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ARTICLE *in* JOURNAL OF MOLECULAR STRUCTURE · MAY 2010

Impact Factor: 1.6 · DOI: 10.1016/j.molstruc.2010.02.014

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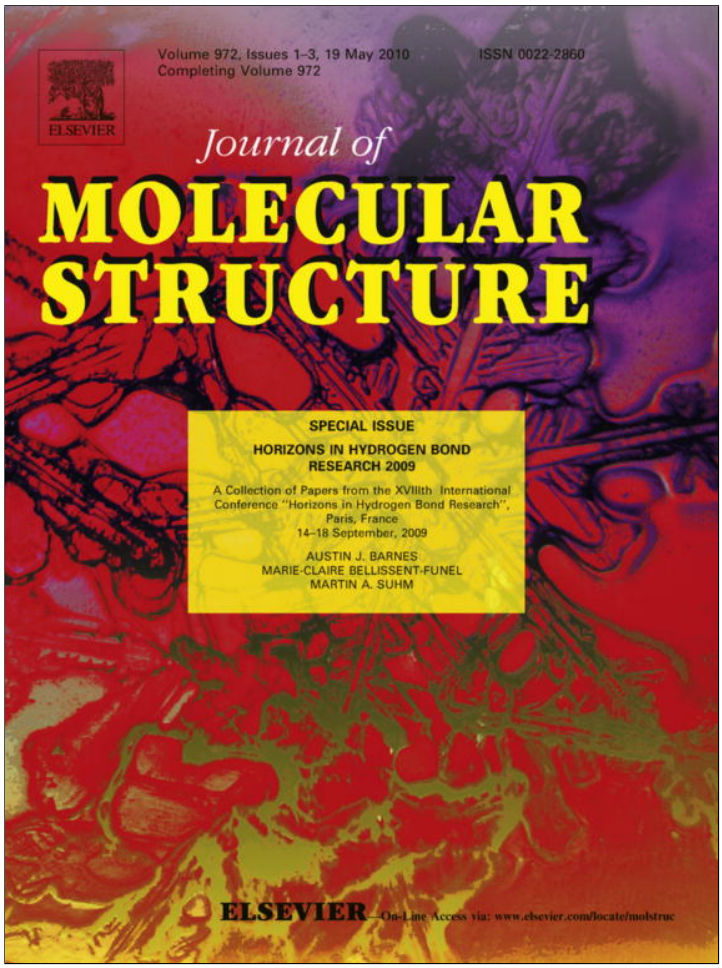

Journal of Molecular Structure 972 (2010) 87–91

Contents lists available at ScienceDirect

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Vibrational and structural investigation of SOUL protein single crystals by using micro-Raman spectroscopy

Barbara Rossi [a,b,*], Marco Giarola [c], Gino Mariotto [c], Emmanuele Ambrosi [d], Hugo L. Monaco [d]

[a] Department of Physics, University of Trento, Via Sommarive 14, 38050 Povo, Trento, Italy
[b] INFM CRS-SOFT, c/o University of Roma "La Sapienza", 00185 Roma, Italy
[c] Department of Computer Science, University of Verona, Strada le Grazie 2, 37134 Verona, Italy
[d] Department of Biotechnology, University of Verona, Strada le Grazie 2, 37134 Verona, Italy

ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form 2 February 2010
Accepted 4 February 2010
Available online 10 February 2010

Keywords:
Protein crystals
Protein SOUL
Secondary structure
Raman spectroscopy
DFT computation

ABSTRACT

Protein SOUL is a new member of the recently discovered putative heme-binding protein family called SOUL/HEBP and, to date, no structural information exists for this protein. Here, micro-Raman spectroscopy is used to study the vibrational properties of single crystals obtained from recombinant protein SOUL by means of two different optimization routes. This spectroscopic approach offers the valuable advantage of the *in-situ* collection of experimental data from protein crystals, placed onto a hanging-drop plate, under the same conditions used to grow the crystals. By focusing on the regions of amides I and III bands, some secondary structure characteristic features have been recognized. Moreover, some side-chain marker bands were observed in the Raman spectra of SOUL crystals and the unambiguous assignment of these peaks inferred by comparing the experimental Raman spectra of pure amino acids and their Raman intensities computed using quantum chemical calculations. Our comparative analysis allows to get a deeper understanding of the side-chain environments and of the interactions involving these specific amino acids in the two different SOUL crystals.

