brief communication

Hydration-dependent far-infrared absorption in lysozyme detected using synchrotron radiation

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ABSTRACT Using the National Synchrotron Light Source (NSLS) at Brookhaven far-infared absorption in the frequency range 15–45 cm^{-1} was detected in samples of lysozyme at different hydrations and in water. The absorption is due to the presence of low-frequency (picosecond timescale) motions in the samples, such as are calculated in molecular dynamics simulations. The form of the transmission profile is temperature independent but varies significantly with the degree of hydration of the protein. At higher hydrations the profile resembles closely that of pure water in the region 20–45 cm^{-1}. At a low hydration marked differences are seen with, in particular, the appearance of a transmission minimum at 19 cm^{-1}. The possible origins of the hydration dependence are discussed. The results demonstrate the usefulness of long-wavelength synchrotron radiation for the characterisation of biologically-important low-frequency motions in protein samples.

INTRODUCTION

Evidence for the existence of 'low-frequency' picosecond timescale motions in proteins and their surrounding water of hydration has been provided by experiments and by theoretical analyses that use methods such as molecular dynamics simulations and normal mode calculations (1, 2). The results of the simulations suggest that the protein dynamics consist of correlated motions of groups of atoms, often delocalized over the protein molecule, and strongly influenced by weak, nonbonded interactions. Harmonic analyses of small proteins such as lysozyme and the bovine pancreatic trypsin inhibitor (2) suggest that the lowest frequency modes are ~5–10 cm^{-1} and that several tens of modes exist with frequencies below ~50 cm^{-1}. Motions at these frequencies contribute a major part of the calculated mean-square displacements of the protein atoms from their vibrational equilibrium positions. They are of special importance in the thermodynamics of protein folding, activity and electron transfer properties (1, 2).

Fundamental questions concern the nature of the picosecond timescale motions in protein–water systems in which the temperature and/or the degree of hydration are varied. The existence of a dynamical and thermodynamical temperature-dependent transition in myoglobin and the surrounding water of hydration has been extensively documented (3–6). For the protein motions the dynamics are approximately harmonic for temperatures below 150 K; at higher temperatures anharmonic motions become important. These can be of a frictional nature or can involve transitions between different minima on the protein potential energy surface (4–6). In principle the dynamical transition should be a phenomenon general to globular proteins.

In hydrated samples both the effect of water on the internal protein motions and of the protein on the water dynamics are of interest. Although the tertiary structural characteristics and presence of activity of lysozyme molecules remain at low hydrations, the picosecond timescale protein motions are reduced (7, 8). The dynamics of water molecules at the surface of a protein might be expected to be modified compared with the dynamics in the bulk (7). Crystallographic studies indicate a spatial ordering of the water molecules close to the surface, although this is not direct dynamical information.

Although several experimental techniques have indicated that motions on the picosecond timescale do exist in proteins (2), few data are available allowing detailed comparison with theory of such properties as the frequencies, forms, and anharmonicity of the motions involved. Some recent progress in this direction has been made using inelastic neutron scattering spectroscopy (4, 5, 8, 9). Comparisons of measured and calculated neutron scattering from small proteins have indicated that the experimentally-derived motional amplitudes and frequencies are in general agreement with those calculated from molecular dynamics simulations and harmonic analyses although the theoretical results are sensitive to the method of representation of the long range electrostatic interactions.

Far-infrared spectroscopy can in principle provide additional information on the nature of motions in proteins and the water of hydration, and may be free of

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the sample size and data collection time restrictions which hamper the development of inelastic neutron scattering. Several difficulties have impeded progress of the far-infrared technique. Sources and detectors have been inefficient below ~100 cm^{-1}, and protein samples absorb weakly in this region. In the case of hydrated (solution or crystalline) proteins significant absorption is expected from water molecules. An early application of the technique (10) was successful in detecting absorption in crystals of lysozyme (with notable peaks at 25 and 56 cm^{-1}), but the problem of separating the water contribution remained, and clear absorption was not visible with dehydrated samples.

The advent of powerful synchrotron far-infrared sources and the increased importance of picosecond timescale dynamical simulations in biology warrants further experimental investigation of this spectroscopical method. While synchrotron radiation is two to three orders of magnitude brighter than conventional sources throughout the infrared spectral region, it is more powerful only in the far-infrared (11). In Fig. 1, a comparison of the power output of the synchrotron radiation with that of a black body of 2,000°K is shown. The synchrotron radiation is on average 10 times more powerful in the region 166 to 1,000 μm (60 to 10 cm^{-1}).

