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Short communication

Raman spectroscopy of Na/K-ATPase with special focus on low-frequency vibrations

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ABSTRACT

Na/K-ATPase powder samples were analyzed, both in lyophilized and rehydrated states, by a latest generation Raman spectrometer mounting Ultra Low Frequency (ULF) filters that allowed investigating even the lower frequencies ($<500\,cm^{-1} = 15$ THz). While several known peaks, which are assigned in the literature to molecular vibrations of chemical groups or amino acids, were recognised in the 500–3500 cm^{-1} range, the analysis of the sub–500 cm^{-1} region, performed with two different resolutions, allowed finding unassigned peaks. Such low-frequency peaks are apparently unknown in the current literature for Na/K-ATPase. Recent studies suggested that low-frequency collective vibrations that involve large protein portions are strictly correlated to protein functions. Thus, the present results could be of interest with regard to possible connections between low-frequency mechanical vibrations and the operating mechanism of the sodium-potassium pump.

1. Introduction

Protein dynamics is strictly connected with protein biological function. Given the high complexity of biological systems, both experimental and numerical techniques are intensively used, in combination or independently, to investigate protein structural vibration. At present, most of the attention is focused on low-frequency collective modes, i.e., those thought having a great influence on controlling structural configuration changes (folding) [1–4]. Delocalized vibration modes of proteins, both crystallized and in solution, were found in the fields of GHz and THz, e.g. see [5,6]. Many attempts to measure protein vibrational spectra in the Terahertz range were done aiming at finding peaks that could be assigned to biochemically relevant motions [1]. However, measuring such low-frequency vibration modes in proteins is very complicated.

On the other hand, interesting theoretical results were obtained by the thermodynamic approach to biosystems, with possible important implications for anticancer therapy [7,8]. Recent studies highlighted the concept of thermodynamic resonance, for which low-frequency electromagnetic waves have important effects on biological systems since they interfere with the biological structures [9–14]; results indicate a reduction in tumor growth. In particular: [9] furnishes metabolic consequences; [10,11] discuss the growth and diffusion of metastases, showing how the low-frequencies reduce the tumor growth; [12] provides experimental evidence of such minor growth; [11,13,14] develop a theoretical thermodynamic model.

In a previous study, the authors investigated experimentally (by Raman spectroscopy) and numerically (by normal-mode calculations) low-frequency vibrations in lysozyme [15]. Here we present Raman spectra obtained on lyophilized and rehydrated powder samples of sodium-potassium pump (Na/K-ATPase), focusing the attention on the sub-500 cm^{-1} region. As a matter of fact, to the best of the authors' knowledge, experimental results on low-frequency vibration of the sodium-potassium pump are not easy to find in the literature. Therefore, they could be of interest, given the biological relevance of such protein.

Na+/K+-adenosine triphosphatase (Na/K-ATPase) is the most prominent member of the P-type adenosine triphosphatase (ATPase) family, that includes sarcoplasmic reticulum Ca2+-ATPase and gastric H+/K+-ATPase, among others [16]. This protein, also known as sodium potassium pump, is found in the plasma membrane of all mammalian cells and it is finely regulated. Na/K-ATPase pumps three Na+ ions out and two K+ ions into the cell per molecule of ATP hydrolysed; as a consequence, it creates concentration gradients across the plasma membrane. These gradients of Na + and K + ions, created and maintained by Na/K-ATPase, are used for many purposes, including the generation of action potentials along nerves, or acting as an energy source for

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secondary active transport. In addition to the catalytic unit (the α-subunit) of approximately 1000 residues, Na/K-ATPase contains a heavily glycosylated β-subunit of about 300 residues and a tissue-specific auxiliary regulatory subunit of approximately 70–180 residues. The latter are known as FXYD proteins and modulate Na/K-ATPase function according to the specific demands of a given tissue. In humans, there are four isoforms for the α-subunit, three for the β-subunit, and seven for FXYD, leading to different Na-K ATPase variants. Furthermore, Na/K-ATPase is now regarded as a key player in cell adhesion, as the β-subunit was shown to act as a K+-dependent lectin. Finally, aberrant expression and activity of Na/K-ATPase may be implicated in the development and progression of various cancers [17]. Thus, Na/K-ATPase is now recognized as an important therapeutic target [18]. As it can be seen from the previous brief discussion on Na/K-ATPase physiological function, the sodium potassium pump is a complex system that is pivotal in the regulation of cell homeostasis.