1. Introduction

Protein SOUL (or heme-binding protein 2, HEBP2, or placental protein 23, PP23) is a new member of the recently discovered putative heme-binding protein family called SOUL/HEBP [1]. The first member of this family was p22HBP (heme-binding protein 1, HEBP1), a protein of 190 amino acid residues purified from mouse liver cytosol with no apparent homology to any other known heme-binding protein. Human and mouse HEBP1 can bind Fe–hemin with a binding stoichiometry of one heme molecule per protein [2]. Subsequent studies suggest that these proteins may probably be generic tetrapyrrole-binding proteins [3]. Heme proteins play a critical role in O_2 transport (hemoglobin) and storage (myoglobin) as well as in electron transfer (cytochromes) and in the activation of the O–O bond (cytochrome p450 and peroxidases). Specifically, all these heme-based proteins are able to respond to changes involving the electronic and the ligand-binding states of heme molecules thus playing a variety of physiological functions inside the cells [4].

Protein SOUL was identified at the transcriptional level in chicken retina and pineal gland by suppression subtractive hybridization. Related genes were also found in other living organisms, such as human, mouse, rice, tobacco, *Arabidopsis thaliana* and *Escherichia coli* [1,5]. Because of its sequence homology with p22HBP, a well recognized heme protein, and its heme-binding properties, protein SOUL has also been called with the alternative name heme-binding protein 2 [4].

Functional studies on HEBP2 are not numerous and the role of SOUL protein is still unknown. On the basis of its binding ability and expression pattern, HEBP2 may function as a heme sensor by interacting with other proteins [4]. However, sequence analysis showed that the putative hydrophobic heme-binding region on HEBP1 is not present in HEBP2 due to the presence in its homologous stretch of three charged amino acids [1]. An important piece of information missing is the three-dimensional structure of SOUL which could provide insight into its function.

Investigations of protein structure by Raman spectroscopy have been run for more than three decades, during which specific band assignments, signatures of secondary structure and Raman markers of side-chain environments have been established [6]. Several studies have demonstrated that new insights into protein folding, assembly and aggregation can be obtained by Raman spectroscopy and the technique has been also used to characterize intrinsically unstructured proteins [7–9]. During the last years, micro-Raman spectroscopy became a versatile tool in proteomics and biotechnology and was extensively used in structural biology

* Corresponding author. Address: Department of Physics, University of Trento, Via Sommarive 14, 38050 Povo, Trento, Italy. Tel.: +39 0461 88294.
E-mail address: rossi@science.unitn.it (B. Rossi).

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doi:10.1016/j.molstruc.2010.02.014

investigations, in particular for the study of ligand-binding and for the assignment of protein secondary structure. Recent papers have shown that Raman microscopy can provide useful details on molecular processes occurring in protein crystals and in other protein-based samples [10–12]. In fact, an important advantage of Raman microscopy is that the data can be collected from protein crystals *in-situ*, in a typical hanging-drop plate, under the same conditions used to grow the crystals [10,11], thereby ensuring sample stability and allowing its subsequent use in other experiments. Raman microscopy has been applied to non-invasively monitor protein crystal quality during its growth [13] and to characterize the state of protein crystallization [14], showing that the integrated spectroscopic/crystallographic approach is a powerful new tool to investigate these systems [15].

