UV resonance Raman spectroscopy reveals details of the "random coil" state of polypeptides

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Introduction

The question of how proteins fold to their unique and highly organised three-dimensional structure is one of the most challenging questions investigated in biological research. In recent years, the investigation of *dynamic* aspects of this folding process has been extended to the fastest processes, occurring on the nanosecond time scale. Such measurements require fast spectroscopic techniques for following structural changes. Although fluorescence methods offer many advantages in this context, they usually report only on local changes and/or require non-native chromophores, whereas intrinsic methods, such as UV circular dichroism (UVCD), IR or UV Resonance Raman (UVRR) spectroscopy, can be used for all proteins and report on the structural changes of the whole polypeptide backbone.

Time-resolved IR spectroscopy has been used with great success to follow protein folding with high signal-to-noise (S/N) [1,2]. However, we have shown recently that IR spectroscopy is not able to quantitatively measure the secondary structural content even of 'simple' α-helical model peptides [3]. UVCD spectroscopy has only very recently been implemented on the ns-time scale [4] and suffers from low S/N as well as low sensitivity for distinguishing between different helical conformations. UVRR spectra, on the other hand, can be taken with ns-time resolution and are greatly more sensitive towards secondary structures than IR or UVCD spectra, thus potentially providing more detailed insight into the processes occurring during the folding of a protein [5-8].

Here, we report results of cw-UVRR spectroscopic measurements on a simple polypeptide, namely polyglutamic acid (PGA). The spectra are interpreted using the theoretical framework developed by Asher and coworkers [5-8]. It is shown that in the so-called 'random coil' state the residues of PGA adopt a mixture of different local structures which, however, span only part of the Ramachandran-allowed region. These structures include polyproline II (PPII), extended β-strand and helical conformations. UVRR spectroscopy allows quantification of the relative contributions of these different conformations and it was found that these do not change greatly between 10 and 60°C.

Experimental

PGA (MW 64,000, Aldrich-Sigma, used as supplied) was dissolved in H_2O to a concentration of 5 mg/ml and the pH was measured using a pH meter equipped with a
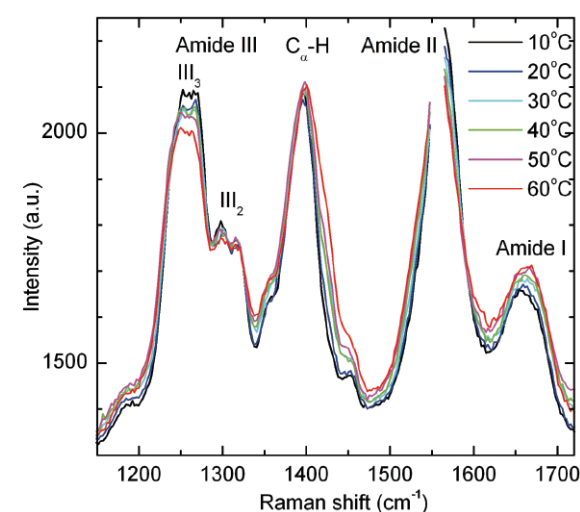

Figure 1. UVRR spectra of PGA at pH 7.6, 10 to 60°C, excited at 213 nm (data in the range 1547-1565 cm^{-1} were distorted by an experimental artifact and have been excised).

microelectrode. The sample was flowed through a home-built temperature-controlled IR cell (pathlength 0.25 mm, UV-grade CaF_2 windows) using a peristaltic pump. The whole cell was continuously moved orthogonal to the laser beam to avoid sample precipitation onto the window at the laser spot.

UVRR spectra were excited at 213 nm using the fifth harmonic of an amplified pulsed Nd:YVO_4 laser (AOT-YVO 20 QSP/MOPA, pulse width approx. 1 ns, repetition rate 12.5 kHz, 2 mW at the sample). Scattered light was collected in back reflection geometry, filtered in a subtractive double spectrometer, dispersed using a 3600 grooves/mm grating (Spex Triplemate 1877 B, f = 0.6 m) and detected with a CCD camera back illuminated and UV optimised (Andor iDus 420/BU2), yielding a spectral resolution of 7 cm^{-1}.