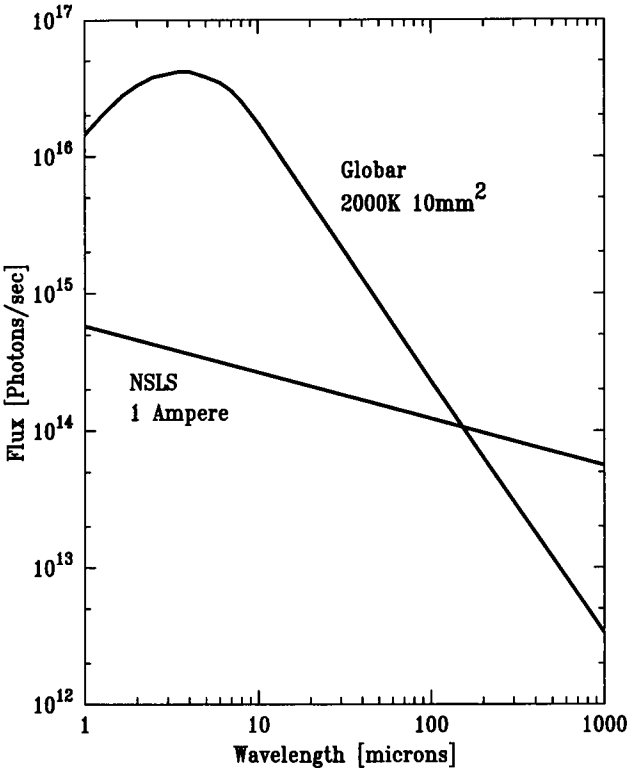

FIGURE 1 Comparison of the power output of the synchrotron (NSLS) and conventional globar sources in the infrared spectral region.

In this paper, we present spectra from lysozyme samples using a commercial instrument on the far-infrared beamline at the NSLS (11). The detector used enabled reliable spectra to be obtained over the frequency range 15–45 cm^{-1}. This is the frequency range over which the lowest frequency underdamped vibrations occur in proteins (9, 12). We investigate the temperature and hydration dependence of the far-infrared absorption from lysozyme samples containing different proportions of water: a 'low hydration' sample, containing 0.1 ± 0.05 g H_2O/g protein; and a 'high hydration' sample, containing 0.3 ± 0.1 g H_2O/g protein. The transmission of these samples was measured at three different temperatures, 150, 210, and 300°K. These temperatures cover the range over which the dynamical transition occurs in myoglobin. Spectra were also taken from pure water at 300°K in the same frequency range. A comparison between the water and protein spectra allows suggestions to be made as to the origins of the observed absorption features. A significant contribution to the far-infrared absorption is expected to come from the water molecules; the absorption from the protein samples is expected to be a combination of the water and protein absorption. We find the form of the transmission profile is temperature independent but varies significantly with the degree of hydration of the protein. At higher hydrations the transmission profile resembles that of pure water. At a low hydration marked differences are seen with, in particular, the appearance of a transmission minimum at 19 cm^{-1}.

MATERIALS AND METHODS

Hen egg-white lysozyme was purchased from Boehringer Mannheim Diagnostics, Inc. (Houston, TX) and, for the low-hydration powder sample, was lyophilized to a hydration of 0.1 ± 0.05 g H_2O/g protein, as determined by dry weight analysis. The high-hydration sample was made by thoroughly mixing in 0.3 ± 0.1 g H_2O/g protein. Immediately after lyophilization/hydration the samples were transferred to sealed containers. The stated hydrations did not change significantly during these handling procedures. For the infrared measurements the samples were enclosed in vacuum-tight polyethylene cells with wedge-shaped windows so as to avoid fringes. The cells were mounted in a vacuum environment. The transmission spectrum of the sample was measured with a Nicolet rapid-scanning 20F Michelson interferometer-spectrometer used at a full-width half-maximum resolution of 6 cm^{-1}. The detector is a liquid helium cooled boron-doped Si bolometer operated at 1.8°K (by pumping the helium) with a noise equivalent power of 1.5×10^{-13} $Ws/(Hz)^{-1/2}$ derived from the D.C. load curve. For each spectrum three sets of 256 interferograms were recorded, for a total collection time of ~5 min. Each set of interferograms was inspected for artefacts before Fourier transformation and the noise

was estimated by the standard "100% line" method of comparing two supposedly identical spectra. For the spectra shown here the reproducibility was better than 1%. The three sets of interferograms were normalized to the synchrotron beam current, co-added, and divided by the background to obtain the corrected transmission spectrum of the sample.

Two temperature sensors were attached, one on each side of the polyethylene cells. The reported temperatures are averages obtained from these two sensors with an estimated error of a few Kelvins. The reliability of the forms of the transmission profiles was checked by using different sample thicknesses. No temperature hysteresis was seen, i.e., on reheating protein samples to 300°K, recorded spectra were the same as before cooling. Although the absolute value of the protein sample transmission depends on the sample thickness the shapes and features of the curves do not. The absorption coefficients could not be obtained because the quantity of sample in the beam is not precisely known. Thus all data are presented in arbitrary units.

