Vibrational Stark spectroscopy for assessing ligand-binding strengths in a protein

Padmabati Mondal and Markus Meuwly*

Nitrile groups are potentially useful spectroscopic probes in the infrared to characterize the binding and dynamics of ligands in proteins. This opens the possibility of locating and determining the binding mode of suitably labelled ligands in proteins based on optical spectroscopy, without the need for determining an X-ray structure. However, relating structure and spectroscopy requires means to accurately compute infrared spectra. This is investigated for benzonitrile (PhCN) in water, wild type (WT) and two lysozyme mutants in solution. The force field is validated by comparing with experimental data for benzonitrile in water which is the basis for computing the Stark shift and time scale for spectral diffusion of PhCN in WT and the L99A and L99G mutants of T4 lysozyme. The 1-d spectra for PhCN in WT and the two mutant proteins differ in their maximum absorption by up to 4 cm$^{-1}$, which reflects the modified electrostatic environments in the three proteins. It is also tested whether extending from 1-d to 2-d infrared spectroscopy provides further discrimination in the ligand-binding modes. First, for PhCN in solution the frequency fluctuation correlation function (FFCF) decays to zero at short times whereas in the protein a pronounced static inhomogeneous component is found. Secondly, the decay time of the FFCF for the mutant to which PhCN binds most strongly has the longest decay time. It is demonstrated explicitly that the ligand-binding free energy with respect to the three protein variants correlates with the Stark shift. This makes 1-d infrared spectroscopy together with computations a valuable tool for characterizing binding modes and potentially binding locations in proteins.

1 Introduction

Vibrational frequency shifts caused by external electric fields (‘Stark shifts’) are important to study the structure, electrostatics and dynamics of ligands and spectroscopic probes at protein active sites.1–6 The dynamical and spectroscopic response of chemical bonds to changes in the local electric fields can be accurately measured through 1-dimensional spectroscopy. However, relating this information to the structure of the environment surrounding the spectroscopic probe is not straightforward because simultaneous observation of spectroscopy and structure is difficult.7 Under such circumstances, atomistic simulations with sufficiently accurate and validated force fields provide a valuable complement. The preferred use of physics-based empirical force fields over ab initio molecular dynamics (AIMD) simulations derives from the fact that comprehensive conformational sampling for a protein–ligand complex in solution is currently not possible due to the computational expense of AIMD. In addition, recent examples have shown that empirical force fields fitted to suitable reference data and validated vis-a-vis experiments can provide important structural information.14

The nitrile group (–CN) has been used as a spectroscopic probe for the local structure, electric field and solvent dynamics involving proteins and biological molecules.2,3,15–18 Some previously used nitrile probes for proteins are CN-labelled phenylalanine9–13 and the nitrile-containing IDD type inhibitor for human aldose reductase.2,22 Herein, we focus on nitrile-containing probe benzonitrile (PhCN) for probing the interior containing probe benzonitrile (PhCN) for probing the interior of lysozyme to determine the local electrostatic environment. To date, PhCN has been used to probe the local environment in water23 and in ionic liquids.24 For the present work PhCN is a suitable probe because the –CN stretching mode at $\approx$ 2200 cm$^{-1}$ (a) absorbs in a frequency range (i.e. between 1800 cm$^{-1}$ and 2800 cm$^{-1}$) in which proteins containing only naturally occurring amino acids have no vibrational spectral response (except for the –SH group in cysteine), (b) is, to a good approximation, a local mode (i.e. uncoupled from other framework modes) and (c) the dipole moment of PhCN is to a large extent that of the nitrile group itself. On the other hand, the nitrile group may pose additional challenges in concrete experiments due to its low extinction coefficient.25 The previous work on PhCN in water23 provides an ideal benchmark to validate the computational methods used in the present work.

Lysozyme is an enzyme present in most bodily secretion and works as an anti-bacterial agent by catalyzing the hydrolysis of...
specific glycosidic linkage in the peptidoglycan that forms bacterial cell walls. One of the striking properties of lysozyme is its tolerance to changes in amino acid sequences. It has been shown previously for T4 lysozyme that protein cavities with binding affinity to specific ligands can be engineered. In particular, it has been shown that engineering of a benzene-binding cavity is possible by replacing leucine at position 99 with alanine. Since then, there have been several studies on the energetics of benzene bound to the the L99A mutant of T4 lysozyme, which makes it a pivotal system to validate computational protocols. Combining benzene and a nitrile label in the present work, we use PhCN as a spectroscopic probe in T4 lysozyme.

Among all the available experimental and theoretical approaches, including photophysical experiments, neutron diffraction measurements, Raman spectroscopy, or X-ray scattering studies, the response of a single molecular vibration to the local environment via IR spectroscopy is probably the most direct way to probe the local environment. In the past, several 2-dimensional infrared (2DIR) experiments with a nitrile probe incorporated into phenylalanine to form cyano-phenylalanine have been carried out. However, it is challenging to interpret the spectroscopic response in terms of the underlying structures in such complex systems. This is where validated atomistic simulations can provide valuable insights.