Results and discussion

Figure 1 shows the temperature-dependent UVRR spectra, excited at 213 nm, of PGA at pH 7.6, where most of the side chains of PGA are negatively charged, which prevents formation of the regular secondary structure observed at low pH, thus forcing the polymer to adopt the so-called 'random coil' form [9]. Due to the proximity of the amide $\pi \rightarrow \pi^*$ transition, UVRR excitation at 213 nm excites

predominantly the amide backbone vibrations (Amide I-III, see figure 1), and the C_α-H bending vibration, which for the 'random coil' state is strongly coupled to the Amide III vibration [5]. The UVRR spectra and the slight temperature-induced shifts of the bands shown in figure 1 are similar to those reported previously for PGA at pH 9 at 0°C and 70°C, excited at 204 nm, i.e. closer to the amide $\pi \to \pi^*$ transition [6].

As shown in figure 1, the Amide III region consists of several bands. These have been studied extensively and the Amide III_3 frequency has been shown to be particularly sensitive to the polypeptide backbone conformation due to coupling of this vibration with C_α-H bending, which depends on the relative orientation of the N-H and C_α-H bonds. It has been shown that for extended conformations, where the backbone is exposed to hydrogen bonding to water, the Amide III_3 vibrational frequency ν_{III3} (1200-1300 cm^{-1}) directly correlates to the Ramachandran backbone ψ angle according to Eq. (1) [5]:

$$\nu_{III3}(\psi)/cm^{-1} = 1256 - 54 \cdot \sin(\psi + 26°) - 0.11 \cdot T \qquad (1)$$

Here, T is the temperature (in °C) and the last term accounts for the effect of temperature-dependent hydrogen bonding to water molecules. It has to be noted that the dependence of the ν_{III3} frequency on the other Ramachandran angle, Φ, was shown to be essentially negligible. Similar equations have been developed for situations where significant secondary structure, stabilized by intra-peptide hydrogen bonds, is present [5].
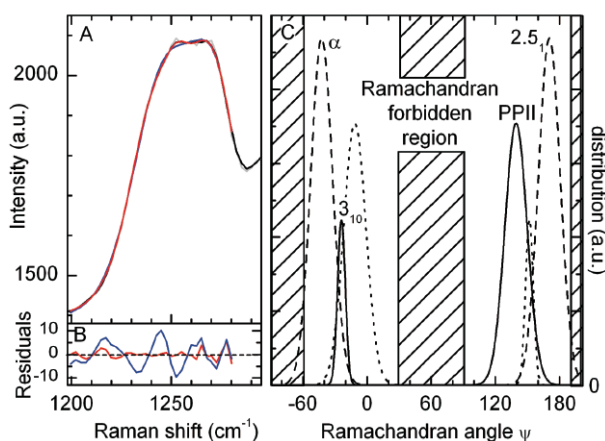

Figure 2. Deconvolution of the UVRR spectrum of PGA at pH 7.6, 10°C, in the Amide III_3 region. (A). Experimental spectrum (grey), 3-point smoothed spectrum (black) and fitted spectrum using two (blue) or three (red) Gaussian curves for the distribution of ψ angles. (B). Residuals of the fits in A. (C). Gaussian curves for the distribution of the Ramachandran angle ψ obtained from the three-Gaussian fit in A; solid lines indicate our preferred assignments, whereas dashed lines show distributions with ambiguous assignments and dotted lines are theoretical fit results which we suggest not to be of relevance, for details see text. Also indicated are the regions of ψ values which are forbidden due to steric repulsion [10].

	Gaussian 1	Gaussian 2	Gaussian 3
Centre angles and possible assignments:			
ψ_{centre} (1)	**140°**	**171°**	152°
	PPII (145°)	2.5_1 (170°)	
ψ_{centre} (2)	–12°	**–43°**	**–24°**
		α (–47°)	3_{10} (–26°)
Width	24.5°	23.9°	8.5°
Relative population	0.40	0.51	0.09

Table 1. Deconvolution fit results for the UVRR spectrum of PGA at pH 7.6, 10°C, in the Amide III_3 region. Given are the centre angles, widths (fwhm) and relative populations of the three Gaussian curves describing the distribution of the Ramachandran ψ angle. Two possible solutions for the centre frequency are presented due to the ambiguity of Eq. (1) for converting the Amide III_3 frequency ν_{III3} to ψ; also given are possible assignments of the fitted centre frequencies to characteristic structural elements, together with the ψ angles of the ideal structures, see text.