Generally speaking, vibrational spectroscopy is a sensitive probe of local structure in biological macromolecules and the structural information obtainable from vibrational spectra can be enhanced by investigating oriented bio-molecules using polarized radiation. In fact, the polarization analysis of the scattered light has long been established as a powerful method to elucidate symmetry degree, preferential orientations, and order/disorder effects in crystalline structures. As it concerns the biological systems, polarized Raman spectroscopy provided, for instance, new insights into the orientations of α-helix structure domains in protein molecules [16] and into protein and DNA residue orientations in the Filamentous Virus Pf1 [17,18]. Moreover, polarized Raman spectroscopy has been used also to monitor hemoglobin protein ordering effect when the red blood cell is stretched by means of optical tweezers [19].

Recently, we used Raman microscopy to investigate the vibrational properties of crystals obtained from wild-type and from selenomethionine-labelled protein SOUL, evaluating the relative amount of selenomethionine replacement in the crystals of SOUL by comparative analysis of the Raman intensity of amino acids marker peaks [20]. In the present work, we report on the results of a Raman investigation aimed at getting deeper insight on two different crystalline forms obtained from wild-type protein SOUL. The spectral ranges of amides I and III have been inspected and a tentative assignment of some spectral components to characteristic secondary structures is hereafter proposed. Moreover, some side-chain marker bands observed in the Raman spectra of SOUL crystals have been investigated; these Raman peaks are assigned to vibrational modes of some specific amino acids, i.e.: tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe). On the basis of the comparison between experimental Raman spectra of the pure amino acids and Raman intensities computed using quantum chemical calculations, an accurate analysis of these normal modes has been done, thus allowing for a deeper understanding of the side-chain environments and of interactions involving these specific amino acids when the SOUL protein is arranged in the two different crystal forms.

2. Materials and methods

Recombinant human protein SOUL was obtained by inserting the coding sequence into the pQE50 vector, which allows the expression of the desired protein with a 6-histidine tag at the C-terminus which is used to purify the protein by affinity chromatography from the bacterial lysate. A thrombin cleavage site was introduced upstream this tag in order to facilitate its removal after protein purification. The resulting construct was transformed into *E. coli* SG13009 (pREP) strain [21]. Protein expression was induced with IPTG (1 mM final concentration) when the OD_{600} reached 0.6 and hold for 16 h at 20 °C. The cells were harvested by centrifugation, resuspended and disrupted by sonication. The soluble fraction of the lysate was loaded onto an IMAC (IMAC = immobilized metal ion affinity chromatography) column and elution was performed with a linear 0–0.5 M imidazole gradient. Fractions showing higher SOUL purity were pooled, concentrated and treated with thrombin. The protein was further purified after tag removal by gel filtration (Superdex G200) and hydrophobic interaction chromatography (Lipidex 1000).

Protein SOUL crystals were obtained with the vapour-diffusion technique, both in hanging-drop and sitting-drop; SOUL crystals grew in two different conditions, later optimized in order to get crystals suitable for X-ray diffraction experiments. The two crystalline forms belong respectively to the hexagonal crystal system (**A1**) and to the orthorhombic crystal system (**A2**).

Raman scattering measurements on protein crystals and pure amino acids were carried out in backscattering geometry at room-temperature using a triple-monochromator (Horiba-Jobin Yvon, model T64000), set in double-subtractive/single configuration and equipped with holographic gratings having 1800 grooves/mm. The exciting radiation at 514.5 nm, provided by a mixed Ar–Kr ion gas laser (Spectra Physics, model Satellite 2018 RM), was focused through a long working distance 80× objective (NA = 0.75) directly on the protein crystals (the power on the sample surface being of the order of 10 mW, while the irradiated spot area was of the order of 10 μm^2). The scattered radiation was detected at the spectrograph output by a charge-coupled device (CCD) detector, with 1024 × 256 pixels, cooled by liquid nitrogen. In this configuration the spectral resolution was about 0.6 cm^{-1}/pixel. During the measurements, a single crystal, previously transferred to a hanging-drop of suitable storage solution in a 24-well plate, was placed, in turn, on the microscope stage and viewed by using the video camera incorporated into the microscope. Polarized Raman spectra were carried out either in parallel (VV) or in crossed (VH) polarization configuration, where, according to usual notation, the first element of the pairs into parentheses refers to the electric field direction of the incident radiation while the second elements refer to that of the scattered radiation. Raman scattering observed from all the samples was generally super-imposed over a continuous, nearly flat luminescence background, which was properly accounted for in the spectra analysis by comparing replicated spectra of each sample over the whole spectral range.