RESULTS

In Fig. 2 *a* are presented far-infrared spectra of lyophilized lysozyme powder at low hydration, taken at three temperatures, 150, 210, and 300°K. The spectra indicate considerable absorption over the reliable frequency range, i.e., 15–50 cm^{-1}. There is a clear minimum centred at 19 cm^{-1}, and a smooth decay to 38 cm^{-1} with an inflection at 26 cm^{-1}. The form of the transmission profile is closely similar at the three temperatures studied, the transmission increasing with decreasing temperature.

The spectra taken from the increased hydration sample are in Fig. 2 *b*. The 19 cm^{-1} transmission minimum seen in the low-hydration sample is now absent. However, the inflection at 26 cm^{-1} is now more clearly visible, and there is a transmission minimum at 38 cm^{-1}. Spectra of lysozyme powders at higher hydrations than that used in Fig. 2 *b* give essentially the same far-infrared curve as that of Fig. 2 *b*. In Fig. 2 *c* a spectrum from pure water is presented. A transmission minimum is seen at 40 cm^{-1} with a shoulder at 27 cm^{-1}. The small differences in positions of the observed features in Figs. 2, *a–c* are insignificant compared with the instrumental resolution. A transmission maximum is seen at 22 cm^{-1}. Additional water spectra were run on very thin samples; although the noise level was not good in these spectra, the 38–40 cm^{-1} transmission minimum was always present.

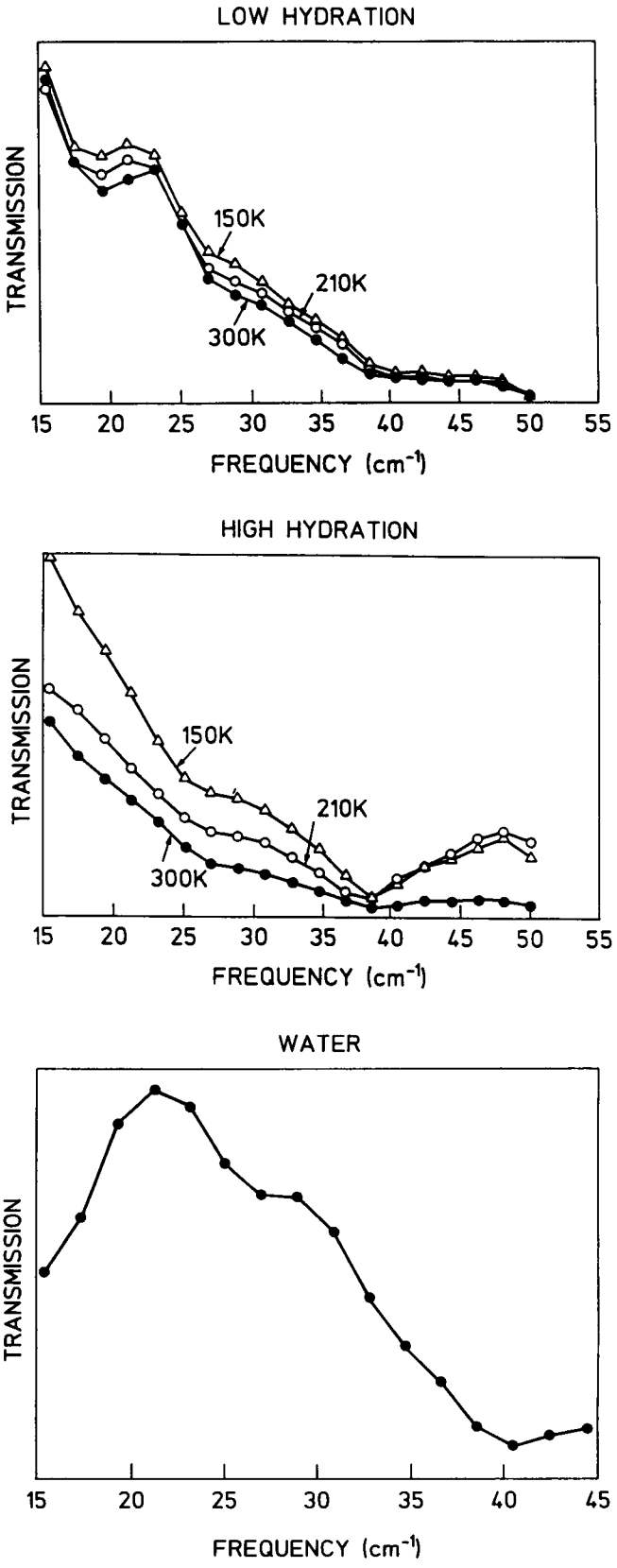

FIGURE 2 (*a*) (*Top*) Far-infrared transmission spectra from low-hydration lysozyme at 150, 210, and 300°K. (*b*) (*Middle*) Far-infrared transmission spectra from high-hydration lysozyme at 150, 210, and 300°K. (*c*) (*Bottom*) Far-infrared transmission spectra from water at 300°K.

