primarily because of the stretching vibrations of the carbonyl group.²⁸ Deuteration of the polypeptide

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FIG. 3. Multiplex CARS spectra of bovine albumin in D_2O (dotted lines), $\alpha = 60^\circ 10'$. From a to d, β is varied from 63.38° to 61.88° with steps of 0.5° as indicated by the insets. Spectra are normalized on the nonresonant background profile of the solvent and FFT filtered. Curves in bold are the best simultaneous fits with parameters listed in Table I. Note the change of CARS intensity scale in going from a to d.

backbone leads to a small overall downshift ($2\text{--}12\text{ cm}^{-1}$, Ref. 31) of the amide I frequency. As shown in Ref. 27 for the model poly-L-lysine, upon deuteration, the three conformations display Raman peaks at, respectively, 1632 , 1658 , and 1660 cm^{-1} . These Raman positions were introduced into the fit as initial parameters. The Raman bandwidth of each contributor was taken to be equal.

As can be seen in Fig. 3a–3d, the spectral features are well reproduced by the curves simulated. The noise contribution in the MCARS spectra, which can be estimated to be within 6%, limits the accuracy of the fits. The noise arises²³ from (1) pulse-to-pulse variations in the spectral energy of the Stokes dye laser, (2) pulse-to-pulse variations in the spatial and temporal overlap of the pump and Stokes pulses, and (3) shot noise of the detector. Since picosecond lasers with a high repetition rate (3.8 MHz) were used, the influence of the first two factors was minimized by accumulating the signal for some 10^9 laser pulses. The detector noise, though normally negligible, became dominant at very low CARS intensities, such as at the edges of spectra in Fig. 3a–3d. The fit quality is also dependent on the signal-to-nonresonant background ratio. A fraction of the background emerges even in "background-free" spectra owing to the stress-induced birefringence of cuvette windows. Such a residual background was experimentally minimized by introducing an opposed ellipticity in the polarization of the Stokes beam, the latter arising upon reflection at the matching mirror.³

The calculated nonresonant depolarization ratio $\rho^{NR} = 0.304$ differs from its expectation value of $1/3$, indicating a slightly elliptical polarization of the nonresonant contribution. This result can be attributed to the contribution of the electronically resonant susceptibility of protein, which arises because of the two-photon absorption. The determination of the ρ_i^R values (and the corresponding amplitudes A_i^R) from the set of background-suppressed spectra is most accurate for those vibrations which have ρ_i^R close to ρ^{NR} , as the heterodyning effect enables one to control their shape most efficiently. On the other hand, the heterodyning technique fails to resolve the modes with $\rho_i^R \approx \rho^{NR}$. There have been attempts^{3,8} to fit polarization CARS spectra of various proteins on the basis of the assumption that protein bands are either purely polarized $\rho_i^R = 0$ or depolarized $\rho_i^R = 0.75$. In Ref. 8 the authors decomposed the amide I' band with the three subelements assuming equal polarization properties (all ρ_i^R taken to be zero). Such an approach, though, appears to be inconsistent with the motivation of using the PS/CARS technique because of its unique sensitivity to the variations of ρ_i^R ratios. Accurate determination of the depolarization ratios was limited⁸ by significant pulse-to-pulse fluctuations ($\sim 30\%$) and was possible only for the $0.2\text{--}0.4$ range.

Because of the higher spectral quality in multiplex experiments as compared to scanning PS/CARS, the spectra with smaller signal-to-background ratio can still be resolved. This capability permits one to enlarge the range of ϵ -angle variations (up to $\pm 2^\circ$ in the given case) and, therefore, to make better use of the heterodyning technique. The latter enhances the accuracy with which the parameters are obtained from the simultaneous fitting of the set of spectra. The estimated fitting accuracy is $\sim 1.5\text{ cm}^{-1}$ for the band positions and is $\sim 8\%$ for the bandwidths. One can see from Fig. 3a–3d and Table I that, for the strongest bands, such as those at 1634 and 1651 cm^{-1} , the magnitude of ρ_i^R can be established with an accuracy of up to 0.05 , thanks to the high spectral quality achieved with the multiplex PS/CARS. The validity of our fits was verified by simulating the polarized Raman spectra of albumin with the best set of MCARS parameters. The simulated profiles are plotted in Fig. 4a and

FIG. 4. Polarized spontaneous Raman spectra of bovine albumin in D_2O (40 mg/mL, background-subtracted) and corresponding spectra (in bold) reconstructed from the best set of CARS fitting parameters.

4b together with the VV and VH spontaneous Raman spectra observed experimentally. Figure 4 shows a good correspondence between the curves obtained independently. The discrepancy, however, remains in the amplitude/depolarization ratio calculated for the relatively weak vibrations at 1421 and 1615 cm^{-1} .

As can be seen from Table I, a negligible amplitude of the mode at 1661 cm^{-1} , corresponding to β -sheet conformation, was calculated. This result agrees well with the data reported for bovine albumin.^{8,28,29} Figure 5 shows the relative fractions of the albumin substructures as calculated from the CARS fit parameters. Estimates obtained with other methods are given in Fig. 5 as well. Comparing the relative content of the conformations evaluated by scanning PS/CARS for human albumin⁸ (52% helical conformation vs. 47% of random coil) and the results of our PS/MCARS on bovine albumin (respectively 56% and 44%), it should be mentioned that, in Ref. 8, the estimates were made by comparing the

FIG. 5. Comparison between estimates of the relative content of albumin secondary structures obtained with multiplex PS/CARS (this work), spontaneous Raman²⁹ spectroscopy, circular dichroism,²⁸ and polarization-sensitive scanning CARS.⁸

amplitude ratios of amide I' contributors, instead of integrated areas. Inspection of Fig. 5 reveals reasonable correspondence of PS/MCARS data to the results obtained with CD and Raman spectroscopy. It shows that multiplex PS/CARS is capable of yielding sufficiently accurate estimates of the protein structural subelements.

CONCLUSION

A picosecond multiplex CARS spectrometer has been developed enabling the acquisition of $\sim 400\text{ cm}^{-1}$ broad spectra with spectral resolution of 3.4 cm^{-1} and high polarization accuracy. An improvement in signal-to-noise ratio, as compared to that with scanning techniques, is achieved by exploiting the statistically superior broadband CARS excitation and detection, and averaging over some 10^9 excitation events.

It has been demonstrated that background-free MCARS spectra of transparent samples can be obtained. Spectra of protein bovine albumin in D_2O have been resolved in the 1370–1730 cm^{-1} Raman range and fitted simultaneously with a single set of vibrational parameters. Major bands have been assigned to protein backbone modes (amide I') and CH_2 , CH_3 deformations. The amide I' band has been decomposed into the contributions of the protein's secondary structures for which the relative content was determined. Relative weights of α -helix and random coil conformations (56 to 44%) agree reasonably well with Raman and CD data available.

ACKNOWLEDGMENTS

The authors wish to thank Dr. B. Toleutaev for his valuable contribution to the design of spectrometer and for stimulating discussions. The experimental contribution of J. Kanger is also gratefully acknowledged.

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