Quantum chemical calculations on the amino acid molecules were performed using the GAUSSIAN 03 program suite [22] and unrestricted DFT [23]; the non-local B3LYP functional hybrid method was employed [24] and the 6-31G(d) basis set [25] was used for geometry optimization and wavenumber analysis. For the plots of theoretical Raman spectra a Lorentzian lineshape with a line width of 4.0 cm^{-1} was used.

3. Results and discussion

Strongly polarized peaks are observed in room-temperature Raman spectra carried out from both SOUL crystals **A1** and **A2**, thus revealing a remarkable dependence of the Raman activity of the related modes on the crystal orientation. However, this dependence needs for further and more careful *ad hoc* investigations and hereafter only the spectra recorded in parallel polarization configuration (VV) will be here shown and discussed.

In Fig. 1 we report the experimental Raman spectra of SOUL crystals **A1** and **A2** in the wavenumber range between 1200–1330 (left panel) and 1570–1740 cm^{-1} (right panel) where the vibrational spectra of proteins are particularly sensitive to changes occurring in polypeptide secondary structure and conformation; in particular amides I and III bands show quite strong intensity and their wavenumbers can be correlated with dihedral angles and hydrogen bonding, thus revealing characteristic features of the protein secondary structure [26–28]. The spectra reported in Fig. 1 have been collected *in-situ* on the crystals transferred in a suitable storage solutions (see Section 2) rather than in mother li-

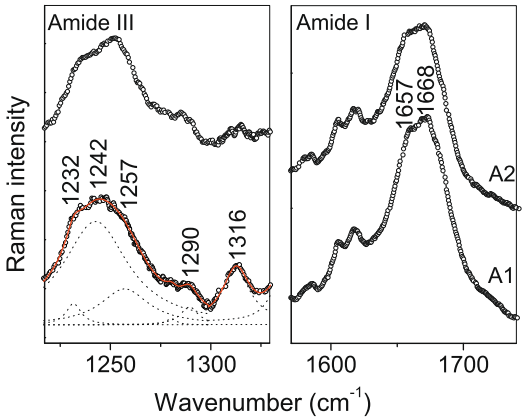

Fig. 1. Experimental Raman spectra of SOUL crystals **A1** and **A2** in the spectral ranges of amides I (right panel) and III (left panel). This one shows both experimental data (empty circles) and Lorentz fit of data (dotted lines).

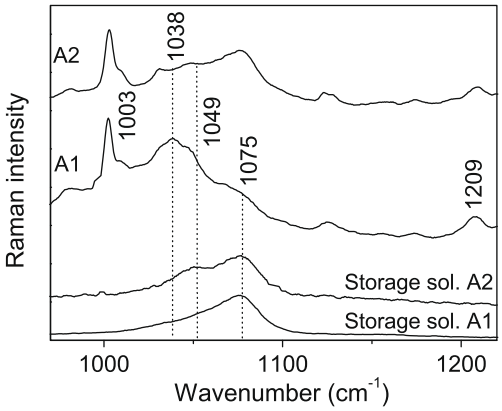

Fig. 2. Experimental Raman spectra of SOUL crystals **A1**, **A2** and of respective storage solution in the spectral regions 950–1220 cm^{-1}.