The aim of the present work is three-fold. First, to provide a quantitative assessment of the expected spectral shifts and changes in the lineshapes for the 1-dimensional infrared (1D-IR) spectrum of a nitrile-substituted probe in different environments ranging from the bulk solvent to a typical protein cavity. Second, to investigate whether 2D-IR spectroscopy has any benefits for more accurately probing the active site dynamics of proteins. And third, to investigate whether there is a relationship between the protein–ligand binding strength and the IR-spectroscopy and dynamics of the ligand in the cavity.

In this work, we use atomistic molecular dynamics simulations with a combination of sophisticated force fields to determine the vibrational Stark shift and spectral diffusion of a nitrile probe of a PhCN ligand in the cavity of the T4 lysozyme and its mutant. The nitrile bond vibration is considered as a Morse oscillator and the change of polarizability of the nitrile probe due to the environment is taken into account using the fluctuating point charge model. To our knowledge, we report here the first computational study of 2DIR spectroscopy in combination with the vibrational Stark shift of a probe-containing ligand in the protein–ligand complex using atomistic MD simulations.

The article is organized as follows. The computational methods containing the simulation methods, intermolecular interactions and methods for the analysis are described in Section 2. The results are described and discussed in Section 3. While Section 3.1 describes the results related to the vibrational Stark shift and spectral diffusion properties corresponding to PhCN in water, the results of PhCN in WT and mutants of lysozyme are discussed and compared with the same of PhCN in water in Section 3.2. The thermodynamic parameters related to the binding of PhCN to lysozyme are provided and discussed in Section 3.3. Finally, Section 4 concludes the work.

2 Computational methods

2.1 Simulation methods

All MD simulations were carried out using the CHARMM MD simulation package. For the protein–ligand simulations, the initial T4 lysozyme structure of the L99A mutant with benzene in the cavity was obtained from the X-ray crystal structure (PDB 1L83). The benzene at the active site was then replaced by PhCN maintaining the position of the carbon atoms. For the L99G mutant, the X-ray structure (PDB code 1LYD), docking of PhCN into the (tight) cavity yielded several clashes. Therefore, the setup for native lysozyme was started from the L99A mutant including PhCN (see above) and alanine was replaced by leucine. As a validation, the structure of the solvated WT apo-lysozyme (i.e. the apo-WT structure generated from the L99A X-ray structure 1L83) from a 1 ns production run was compared with the crystal structure of the WT apo-lysozyme (1LYD) and the root mean square deviation (RMSD) was 1.5 Å. Fig. 1 shows the structure of the L99A mutant of T4 lysozyme with PhCN in the cavity.

Hydrogen atoms were included using CHARMM and the protein with the ligand was solvated in a water box (78 × 78 × 50 Å³) which leads to a total system size of around 30 000 atoms. The systems were minimized, heated (to 300 K) and equilibrated keeping the protein and ligand fixed. Next, the full system was minimized, heated (to 300 K) and equilibrated for 200 ps. All bonds involving hydrogen atoms are constrained via SHAKE. A leapfrog Verlet integrator and the Berendsen thermostat were employed for the NVT simulation. The production run was continued for 5 ns and snapshots were saved every 5 fs for the frequency calculations. For PhCN in water, PhCN was solvated in a cubic water box (25 × 25 × 25 Å³).

2.2 Intermolecular interactions

The force field used in the simulations was CHARMM22 together with the TIP3P water model. For the PhCN ligand, the standard parametrization from Swissparam was used except for the CN-bond and the charges which were determined from electronic structure calculations. All ab initio calculations were performed at the MP2/aug-cc-pVDZ level of theory using the Gaussian09 suite of programs. The CN-bonded parameters were calculated by fitting electronic structure data to a Morse function, \( V(r) = D_e (1 - e^{-\beta (r-r_m)})^2 \), see Fig. 2A.

The CN frequency was calculated from the power spectrum of the CN-bond length from a gas phase simulation using the fitted Morse parameters. From this, an improved Morse potential was obtained by modifying the value for \( D_e \) to reproduce the experimental gas phase frequency of the nitrile stretch at 2242 cm⁻¹ in PhCN. The obtained Morse parameters are \( D_e = 288.3 \text{ kcal mol}^{-1}, r_{eq} = 1.1791 \text{ Å}, \) and \( \beta = 2.0667 \text{ Å}^{-1} \).
To account for the internal polarization of the atoms of PhCN due to the vibration of the nitrile group during the dynamics, a fluctuating point charge (FPC) model was used. In this model, the point charges of PhCN fluctuate as a function of the CN-stretching coordinate. For the FPC model, the quantum mechanically calculated (MP2/aug-cc-pVDZ) electrostatic potential (ESP) - fitted charges of all PhCN atoms around the CN equilibrium bond length ($r_{\text{eq}}$) were represented as a linear function (see Fig. 2B)

$$q_X(r_{\text{CN}}) = q_{X,\text{eq}} + a_X(r_{\text{CN}} - r_{\text{eq}}).$$

Here, $q_{X,\text{eq}}$ is the ESP-fitted charge of atom X at $r_{\text{eq}}$ and $a_X$ is the first-order expansion coefficient, where X refers to each atom of PhCN. The fitting parameters, given in Table 1, were used for the dynamics and frequency calculations. The point charge model obtained from the fitting of ESP at each $r_{\text{CN}}$ using the Merz-Singh-Kollman scheme implemented in Gaussian is referred to as the ESP-fit model.