Asher and co-workers used this correlation for determining the inhomogeneous distribution of ψ, angles in polypeptide backbones by deconvolution of the Amide III_3 band [6-8]. This deconvolution assumes a homogeneous line width of 15 cm^{-1} (fwhm) for the UVRR Amide III_3 band, based on observations on peptide crystals. The method was applied to PGA at pH 9, albeit only for the spectrum at 0°C, and it was found that for this sample the distribution of ψ angles can be described by two Gaussian curves, centered on $\psi \approx 145°$, corresponding to the PPII structure, and $\psi \approx 170°$, which was assigned to a β-strand conformation with 2.5_1-helical structure [6].

We have analysed the UVRR spectra shown in figure 1 following a similar approach. Based on the results by Asher and coworkers, a two- or three-Gaussian distribution of ψ angles was assumed; using equation (1), this was converted to a distribution of ν_{III3} frequencies, from which the UVRR spectrum was calculated by convolution with a 15 cm^{-1} (fwhm) homogeneous Lorentzian band, assuming all conformations to have the same UVRR signal strength. The only free parameters (position, width and relative amplitudes of the Gaussian curves describing the distribution of ψ angles) were then varied to yield an optimum fit with the experimentally observed UVRR spectrum. The resulting fitted spectra and the fit residuals for the UVRR spectrum at 10°C are shown in figure 2.

The inclusion of a third Gaussian curve yields a significantly better fit, see the fit residuals shown in figure 2B. Thus, we conclude that in our sample a small fraction of the PGA backbone (approx. 9% at 10°C) is in a structure different to those found by Asher and coworkers, who did not see any indication for this structure at pH 9, 0°C. As discussed below, we propose these residues to adopt short 3_{10}-helical stretches. It has to be noted that our conditions (pH 7.6) are closer to those required for helix formation than those used by Asher and coworkers (pH 9). However, other factors, such as the significantly

larger MW of the PGA used in the present study, might also contribute to the difference. No helix formation has been observed for PGA at neutral pH using UVCD and related methods;[9] however, UVCD requires coupling between several residues and therefore is well known to not be sensitive to short helical stretches.

Figure 2C shows the individual Gaussian curves describing the distribution of ψ angles obtained from the three-Gaussian fit and Table 1 presents the parameters describing these curves. It is important to note that equation (1) does not yield a unique solution for ψ for a given Amide III_3 frequency v_{III3}; therefore, for each Gaussian two possible centre frequencies are given in Table 1.

The significant width of these distributions (similar to those found for PGA at pH 9, 0°C[6]) indicates that the polypeptide backbone does not adopt a rigid, well-defined structure, but fluctuates around certain preferred conformations. However, even in the "random coil" state not all ψ angles which are sterically allowed (i.e. are in the allowed regions of the Ramachandran plot[10], compare figure 2C) are actually adopted. Non-steric interactions between the side chains or between side chains and backbone seem to lead to a preferential selection of ψ angles in a restricted region of all possible conformations.

It is possible to determine the nature of the preferred conformations by comparing the centre frequencies to the Ramachandran ψ angles of idealized structural elements; for this it has to be kept in mind that, unlike the strongly coupled Amide I vibration, the Amide III_3 vibration is localized on individual residues, [6] so that the distribution of angles mirrors the distribution of *local structure* (i.e. the structure of individual residues) and not necessarily that of more extended *secondary structure*.

A large fraction of residues (~40%) is seen to have a Ramachandran ψ angle around 140° or –12°. In agreement with Asher and coworkers [6], we assign these residues to PPII-like structure, since the ideal PPII structure corresponds to a ψ value of 145° and a bulk of independent evidence has suggested PPII to be widely found in unfolded polypeptides [11].