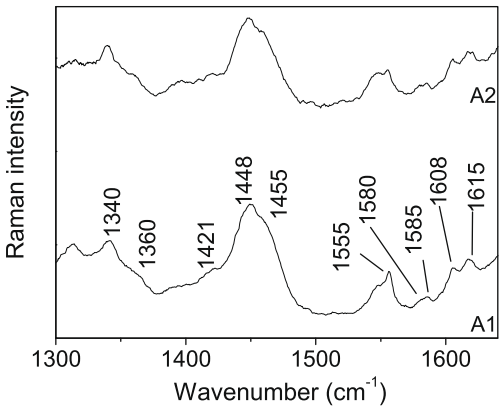

Fig. 3. Experimental Raman spectra of SOUL crystals **A1** and **A2** in the spectral regions 1300–1650 cm^{-1}.

quor since the Raman spectrum of the latter solution showed some interfering bands in the spectral ranges of interest. Nevertheless, we have verified that this procedure does not induce any significant damage on both the protein crystals of SOUL. Raman spectra of the pure storage solutions have been recorded and analysed, in order to distinguish in the spectra of crystals the components related to protein vibrations and those deriving from storage solution. The experimental data have been analysed using Lorentz curve fitting procedure in order to obtain a good deconvolution of the principal spectral components.

For a more reliable comparison between the spectral features of the two crystals, Raman spectra reported in Fig. 1 were normalized to the intensity of the 1460 cm^{-1} Raman band which is assigned to CH-deformation vibrational modes and is commonly used as an internal standard [28]. In the amide I region the major component observed at 1668 cm^{-1} in both the spectra of **A1** and **A2** SOUL crystals is associated with β-sheet and random-coil structures [29,30], while the band at 1657 cm^{-1} is attributed to α-helices [31].

The amide III range (1230–1320 cm^{-1}) is relatively free from side-group vibrations and, is thus highly diagnostic of characteristic protein secondary structure. In both spectra shown in Fig. 1, left panel, the bands at 1232 and 1242 cm^{-1} suggest the presence of β-sheet [31] and random-coil structure, respectively [29,30]. A contribution derived from non-ordered conformations can be inferred by the appearance of a broad amide III component at about 1257 cm^{-1} in all the spectra [31,32], although β-sheet structures could also contribute. The weak Raman feature at 1290 cm^{-1}, observed in both SOUL protein crystals is presumably due to β-turns, according to the theoretical predictions reported for the normal modes of β-turns [33]. Moreover, this assignment can be supported also by the spectra of solid poly-glycine and poly-L-alanine [9], two polypeptides characterized by β-turn structure whose Raman spectrum shows intense bands in the range 1290–1300 cm^{-1}. Finally, the Raman spectra of **A1** and **A2** SOUL crystals exhibit a component at 1316 cm^{-1} related to α-helical structure which has been already observed in the amide I region. Besides the amides I and III bands, a characteristic of α-helical conformation is the appearance of two spectral components at 890 and 945 cm^{-1}, but these peaks were not easily observed in our spectra because they fall in a spectral region with intense interfering bands of the storage solution and therefore they were not included in the figure.

By comparing the spectra of **A1** and **A2** SOUL crystals shown in Fig. 1 and taking into account the differences due to spectral resolution, we can note that the profile is very similar for the two crystals, providing strong evidence, as expected, for equivalent secondary structure in the two different crystal forms.

Figs. 2 and 3 display, respectively, the experimental Raman spectra of SOUL crystals **A1** and **A2** observed in the regions between 950–1220 and 1300–1650 cm^{-1} where, some amino acid side-chain marker peaks, specifically assigned to Trp and Phe, occur. Fig. 2 also shows the Raman spectra of storage solutions of both crystals. In view of an exhaustive discussion of spectra shown in Figs. 2 and 3, we have carried out micro-Raman spectra from Trp and Phe amino acids in solid state phase as reference samples to discuss the spectra of Figs. 2 and 3.