For validation, an independent, point charge-based model (PC-fit) was determined from fitting the ESP of the PhCN equilibrium structure using a recently developed fitting environment. The two fitting procedures (ESP-fit and PC-fit) differ in that the ESP-fit uses the additional constraint to fix the dipole moment of the molecule to the computed value. The true ESP at the MP2/aug-cc-pVDZ level of theory compared with the ESP computed from the ESP-fit and PC-fit charges is reported in Fig. 3.

The average root means square errors (RMSEs) over the entire grid for the two charge models are 1.2 kcal mol$^{-1}$ (ESP-fit) and...
Table 1  The parameters for the fitting of static ESP-fit, FPC (according to eqn (1) and Fig. 2B) and static PC-fit models. The same expansion coefficients \( a_s \) were used for the fluctuating ESP-fit and fluctuating PC-fit models.

<table>
<thead>
<tr>
<th>Atoms</th>
<th>( q_{\text{ECE}} ) (e)</th>
<th>( a_s ) (e Å(^{-1}))</th>
<th>( q_{\text{ECE}} ) (e) (PC-fit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1, C2</td>
<td>-0.333</td>
<td>-0.320</td>
<td>-0.164</td>
</tr>
<tr>
<td>C3, C4</td>
<td>0.154</td>
<td>0.228</td>
<td>-0.164</td>
</tr>
<tr>
<td>C5</td>
<td>-0.373</td>
<td>-0.274</td>
<td>-0.164</td>
</tr>
<tr>
<td>C</td>
<td>0.186</td>
<td>0.681</td>
<td>0.114</td>
</tr>
<tr>
<td>N</td>
<td>-0.425</td>
<td>0.700</td>
<td>0.438</td>
</tr>
<tr>
<td>C6</td>
<td>0.302</td>
<td>-1.081</td>
<td>0.263</td>
</tr>
<tr>
<td>H, H1</td>
<td>0.175</td>
<td>0.079</td>
<td>0.177</td>
</tr>
<tr>
<td>H2, H3</td>
<td>0.070</td>
<td>-0.028</td>
<td>0.177</td>
</tr>
<tr>
<td>H4</td>
<td>0.182</td>
<td>0.055</td>
<td>0.177</td>
</tr>
</tbody>
</table>

0.43 kcal mol\(^{-1}\) (PC-fit), respectively. The PC-fit charges represent the region around the \(-CN\) probe particularly well whereas the ESP-fit charges lead to differences of \( \sim 1 \) kcal mol\(^{-1}\) in this region, see the right hand column of Fig. 3, which reports the difference between the true ab initio ESP and the ESP from the two point charge models.

2.3 Analysis

Calculation of the 1D absorption spectra. The instantaneous (harmonic) frequencies for each of the \( 10^6 \) snapshots of the trajectory (separated by 5 fs) from the NVT simulation were calculated from a normal mode analysis by minimizing the PhCN structure while keeping the environment (protein plus solvent) fixed. This procedure is referred to as “vibrational analysis” in the following. The normal mode analysis on the DFT-optimized structure suggests that the CN-stretch mode is only weakly coupled to the C–C6 stretching mode and hence is, to a good approximation, a local mode. The frequency fluctuation correlation function (FFCF), \( C(t) \), was then calculated according to

\[
C(t) = \langle \delta \omega(t_0) \delta \omega(t_0 + t) \rangle_{T_0}.
\]

Here, \( \delta \omega(t_0) = \omega(t_0) - \langle \omega \rangle \) and \( \delta \omega(t_0 + t) = \omega(t_0 + t) - \langle \omega \rangle \) with \( \langle \omega \rangle \) the average frequency over the entire frequency trajectory and \( \omega \) is the frequency of the CN-stretching mode. The FFCF was then fitted to a multi-exponential function

\[
C(t) = \sum_{i=1}^{3} a_i \exp(-t/\tau_i) + A_s^2,
\]

where \( A_s \) is the static component related to the slow CN-frequency fluctuation connected to the structural changes occurring on a longer timescale and often widely used for the study of structural dynamics of protein. The amplitudes and timescales, respectively, of the vibrational decay. The 1D absorption spectrum was calculated from the 1D-Fourier transform of the response function \( R^{(1)}(t) \). The linear response function \( R^{(1)}(t) \propto e^{-t/\tau} \) can be determined from the lineshape function \( g(t) \) which is obtained by double integration of the FFCF (eqn (4)),

\[
g(t) = \int_{0}^{\infty} \int_{0}^{\infty} d\tau' d\tau'' C(t),
\]

The contribution of the short lifetime of the vibrationally excited state, \( T_1 \), to the lineshape was taken into account by multiplying the linear response function, \( R^{(1)}(t) \), with a phenomenological factor \( e^{-t/T_1} \).