The three-dimensional structure of Na/K-ATPase was described by Shinoda and Cornelius, who obtained a 2.4-ångström (240 pm) resolution model via X-ray crystallography [19]. The shape of this enzyme is elongated, with two sort of horns that extend inside the cell and a globular part outside the cell. The entire protein has a main size of 15–16 nm, and counts 10131 atoms without hydrogen. Raman spectroscopy was mainly used to monitor the changes of the secondary structure in the Na/K-ATPase (the E2 —› E1 transition induced by changes in the concentration of Na^+ and K^+) occurring in the amide I region [20,21], but with conflicting results. Raman measurements in the above–300 cm^{-1} region were performed in [22], where conformational changes in membrane-bound Na/K-ATPase are investigated.

In the present paper, we present Raman measurements on lyophilized and rehydrated powder of Na/K-ATPase from porcine cerebral cortex. After showing the results of broad-range Raman measurements and comparing them to literature data, we present the results of measurements made in the sub-500 or sub–300 cm^{-1} region with the purpose of investigating vibrations involving large protein portions. Normal-mode calculations on a Na/K-ATPase all-atom model are in progress: the results will be presented elsewhere.

2. Materials and methods

Tests were conducted on samples of Adenosine 5′-Triphosphatase from porcine cerebral cortex (Sigma-Aldrich), a lyophilized powder containing ≥0.3 units/mg protein, pH 7.8. In addition, samples were also analyzed in the rehydrated state. Rehydration was obtained adding a drop (0.05 ml) of Milli-Q water (Millipore) on a powder sample of 0.05 g.

The experimental apparatus consisted in a LabRAM HR evolution Raman spectrometer (Horiba Scientific), mounting a confocal microscope equipped with three objectives (20×, 50×, 100×) coupled to a 633 nm wavelength He-Ne laser. This spectrometer is endowed with two diffraction gratings having resolutions respectively equal to 600 lines/mm (<3 cm^{-1}) and to 1800 lines/mm (<1 cm^{-1}); moreover, it mounts Ultra Low Frequency (ULF) filters of the latest generation allowing Raman spectroscopic information in the sub–100 cm^{-1} region, with measurements down to 5 cm^{-1} routinely available, without any limitation in the higher wave-number region. Stokes and Anti-Stokes spectral features can be simultaneously measured [23].

A preliminary calibration of the spectrometer was done prior to the measurements, adopting the emission spectrum of a Neon lamp as a reference. The experiments were conducted in room conditions, at the temperature of 22 °C.

Acquisitions were made using 600 or 1800 lines/mm diffraction grating, 100× objective and 200 μm confocal hole. For each measure, different spectral ranges were analyzed and then results were collected in a unique spectrum. Each result was the mean of 10 measures of 60 s each. Polynomial baseline correction (polynomial degree ≤3) was applied to each original spectrum in order to eliminate the background noise. Data were processed with LabSpec software, supplied with the spectrometer adopted.

3. Results and discussion

Fig. 1 shows a broad-range Raman spectrum obtained analyzing the lyophilized sample with the 600 lines/mm diffraction grating. The depicted spectrum was collected merging 5 spectral ranges (grating centered at 750, 1400, 2100, 2850, 3400 cm^{-1}). Several known peaks that represent the protein fingerprint can be recognized in the above–500 cm^{-1} region. The most important ones are indicated in the same figure, while the corresponding assignments to chemical groups/amino-acids, reconstructed from [24], are listed in Table 1. At the same time, peaks were found even in the sub–500 cm^{-1} region. Such lower frequency peaks correspond to delocalized or collective vibrations that involve large protein portions, not just single chemical groups.