The Raman spectrum of pure amino acids Phe and Trp, in solid state form, was shown in the wavenumber range 900–1700 and 1200–1700 cm^{-1} in Fig. 4(a) and (c), respectively, as compared with the theoretical spectrum of Phe (4(b)) and Trp single-molecule (4(d)) obtained by quantum chemical calculations. A systematic downward shift in the computed Raman line positions with respect to the experimental ones can be observed, this mismatch probably deriving from anharmonicity effects and from the general tendency of the quantum chemical methods to overestimate the force constants of bonding among light atoms at the equilibrium configuration [34]. Despite this, the wavenumber and relative intensities of the computed peaks allows for a reliable attribution of the observed main spectral components. The amino acid Phe exhibits a number of aromatic ring vibrations, the intensities of which cannot be influenced by external factors [35]. By comparing the experimental and computed Raman spectrum of Phe in Fig. 4(a) and (b), on the basis of the wavenumber and relative intensities of the computed peaks, the most intense experimental peaks of Phe at 1003 and 1038 cm^{-1} can be identified with the computed ones at 1018 and 1059 cm^{-1}. They correspond to CH

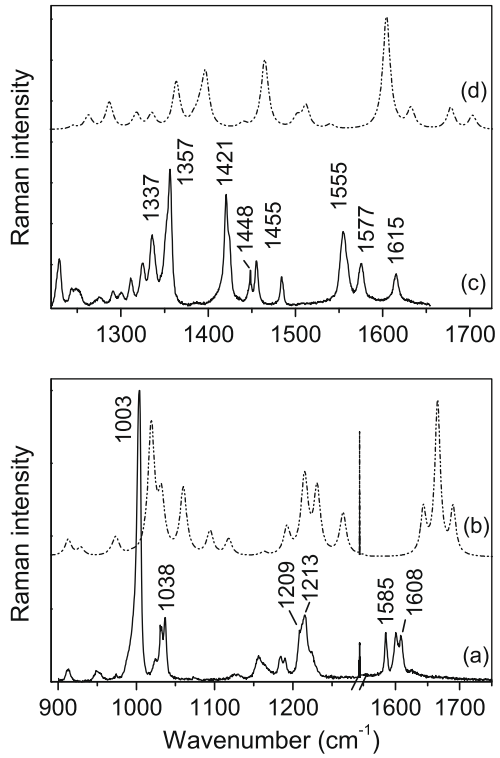

Fig. 4. Experimental Raman spectrum of pure solid state Phe (a) and Trp (c) and DFT-computed Raman spectrum of a single-molecule of Phe (b) and Trp (d).

bending and ring breathing modes of the aromatic ring, as it can be inferred from the analysis of the corresponding normal modes, and definitively agree with the assignments of Harada and Takeuchi [35]. The sharp peak at 1585 cm^{-1} and the spectral components observed at 1608, 1209 and 1213 cm^{-1} in the experimental spectrum of Phe (which are identified with the peaks at 1644, 1690, 1216 and 1231 cm^{-1}, respectively, in the computed spectrum) also correspond to modes related to vibration mainly localized on the aromatic ring. A detailed representation of the atomic displacements vectors of these vibrational modes is found in [35], where they have been considered to be insensitive to the environments.

All the vibrational modes assigned to phenylalanine are present in both the Raman spectra of SOUL crystals **A1** and **A2** (Figs. 2 and 3), where they show the same intensity, with the only exception of the peak at 1038 cm^{-1} which appears much more evident in the **A1** crystal spectrum. However, this spectral difference is of difficult interpretation since in this wavenumber range also the interfering bands of storage solutions at 1049 and 1075 cm^{-1} fall; these bands are observed at the same wavenumber in both the Raman spectra of storage solutions (Fig. 2) used for crystals **A1** and **A2**, but they show different intensities, making difficult a reliable deconvolution of the protein peaks in this spectral range.