Frequency calculation using the QM/MM method. For validation, frequencies along the trajectories were also calculated using the QM/MM method. For this, 5000 snapshots were extracted every 1 ps from an NVT simulation of PhCN in water. For each snapshot, the 1-dimensional potential energy curve along the CN bond was computed from B3LYP/6-31G** calculations on a grid of CN-bond lengths ranging from 0.9 Å to 1.5 Å with PhCN in the QM part and the rest of the system as MM, represented as point charges (TIP3P water). The frequencies for the 0–1 and 1–2 transitions were then calculated by solving the 1-dimensional Schrödinger equation using LEVEL. This procedure (combining snapshots from MD simulations, mixed quantum mechanics/classical mechanics (QM/MM) electronic-structure calculations and quantum-bound-state calculations for the vibrational transitions) is referred to as “QM/MM” in the following.

3 Results and discussion

3.1 PhCN in water

For the validation of the computational approach, PhCN in water was considered first. Experimentally, the CN-atom of PhCN in water is found at 2235 cm\(^{-1}\), which corresponds to a red shift of 7 cm\(^{-1}\) with respect to the gas phase CN frequency. The instantaneous (harmonic) frequencies for each of the \( 10^6 \) snapshots of the trajectory (separated by 5 fs) from the NVT simulation were calculated from a normal mode analysis by minimizing the PhCN structure while keeping the environment (protein plus solvent) fixed. This procedure is referred to as “vibrational analysis” in the following. The normal mode analysis on the DFT-optimized structure suggests that the CN-stretch mode is only weakly coupled to the C–C6 stretching mode and hence is, to a good approximation, a local mode. The frequency fluctuation correlation function (FFCF), \( C(t) \), was then calculated according to

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C(t) = \langle \delta \omega(t_0) \delta \omega(t_0 + t) \rangle_{T_0}.
\]

Here, \( \delta \omega(t_0) = \omega(t_0) - \langle \omega \rangle \) and \( \delta \omega(t_0 + t) = \omega(t_0 + t) - \langle \omega \rangle \) with \( \langle \omega \rangle \) the average frequency over the entire frequency trajectory and \( \omega \) is the frequency of the CN-stretching mode. The FFCF was then fitted to a multi-exponential function

\[
C(t) = \sum_{i=1}^{3} a_i \exp(-t/\tau_i) + A_s^2,
\]

where \( A_s \) is the static component related to the slow CN-frequency fluctuation connected to the structural changes occurring on a longer timescale and often widely used for the study of structural dynamics of protein. The amplitudes and timescales, respectively, of the vibrational decay. The 1D absorption spectrum was calculated from the 1D-Fourier transform of the response function \( R^{(1)}(t) \). The linear response function \( R^{(1)}(t) \propto e^{-t/\tau} \) can be determined from the lineshape function \( g(t) \) which is obtained by double integration of the FFCF (eqn (4)),

\[
g(t) = \int_{0}^{\infty} \int_{0}^{\infty} d\tau' d\tau'' C(t),
\]

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Frequency calculation using the QM/MM method. For validation, frequencies along the trajectories were also calculated using the QM/MM method. For this, 5000 snapshots were extracted every 1 ps from an NVT simulation of PhCN in water. For each snapshot, the 1-dimensional potential energy curve along the CN bond was computed from B3LYP/6-31G** calculations on a grid of CN-bond lengths ranging from 0.9 Å to 1.5 Å with PhCN in the QM part and the rest of the system as MM, represented as point charges (TIP3P water). The frequencies for the 0–1 and 1–2 transitions were then calculated by solving the 1-dimensional Schrödinger equation using LEVEL. This procedure (combining snapshots from MD simulations, mixed quantum mechanics/classical mechanics (QM/MM) electronic-structure calculations and quantum-bound-state calculations for the vibrational transitions) is referred to as “QM/MM” in the following.
but the line widths are only in qualitative agreement with experiment without rotational contributions.

Next, the spectroscopy of PhCN in solution was investigated using the QM/MM protocol. For this, 5000 snapshots were extracted from the 5 ns simulation run using the fluctuating ESP-fit charge model. Depending on the level of theory at which the frequencies are calculated (DFT or MP2), the maximum of $P(\omega)$ occurs at 2240 cm$^{-1}$ or 2308 cm$^{-1}$. The calculated red shift from the gas to condensed phase using the QM/MM method is 2 cm$^{-1}$ and 9 cm$^{-1}$ for DFT and MP2, respectively. Hence, the MP2 calculations reproduce the experimentally observed red shift of 7 cm$^{-1}$ almost quantitatively. For both frequency distributions the FWHM is consistent with the simulations using the fluctuating point charge models and confirms their validity. One shortcoming of the QM/MM method to determine absolute frequencies is the local mode approximation which does not account for the weak coupling between the -CN and C–C6 stretching modes. On the other hand, anharmonic effects are included in such an approach. Fig. 5A shows a comparison of $P(\omega)$ calculated using vibran and the QM/MM method using the fluctuating ESP-fit model for PhCN in water.