Fig. 2a,b show Stokes and Antistokes Raman spectra of lyophilized ATPase obtained respectively with 600 lines/mm grating (spectral resolution <3 cm^{-1}) and 1800 lines/mm grating (spectral resolution <1 cm^{-1}). The central part, which suffers of Rayleigh scattering, was cut from −15 to 15 cm^{-1} and from −8 to 11 cm^{-1} for Fig. 2a and b, respectively. In Fig. 2a, smaller peaks can be recognized in correspondence to 27, 190, 300 cm^{-1} (0.81, 5.7 and 9 THz), whereas larger ones are clearly visible at 370, 400, 454 and 468 cm^{-1}. All the previous peaks but for the lowest one (27 cm^{-1}), can be seen also in Fig. 1. The three low-frequency peaks at 27, 190, and 300 cm^{-1} are also visible in the spectrum with higher resolution of Fig. 2b.

Fig. 1 and Table 1 show that even the lyophilized sample contains spectroscopic signals compatible with water: see the high-frequency band at 3200–3600 cm^{-1}. This band is due to the hydration water around the protein, the so-called water shell. This is confirmed in Fig. 3, where the abovementioned water peak of the lyophilized sample spectrum (blue, lower curve) is compared to a Raman measure (black, upper curve) made on a hydrated sample obtained by depositing a drop (0.05 ml) of Milli-Q water on the lyophilized powder.

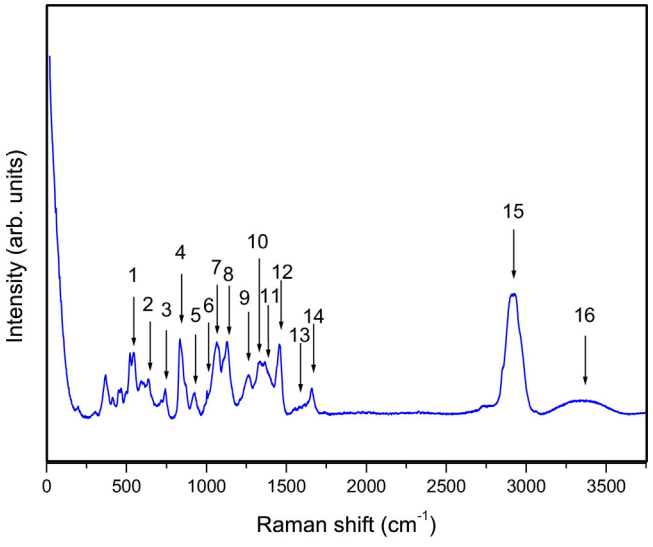

Fig. 1. Broad-range Raman spectrum of Na/K-ATPase lyophilized powder.

Table 1
Assignments of peaks indicated in Fig. 1.

N.	Raman shift (cm^{-1})	Assignment	N.	Raman shift (cm^{-1})	Assignment
1	524	S—S stretching	9	1260	Amide III
2	640	C—C twisting in Tyr	10	1335	CH_3—CH_2 wagging
3	740	C—S stretching	11	1365	Trp
4	831	Tyr	12	1454	C—H bending
5	930	C—C stretching	13	1585	Tyr, Phe
6	1004	Phe	14	1620–1670	Amide I
7	1067	Pro	15	2900–3000	CH_2—CH_3 stretching
8	1125	Trp	16	3200–3600	H_2O stretching