The ratio between the intensity of the Raman bands observed at 622 and 644 cm^{-1} in the crystals of SOUL (not shown in this report [20]), and assigned to ring bending vibrations of Phe and Tyr, respectively [30] can be used to verify the ratio between the number of Phe and Tyr present in the sequence of the protein [28]. The molar ratio between Phe and Tyr derived from the known amino acid composition of SOUL is 0.912; this value is very close to the one obtained by Raman data (0.906) following the known equation $(I_{622}/I_{644}) \times 1.25$ = Phe/Tyr [28] (where I is the intensity of the corresponding Raman bands), confirming the reliability of our Raman spectra to get structural information.

The amino acid Trp displays a number of characteristic bands, most of them due to indolic ring vibrations, as deducted by comparing the experimental Raman spectrum of pure Trp 4(c) and the DFT-computed one 4(d). The experimentally observed peaks at 1421, 1448, 1555, 1577 and 1615 cm^{-1} have been identified with vibrational modes mainly involving the C=C and C–H bonds of indolic ring of Trp, as confirmed also from the assignment made by other author [35]. All these modes are also present in the spectra of both SOUL crystals (see Fig. 3) and therefore these bands should be ascribed to side-chain vibrations of Trp.

Moreover, the peak observed at 1455 cm^{-1} in the spectrum of pure Trp and appearing in SOUL crystal spectra as a shoulder of the more intense band at 1448 cm^{-1} should be assigned to the CH_2 bending vibrational mode.

As for the characteristic Raman peaks of Trp, the doublet falling at 1337 and 1357 cm^{-1} in the spectrum of the pure amino acid (Fig. 4(c)) and occurring at 1340 and 1360 cm^{-1} in the spectra of crystals (Fig. 3) deserves a further discussion. These experimentally observed peaks, identified with the DFT-computed normal modes at 1363 and 1396 cm^{-1}, are related to bending vibrational modes of CH and CH_2 groups and their relative intensity is influenced by the environment; the reason for the high sensitivity is that these modes arise from Fermi resonance. Therefore, the spectral profile of this doublet reveals whether the Trp side-chain is buried or exposed [28,35]. For example, the occurrence of a sharp and intense band at 1360 cm^{-1} is an indication of a buried Trp side-chain or of an increase of the hydrophobicity of the Trp environment [35,36]. Thus, in the spectra of SOUL crystals the presence of the band at 1360 cm^{-1} in the form of a small shoulder of the more intense peak at 1340 cm^{-1} might be interpreted as the Trp side-chains being exposed, in both the protein arrangements within the investigated crystals.

On the other hand, the comparative analysis of the spectra reported in Figs. 2 and 3 seems to suggest no significant difference between the hydrogen bonding state and the micro-environment of Phe and Trp amino acid side-chain, despite the different conditions of pH and precipitant in which the crystals are grown and stored. This is consistent on what found in the electron density maps, where we observe some Trp and Phe residues are exposed to the protein surface and the other are buried, substantially showing the same micro-environment in the two crystals. This suggests that the packaging of SOUL protein in the two crystal forms does not significantly affect the micro-environment of these amino acids.

Important insights on protein structures can be also obtained from the analysis of some specific vibration modes assigned to the amino acid Tyr; in fact, the hydroxyl group of Tyr can be easily involved in hydrogen bond formation. Among many Raman bands of Tyr, the peaks at 828 and 851 cm^{-1}, assigned to the out-of-plan ring bending and to the ring breathing vibrations of the phenolic ring of Tyr (generally referred as tyrosine Fermi doublet [9,28,35]) are considered to be particularly useful for investigating the tyrosine side-chain environment, since the intensity ratio of these peaks has been found to be sensitive to the nature of hydrogen bonding, or to the ionization state of the phenolic hydroxyl group. Therefore, this intensity ratio can be used to identify "buried" and "exposed" Tyr moieties [9,28,35]. It is well recognized that when the I_{830}/I_{850} ratio is found to be small this can be interpreted as the tyrosine side-chains being exposed or hydrogen bonded. Of course, there could be a slight wavenumber shift of these peaks, depending on the protein.