Although the red shift calculated at the MP2 level (QM/MM method) was found to be 9 cm$^{-1}$, the absolute gas (solution) phase MP2 frequency deviates by 75(66) cm$^{-1}$ from the experimental gas phase frequency. The correlation between the frequency calculated using DFT and the MP2 method was found to be close to 1:1 with a constant shift of 68 cm$^{-1}$ to the blue region. This indicates that MP2 overestimates the curvature.
around the minimum energy and explains why the Morse parameters of the fitted MP2/aug-cc-pVDZ calculations need to be adjusted (see above) to reproduce the gas phase experimental –CN frequency.

Finally, the effect of treating the immediate water environment entirely with an empirical force field (TIP3P) or including several water molecules in the QM part was assessed. For this, the same 5000 snapshots were re-analyzed by retaining all water molecules within 4 Å (typically 6 to 9 water molecules) in the QM part. Fig. 5B probes the difference between treating water molecules fully as MM and having a few water molecules treated with QM. The correlation indicates that the mean frequency of the distribution remains unchanged when the surrounding atoms are considered as MM point charges or treated using the full QM method. Also, the anharmonic shift obtained from the QM/MM calculation is 23 cm\(^{-1}\) which is in good agreement with the experimental anharmonic shift of 22 cm\(^{-1}\) for PhCN in water.\(^{23}\)

Overall, for PhCN in the gas phase and in water it is found that a FPC model faithfully reproduces the observation of a red shift on going from the gas- to condensed (water) phase. The frequency distributions from vibran and QM/MM analyses on the same 5000 snapshots yield comparable distributions \(P(\omega)\) and explicitly including water molecules in the immediate environment of the spectroscopic probe (–CN) does not affect or improve the frequency distribution. Hence, the frequency trajectory determined using the vibran procedure provides a meaningful starting point for further analysis. Table 2 provides a quantitative summary of peak frequencies, line widths and peak shifts for different methods/models.

Next, the intermolecular dynamics is considered by analyzing the FFCF, \(C(t)\). Fitting \(C(t)\) to a multi-exponential decay suggests that three time scales are required to describe the temporal evolution of the FFCF of PhCN in water, see Fig. 6A and Table 3. The fitting parameters in Table 3 indicate that no static
component ($\Delta s^2$) is required and the short and intermediate time scales for simulations with the fluctuating ESP-fit and PC-fit models are close to one another. For the picosecond time scale, the decay times differ by about 10%.

The FFCF decays for both the fluctuating ESP-fit and the fluctuating PC-fit models are comparable. The slowest decay time is $\tau_3 \approx 1$ ps whereas the two shorter sub-ps time scales are 0.01 ps and 0.1 ps. Experimentally, 2D-IR spectroscopy for PhCN has been performed in methanol for which decay times of $\tau_p = 2.1$ ps and $\tau_{HI} = 2.6$ ps were obtained, respectively, for the free and hydrogen bonded $\text{CN}$. No data for PhCN in water are available to the best of our knowledge. However, for a Fe(CN)$_5$NO complex the decay times of the CN-stretch in MeOH and water are 2.6 ps and 0.84 ps, respectively. Using the observation that the hydration dynamics around a solvent-exposed $\text{CN}$ group is faster in water than in MeOH by a factor of $\approx 3$, we anticipate a $\tau \sim 1$ ps for PhCN in water which agrees quite closely with the computed value for $\tau_3$, see Table 3. No static inhomogeneous component ($\Delta s^2$) was found for the FFCF decay. The rapid spectral diffusion of the nitrile mode may be attributed to a relatively weak nitrile–water interaction. The hydrophobicity of the benzene ring as well as the largely neutral character of the nitrile group makes the hydrogen bonding significantly weaker than for solvated CN$^-$ for which considerably slower decay constants ($\sim 10$ ps) were found. This also agrees with the observation that the FFCF for PhCN does not show any anticorrelation as found for HOD, N$_3^-$ or CN$^-$ in water. The calculated hydration free energy of PhCN using thermodynamic integration and the ESP-fit and PC-fit models are $-4.6$ kcal mol$^{-1}$ and $-6.1$ kcal mol$^{-1}$, respectively. This is compared with the calculated values of $(-4.8 \pm 0.5$ kcal mol$^{-1}$, $-4.3$ kcal mol$^{-1}$) from earlier work.

For a structural analysis and comparison of the two electrostatic models (ESP-fit and PC-fit), the pair correlation function $g(r)$ and the average number of water molecules $N(r)$ were determined. The $N(r)$ is obtained from the pair correlation function according to $N(r) = 4\pi r^2 g(r)dr$. The $g(r)$ and $N(r)$ for the ESP-fit model are shown in Fig. 6B and show that there are 6 to 7 water molecules around the N of PhCN within 4 Å. The water distribution around the nitrile group was indistinguishable for the two electrostatic models (ESP-fit and PC-fit). Moreover, visual inspection of the trajectories confirms that the water molecules are too labile to form strong hydrogen bonds with the nitrile group of PhCN.