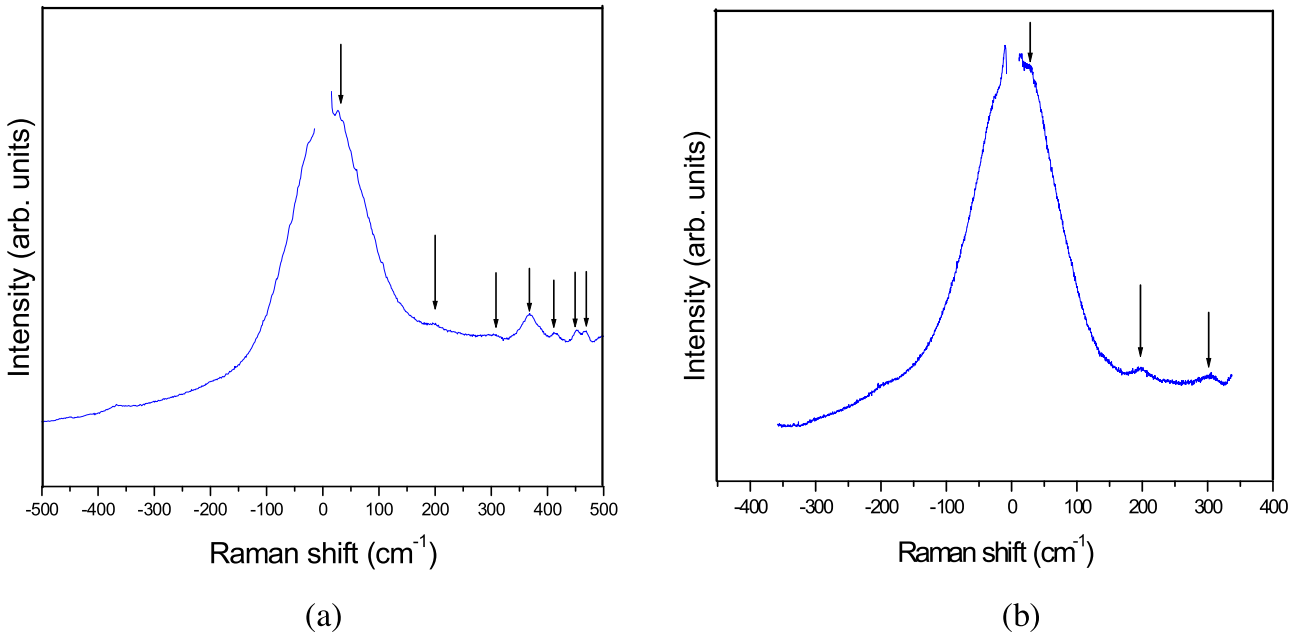

Fig. 2. Lyophilized Na/K-ATPase Raman spectrum centered at 0 cm^{-1}: (a) 600 lines/mm grating; (b) 1800 lines/mm grating.

Broad-range measurements were also conducted on the sample in aqueous solution. As in the previous case, the complete spectrum was collected merging 5 spectral ranges, obtained again with grating centered at 750, 1400, 2100, 2850, 3400 cm^{-1}. In Fig. 4, the complete spectra obtained for the ATPase in water solution (red, upper line) and in lyophilized powder (blue, lower line) are

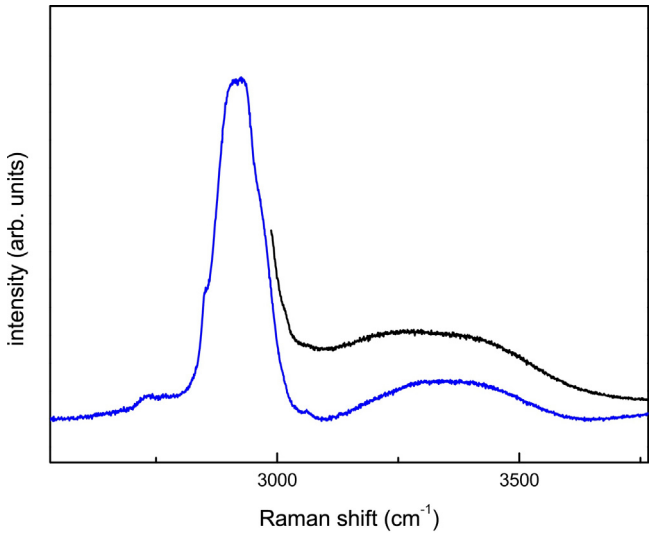

Fig. 3. Comparison of water peaks between lyophilized powder (blue, lower) and hydrated sample (black, upper). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