In Fig. 5, the Raman spectra of SOUL crystals **A1** and **A2** are shown in the wavenumber ranges 790–880 cm^{-1}, where the characteristic Fermi Tyr doublet occurs. In order to allow a comparative analysis, the spectra of Fig. 5 were preliminarily normalized to the total intensity of the peaks centred at 622 and 644 cm^{-1}, which were assumed by us to be reliable internal standards being related to the ring bending vibration of Phe and of Tyr, respectively. This normalization procedure assumes, in fact, that the total number of Phes and Tyrs is the same in both crystals of SOUL.

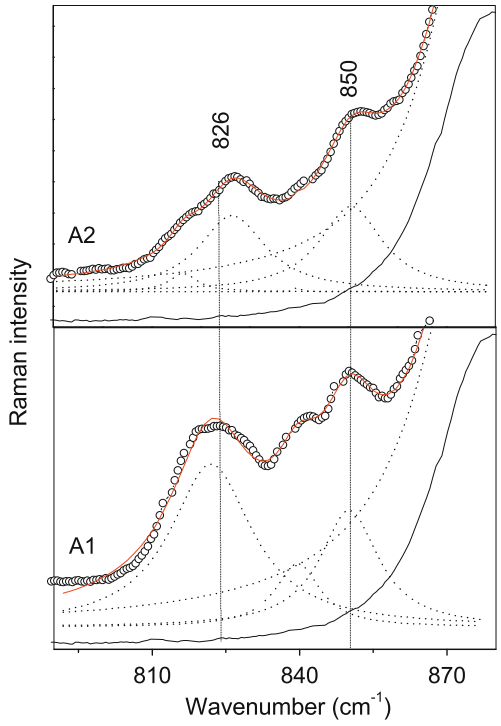

Fig. 5. Experimental Raman spectra of SOUL crystals **A1** and **A2** in the region between 790 and 880 cm^{-1}; experimental data (empty circle) and Lorentz fit of data (dotted and red line). The black full line represents the spectral component of storage solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

The intense spectral component observed in both spectra in Fig. 5 at 897 cm^{-1} is due to the presence of storage solution whose contribution can be isolated (black full line) from the other spectral components. Using a Lorentz fit of the experimental data (dotted line in Fig. 5), clearly it shows that the tyrosine Fermi doublet intensity ratio is very different for the SOUL protein in the two crystal forms, obtaining from our data a I_{826}/I_{850} ratio of 1.4 for **A1** and of 0.98 for **A2** crystal. This results seem suggest a different micro-environment for Tyr side-chain residues or a different hydrogen bond network involving the hydroxylic groups of this amino acid in the two SOUL crystals, probably due to the different arrangement of the protein molecules in the crystal unit cells. In particular, the increasing of the intensity of the peak at 850 cm^{-1} in the spectrum of **A2** crystal respect to that observed for **A1** crystal reveals that in the former crystal arrangement SOUL protein shows a major exposition to the solvent of the Tyr side-chain residues.

4. Conclusion

In-situ Raman scattering experiments were performed on two different crystals, placed in a typical hanging-drop plate. The sample were obtained from wild-type protein SOUL, a new member of the recently discovered putative heme-binding protein family with the aim of investigating their vibrational properties. The spectral regions of amides I and III Raman modes were inspected in order to identify characteristic secondary structures of this protein. Moreover, we also focused our attention on some side-chain marker bands observed in the Raman spectra of SOUL crystals which were assigned to specific vibrational modes of Tyr, Phe and Trp. By using a combined experimental–numerical approach, based on the comparison between the experimental Raman spectra of the pure amino acids and the Raman intensities computed using quantum chemical calculations, an accurate analysis of the normal modes in the spectral range of interest was made. The method yielded a deeper understanding of the side-chain environments and the interactions involving these specific amino acids in the two different SOUL crystals.

Acknowledgements

This work has been supported by P.A.T. (Provincia Autonoma di Trento). The financial support of the Fondazione Cariverona is also gratefully acknowledged.

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