To quantify the residence times of water molecules within the first solvation shell, the solvent-shell occupation fluctuation

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**Table 2** Maximum of the 1DIR absorption spectrum ($\omega_{\text{max}}$), full width at half maximum of $\Delta \omega$ and frequency shift ($\Delta \omega$) with respect to gas-phase frequency for PhCN in water using different models. The number in brackets refers to FWHM including effects due to explicit inclusion of rotational motion and avoiding the cumulant approximation based on comparison with CN$^-$ in solution (see text). Experimental data are those from ref. 23.

<table>
<thead>
<tr>
<th>Models/method</th>
<th>$\omega_{\text{max}}$ (cm$^{-1}$)</th>
<th>FWHM($\Delta \omega$) (cm$^{-1}$)</th>
<th>$\Delta \Delta \omega$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESP-fit static (at $r_n$)</td>
<td>2267</td>
<td>3 (5)</td>
<td>$+25$</td>
</tr>
<tr>
<td>Fluctuating ESP-fit</td>
<td>2236</td>
<td>4 (7)</td>
<td>$-6$</td>
</tr>
<tr>
<td>Fluctuating PC-fit</td>
<td>2241</td>
<td>4 (7)</td>
<td>$-1$</td>
</tr>
<tr>
<td>QM/MM (DFT)</td>
<td>2240</td>
<td>—</td>
<td>$-2$</td>
</tr>
<tr>
<td>QM/MM (MP2)</td>
<td>2308</td>
<td>—</td>
<td>$-9$</td>
</tr>
<tr>
<td>Experiment</td>
<td>2235</td>
<td>9</td>
<td>$-7$</td>
</tr>
</tbody>
</table>

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**Table 3** Parameters of multi-exponential fit of the FFCF for the nitrile probe of PhCN in water using the fluctuating ESP-fit and the fluctuating PC-fit models.

<table>
<thead>
<tr>
<th>Charge model</th>
<th>$a_1$ (ps$^{-1}$)</th>
<th>$\tau_1$ (ps)</th>
<th>$a_2$ (ps$^{-1}$)</th>
<th>$\tau_2$ (ps)</th>
<th>$a_3$ (ps$^{-1}$)</th>
<th>$\tau_3$ (ps)</th>
<th>$\Delta s^2$ (ps$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESP-fit</td>
<td>0.786</td>
<td>0.012</td>
<td>0.505</td>
<td>0.141</td>
<td>0.101</td>
<td>0.884</td>
<td>—</td>
</tr>
<tr>
<td>PC-fit</td>
<td>0.786</td>
<td>0.012</td>
<td>0.506</td>
<td>0.145</td>
<td>0.068</td>
<td>1.096</td>
<td>—</td>
</tr>
</tbody>
</table>

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**Fig. 6** (A) The FFCF decay of the nitrile group of PhCN in water for fluctuating ESP-fit model (red). The solid and dashed green lines correspond to the tri-exponential and bi-exponential fit, respectively. (B) The $g(r)$ (solid lines) and $N(r)$ (dashed lines) for the N(PhCN)–O(water) distance for PhCN in water for the fluctuating ESP-fit model. The water molecules within 4 Å of the N atom and the coordinate $r$ are shown in the inset. Note that the scaling of the $x$-axes ($r$-N$_{\text{PhCN}}$–O$_{\text{water}}$) corresponding to either $g(r)$ (bottom) or $N(r)$ (top) differ.
correlation function, \( C_{\phi}(t) = \langle \delta n(0) \delta n(t) \rangle / \langle (\delta n(0))^2 \rangle \) was calculated for the simulation with the ESP-fit model and fitted to a bi-exponential function (Fig. 7) which was found to be sufficient for a faithful representation. Here, \( \delta n(t) = n(t) - \langle n \rangle \) and \( \langle n \rangle \) are the average occupation numbers over the entire trajectory.58,55

The fit in Fig. 7A yields two time constants, 0.15 ps and 1.5 ps, with amplitudes of 0.54 and 0.43, respectively. The sub-ps timescale (i.e. 0.15 ps) is usually assigned to the librational motion of the water molecule, i.e. its inertial rotational motion, hindered by the interactions with the nearest water molecules.56 The longer timescale (1.5 ps), which is closer to \( \tau_3 \) of the vibrational decay (see Table 3), is attributed to the lifetime of the water molecules to reside close to the nitrile group (i.e. \( N_{\text{PhCN}}-O_{\text{water}} < 3.5 \text{ Å} \)) inside the first solvation shell. This assignment was made by explicitly determining the residence times of individual water molecules within a distance of 3.5 Å. Such time series also confirms that the water molecules in the first solvation shell interact weakly with the –CN group.

The lifetime of the first water shell can be estimated from the correlation function \( C_{\phi}(t) = \langle \delta H(0) \delta H(t) \rangle / \langle (\delta H(0))^2 \rangle \), which is shown in Fig. 7B. Here, \( H(t) = 1 \) and \( H(t) = 0 \) when a particular water molecule is present or absent in the first solvation shell, respectively. The time decay of \( C_{\phi}(t) \) also follows a multi-exponential decay with time constants of 1.4 ps, 8 ps and 50 ps.