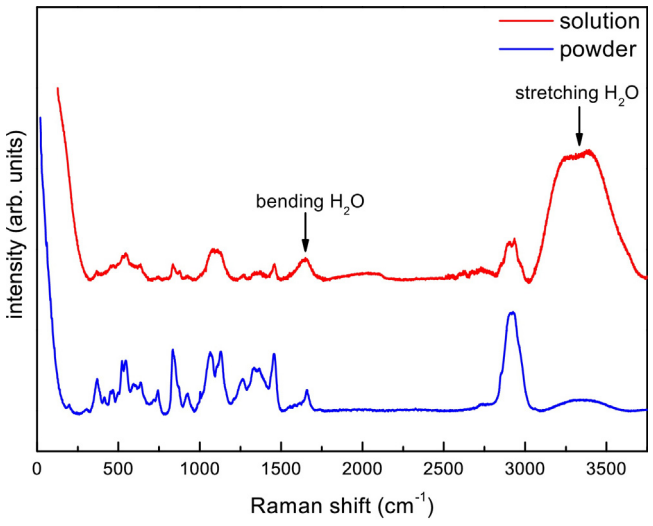

Fig. 4. Comparison between broad-range Raman spectra of Na/K-ATPase in water solution (red, upper line) and in lyophilized powder (blue, lower line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

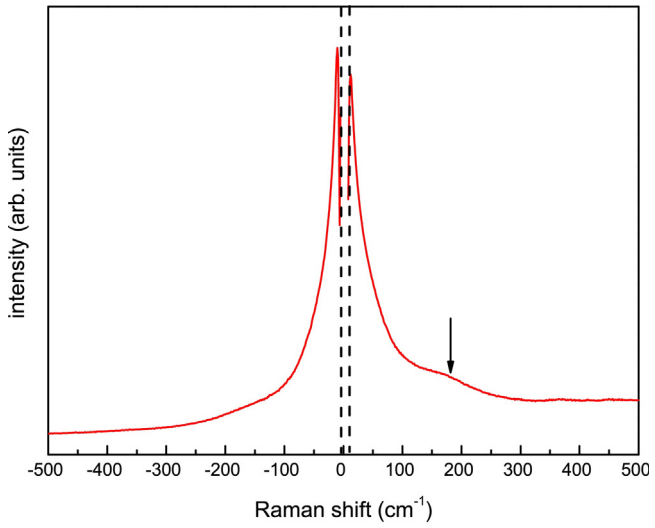

Fig. 5. Hydrated Na/K-ATPase Raman spectrum centered at 0 cm^{-1} (600 lines/mm grating).

compared. As one can see, water acted like a sort of dumper, lowering peaks corresponding to protein vibrations without shifting their frequency. On the other hand, as expected, adding water to the powder increased the peaks corresponding to vibrations modes of water molecules.

A Raman spectrum of the hydrated ATPase centered at 0 cm^{-1} with 600 lines/mm grating was also collected. The result is presented in Fig. 5, where can be seen how, in general, the low-frequency peaks found for the lyophilized sample are no more present; this fact can be ascribed to the strong absorbance of water. Anyhow, we notice that a shoulder around 180 cm^{-1}, like the one seen on the lyophilized powder, is still visible.

4. Conclusions

The experimental measurement of low-frequency vibration modes (i.e., those corresponding to delocalized/global vibrations) in proteins is of prime relevance in understanding crucial biological functions as well as their potential relations with several human diseases. In addition to their direct usefulness, measurements also represent the experimental counterpart of computational simulations. At the same time, low-frequency protein vibrations are difficult to measure.

In this study, the mechanical vibrations of Na/K-ATPase were investigated by Raman spectroscopy measurements conducted on lyophilized and rehydrated powder samples. The use of ULF filters allowed measurements below 5 cm^{-1} (0.15 THz). In particular, three peaks at 27, 190, 300 cm^{-1} were found experimentally analyzing the lyophilized powder; other peaks in the sub–500 cm^{-1} region were found at 370, 400 and around 450 cm^{-1}. All the previous peaks disappeared in the spectrum of the rehydrated sample because of water absorbance, with the only exception for the one at 190 cm^{-1}: a broad shoulder was observed around 180 cm^{-1}. However, the possible biological relevance of such frequency peaks was not discussed in the paper, it being beyond the aim of the present study: this point should be addressed somewhere else.

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