Asher and coworkers assigned those residues with a ψ angle around 171° or –43° exclusively to β-strand conformation (ideal value of ψ: 170°, corresponding to a 2.5_1-helix). However, we note that an alternative interpretation is the assignment of these residues to a *local* α-helical like conformation ($\psi = –47°$). Although PGA at pH 7.6 does not form any (extended) helical secondary structure due to the repulsive interaction of its charged side chains, [9] there is no reason why individual residues should not adopt a local structure with this conformation; provided that only a few neighboring residues have a ψ angle near –47°, i.e. no full α-helical turn is formed, no side chains are in closer proximity than they would be in PPII or β-strand conformation. Therefore, we conclude that ~50% of residues are either in (local) α-helical or in β-strand conformation. It seems likely that both conformations are present to some extent at the same time, in addition to residues in PPII structure, thus approaching a true 'random coil'. No quantitative information on the
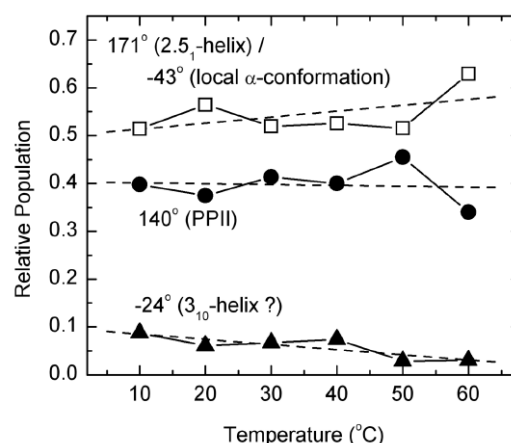

Figure 3. Temperature dependence of the relative populations of the different structures found for PGA at pH 7.6, calculated from the areas under the Gaussian curves describing the distribution of the Ramachandran ψ angle. Dashed lines are linear fits and are shown to guide the eye.

relative prevalence of α-helical and β-strand structure is available from our data, although we note that the large C_α-H bend band near 1400 cm^{-1} would be expected to be smaller if 50% of the residues were in local α-helical conformation, since coupling of the C_α-H bend to the Amide III vibration is greatly reduced in this conformation. However, a smaller contribution (<15-20%) of α-helical structures would be well compatible with the observed C_α-H bend band.

Finally, the additional structure found in our sample, which was not observed in PGA at pH 9[6], corresponds to a ψ angle of 152° or –24°. We tentatively assign this to 3_{10}-helical structure, which has a ψ angle of –26° [12]. These structures may be local (i.e. individual residues which are in 3_{10}-helical conformation) or may even be formed in short 3_{10}-helices, most likely consisting of not more than one turn, thus avoiding excessive electrostatic repulsion between the side chains and not detectable by UVCD and related methods. The latter interpretation is supported by the narrow width of this angle distribution and the decrease of its relative population with increasing temperature, see below. 3_{10}-helices are widely accepted as 'precursors' to the more widely found α-helical structure [13,14], so that it is intriguing to speculate that the structure observed here at pH 7.6, which was not seen at pH 9, indicates the onset of helix formation upon lowering pH. Further measurements, at different pH in the neutral region, will be required to investigate this issue further.

Fits of the UVRR spectra at higher temperatures yield very similar results. In all cases, inclusion of a third Gaussian curve in the ψ angle distribution significantly improves the quality of the fit. The centre frequencies of the three curves do not shift significantly with temperature, confirming their assignment to well-defined residue conformations which correspond to local minima on the potential energy surface on which the polypeptide backbone folds. Figure 3 shows the temperature dependence of the relative populations of these different

structures. The populations of the two majority structures do not change much over the temperature range 10-60°C. The population of the minority structure ($\psi = -24°$), on the other hand, decreases from 9% at 10°C to 3% at 60°C. This further supports the assignment of this structure to nascent 3_{10}-helices, since these would be expected to be stabilized by intrapeptide hydrogen bonds at low temperature, whereas at higher temperatures these hydrogen bonds should be overcome by entropy favoring a more disordered structure. However, as discussed above, more measurements will be required to confirm this assignment.

Conclusions

We have used UVRR spectroscopy to investigate the polypeptide backbone conformation of PGA under conditions where most of its side chains are charged. The results allow us to determine the distribution of Ramachandran ψ angles in the sample. We found that the residues of PGA in the so-called 'random coil' state are in a mixture of conformations, including PPII and extended β-strand, as previously reported. However, some ambiguity remains due to the properties of Eq. (1), which were ignored in previous publications; this leads us to suggest the presence of some residues in (local) α-helical conformation. We also found that at pH 7.6 a minority of residues at low temperatures are in a conformation tentatively assigned to nascent 3_{10}-helices which were not observed at pH 9; the population of this structure decreases with increasing temperature, as would be expected for a structure stabilized by intra-peptide hydrogen bonds.

Acknowledgements

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