3.2 PhCN in protein

Next, PhCN was used as a spectroscopic probe in WT and mutant lysozyme to probe the local electrostatic fields. For this, 5 ns of MD simulation of the solvated protein–ligand complex was carried out using the fluctuating ESP-fit and fluctuating PC-fit (for WT only) models for PhCN. Snapshots were extracted every 5 fs and analyzed in the same fashion as for PhCN in water and as described in the method section. All atoms in the environment were treated as MM point charges for the simulations as well as for the vibran analysis.

The frequency trajectory of the nitrile group of PhCN in L99A was also calculated using the QM/MM method for comparison.

Fig. 8 compares the \( P(\omega) \) calculated using vibran analysis (A) with \( P(\omega) \) obtained using the QM/MM method for 1000 equispaced snapshots extracted from 5 ns simulation (fitted to a Gaussian distribution) (B) for PhCN in water (red) and in L99A (green).

Fig. 8B reports a \( \sim 2 \text{ cm}^{-1} \) red shift of the nitrile frequency from water to L99A which is in good agreement with what was obtained using vibran (Fig. 8A). It should also be mentioned here that test calculations by taking the amino acids one by one into the QM part reveal that different amino acids can have canceling effects on the peak shifts depending on the interactions they provide. This means that the residues, depending on their dominant interaction properties, impose the shift of \( \delta \omega_{\text{max}} \) to the lower or higher frequency and the more different residues the PhCN interacts with, the more probable is the quenching of the Stark shift as is found in the case of WT and L99G.

The effect of the intermolecular interactions on the frequency distributions \( P(\omega) \) and the location of its maximum can be determined by specifically retaining certain contributions in the computational model. For this, simulations of PhCN in L99A were carried out whereby only (a) van der Waals interactions (i.e. no electrostatics at all on the ligand) and (b) van der Waals and point charge electrostatics (i.e. no fluctuations) were present. As is shown in Fig. 8A, a pure van der Waals description leads to a peak absorption at 2285 cm\(^{-1}\) (cyan solid line) which shifts by 7 cm\(^{-1}\) to the red (violet solid line) when static point charges are included and changes the shape of \( P(\omega) \) on the red side. Adding charge fluctuations contributes an appreciable additional shift of 42 cm\(^{-1}\) to the red region (green solid line in Fig. 8A) and further contributions to \( P(\omega) \) on the red side (inset in Fig. 8A). Hence, electrostatic interactions contribute significantly through both, static and fluctuating charges. The individual contributions of intermolecular interactions to frequency shifts have recently been analyzed in detail57 and revealed significant contributions of Coulomb, induction, dispersion and repulsion interactions with positive or negative contributions to the frequency shift of the nitrile stretching mode in MeCN and MeSCN. Effects of different electrostatic models and LJ parameters on the 1d-
2d-IR spectral properties were also discussed in previous work on hydrated cyanide\textsuperscript{29} and hydrated N-methyl-acetamide.\textsuperscript{32}

Next, the 1D absorption spectra for the nitrile group of PhCN in the protein were calculated. Fig. 8C shows the 1D absorption spectra of PhCN in WT, L99A and L99G using the fluctuating ESP-fit model. For comparison, the 1D spectrum of PhCN in WT lysozyme is also shown using the fluctuating PC-fit model (Fig. 8C). The absorption maximum $\omega_{\text{max}}$ for PhCN in WT lysozyme is found at 2236.5 cm$^{-1}$ using the fluctuating ESP-fit model compared to 2236 cm$^{-1}$ for PhCN in water. The peak frequency for the nitrile group of PhCN in L99G is shifted by 1 cm$^{-1}$ towards red compared to the $\omega_{\text{max}}$ of PhCN in WT whereas the $\omega_{\text{max}}$ of PhCN in L99A is at 2233 cm$^{-1}$ i.e. shifted to the red by 3.5 cm$^{-1}$. The $\omega_{\text{max}}$ of PhCN in WT lysozyme using the PC-fit model is 3 cm$^{-1}$ red shifted than the $\omega_{\text{max}}$ of PhCN in water. This indicates that the fluctuating PC-fit model is more sensitive to the vibrational Stark shift than the fluctuating ESP-fit model. This could be attributed to the more polar nitrile group and the relatively less polar benzene ring for the PC-fit model than for the ESP-fit model (see Table 1).

Although no experimental data are available on nitrile probes in lysozyme, the present results are in line with those from experiments on a nitrile probe at the active site of human aldose reductase which reports vibrational Stark shifts of 1–3 cm$^{-1}$ in the absence of a hydrogen bond between the CN-group and the amino acid residues.\textsuperscript{2,22} Previous studies suggest that the vibrational Stark effect is stronger in the presence of hydrogen bonding.\textsuperscript{22}

To characterize spectral diffusion, the FFCF of the CN-group of PhCN in the protein is analyzed. The time constants of the FFCFs (see Fig. 8D) from simulations of PhCN in WT, L99A and L99G T4 lysozyme with the fluctuating ESP-fit model and fitting to eqn (3) are given in Table 4.