CONCLUDING DISCUSSION

The results presented here demonstrate the application of long-wavelength synchrotron radiation to the measurement of far-infrared absorption spectra from protein-water samples. The spectra, which are of a good signal-to-noise ratio, show significant features originating from motions at frequencies corresponding to those of large-amplitude fluctuations of the protein molecules and the surrounding water. These motions can be simulated using molecular dynamics and normal mode techniques.

Although it is difficult to assign the contributions from water and protein absorption, clear differences were seen in the transmission profiles as a function of hydration. The transmission profile of the high hydration sample strongly resembles that of pure water in the region 20–45 cm^{-1}, with a shoulder at 26 cm^{-1} and a minimum at 38 cm^{-1}. The presence of these features in all the protein and water samples suggests that they originate from water molecules in the samples. Whether they originate from water molecules associated with the protein or in regions of bulk water in the powder samples is not known. The presence of very low-frequency absorption (<20 cm^{-1}) is seen in the pure water sample but not in the protein.

In the low-hydration sample the 26 and 38 cm^{-1} features are still present, but there is in addition a clear transmission minimum at 19 cm^{-1}. The absence of the 19 cm^{-1} minimum in all but the low-hydration samples may be taken as evidence that it originates from low-frequency protein vibrations and/or from water molecules perturbed with respect to the bulk. For all protein samples there is a decrease in the overall transmission intensity with frequency over the range studied. This was also seen in the previous far-infrared work on lysozyme crystals (10). The overall absorption increase resembles the form of the experimentally-derived density of states for a protein molecule (the vibrational frequency distribution) (12, 13).

It is of interest to compare the form of the far-infrared profiles of the low-hydration lysozyme sample with those seen with inelastic neutron scattering (9, 12). In the latter case a single broad scattering peak is seen centered at 25–30 cm^{-1} originating from the protein vibrations. Differences in the infrared and neutron spectra arise from the relationship between the measured intensity and the dynamics of the sample. In the case of neutron scattering the intensity depends on the hydrogen atom vibrational amplitudes, whereas the infrared intensity is determined by dipole moment reorientations. Therefore, one possibility is that among the low-frequency protein/water motions there are one or several with relatively large dipole fluctuations and effective frequencies of ~ 19 cm^{-1}.

In the protein-water samples the form of the transmission profiles shows no significant temperature dependence over the range measured (150–300°K), although the transmission systematically decreases with increasing temperature, consistent with increased motional amplitudes. It is known that there is a transition in the internal dynamics of myoglobin as a function of temperature (3). This transition was seen in a sample of approximately the same hydration as the 'high hydration' sample in this work. However, as yet there is clear evidence (4–6) for a change in only the diffusive, activated motions on the timescale >1 ps. The effect on the low-frequency vibrations is less well characterized. Any change with temperature of the low-frequency vibrations is undetected in the experiments described here.

The origin of the intensity profiles can be investigated with dynamical simulation techniques. The calculation of infrared intensities from molecular dynamics simulations can be performed using the time correlation function formalism (14). A comparison of the results with spectra such as that presented here may provide more specific information on the origins of the measured far-infrared absorption. Correspondingly, comparisons of such calculations with the measured spectra may allow further conclusions to be drawn as to the accuracy of our representation of electrostatic interactions in proteins, as was the case with the neutron studies (9, 12). In this respect the far-infrared method might be more easily addressed than in Raman spectroscopy, where a knowledge of induced dipole fluctuations is certainly required.

An experimental advantage of the synchrotron far-infrared technique is that, in contrast to neutron spectroscopy, the intensity of the beam allows good quality picosecond timescale spectra to be taken from small samples. Spectra of the quality shown here can be collected in times of the order of a minute compared with times of the order of a day required by the neutron technique. An obvious small disadvantage of the method is that data can only be collected at a synchrotron ring and not in one's own laboratory. In addition, detection at higher resolution and lower frequencies than presently available would be useful. Nevertheless, we feel that the results presented here demonstrate that synchrotron far-infrared spectroscopy can provide unique information on the low-frequency dynamics implicated in protein activity. Future work will include finding the range of biological samples that can be investigated using the synchrotron source.

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