<table>
<thead>
<tr>
<th>Environment</th>
<th>Charge model</th>
<th>$a_1$ (ps$^{-2}$)</th>
<th>$\tau_1$ (ps)</th>
<th>$a_2$ (ps$^{-2}$)</th>
<th>$\tau_2$ (ps)</th>
<th>$\Delta\tau^2$ (ps$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>ESP-fit</td>
<td>0.387</td>
<td>0.088</td>
<td>0.124</td>
<td>1.649</td>
<td>0.247</td>
</tr>
<tr>
<td>WT</td>
<td>PC-fit</td>
<td>0.620</td>
<td>0.079</td>
<td>0.240</td>
<td>1.366</td>
<td>0.297</td>
</tr>
<tr>
<td>L99A</td>
<td>ESP-fit</td>
<td>0.266</td>
<td>0.087</td>
<td>0.079</td>
<td>1.989</td>
<td>0.179</td>
</tr>
<tr>
<td>L99G</td>
<td>ESP-fit</td>
<td>0.472</td>
<td>0.071</td>
<td>0.134</td>
<td>1.409</td>
<td>0.178</td>
</tr>
</tbody>
</table>
All FFCFs for PhCN in the protein exhibit two time scales, compared with three timescales for PhCN in water. The longer decay times $\tau_2$ range from 1.4 to 2.0 ps, which is almost twice as long as the longest time scale for PhCN in water ($\tau_3$). On the contrary, the $\tau_1$ of the nitrile group of PhCN in protein is comparable to the $\tau_2$ of the PhCN in water. A comparison of the time constants for different proteins shows that $\tau_2$ for PhCN in L99A is longer by 30% compared to the WT or L99G mutant. In order to quantify the sensitivity to the electrostatic model used, simulations and analyses were also carried out using the PC-fit model for PhCN in the WT protein. The short decay time is very similar whereas the long decay time and the static component differ by about 20% for the two models.

The most significant difference between PhCN in water and in the protein is the existence of a nonvanishing static component in the case of protein, see Table 4. The value of $\Delta_s$ is in the range of 0.42–0.5 ps$^{-1}$ ($\Delta_s^2 = 0.18–0.25$ ps$^{-2}$) using the fluctuating ESP-fit model. The value of $\Delta_s$ for PhCN in L99A is $\approx 0.42$ ps$^{-1} = 2.21$ cm$^{-1}$. The significant static inhomogeneous components for the FFCF of nitrile in the protein indicate that there is slower dynamics involved which must be related to coupling between the ligand and the protein dynamics significantly contributed by the non-bonded interactions. Hence, the dynamics of PhCN is coupled to the slower conformational dynamics of the surrounding protein. Compared to simulations with the fluctuating ESP-fit, the $\Delta_s^2$ for the fluctuating PC-fit in WT lysozyme is only insignificantly larger (0.25 ps$^{-2}$ vs. 0.3 ps$^{-2}$). It was found from calculations that the $\Delta_s$ contributes significantly to the width of the 1D spectrum. The FWHM of the 1D spectra of PhCN in proteins (Fig. 8C) is around 8–10 cm$^{-1}$.

These results are in line with previous experimental studies which showed that the spectral diffusion dynamics of nitrile probes in the hydrophobic core of a protein HP35 is slowed down,58,59 The static inhomogeneous components were also observed in the spectral diffusion of the azide probe in a peptide bound to a protein.60 The $\Delta_s$ value for PhCN in L99A found in the present simulations can be compared with experimentally determined values for the static components for a nitrile probe (cyanophenylalanine) in HP35 (2.2 cm$^{-1}$) and in S-peptide (2.4 cm$^{-1}$).20,58

### 3.3 Protein–ligand binding free energy

To complement the analysis, the ligand binding free energy for PhCN was determined for the WT and L99A and L99G mutants of lysozyme. This potentially allows linking spectroscopic (1DIR, 2DIR) information with ligand binding affinity which is the relevant quantity for activity studies. The protein–ligand binding free energy was calculated using the MM-GBSA method.61 The binding free energies are determined from the following equation:

$$
\Delta G_{\text{bind}} = \langle \Delta G_{\text{bind}} \rangle + \langle \Delta G_{\text{desolv}} \rangle - \langle T \Delta S \rangle = \langle \Delta H \rangle - T \langle \Delta S \rangle,
$$

(5)

where $\Delta G_{\text{bind}}$, $\Delta G_{\text{desolv}}$ and $T \Delta S$ are the gas-phase contributions, the desolvation energy of the system upon binding and the entropic contribution, respectively. While the ensemble average for the enthalpic contribution ($\langle \Delta H \rangle$) was taken over 10000 equispaced structures over 5 ns simulations, the ensemble average over the entropic contribution ($\langle \Delta S \rangle$) was only computed from 50 equispaced structures due to the considerable computational expense in estimating this quantity. All free energies were calculated from the single trajectory of the protein–ligand complex.62 The gas phase contribution to the binding free energy is

$$
\Delta G_{\text{bind}}^D = \Delta G_{\text{intr}} + \Delta G_{\text{vdW}} + \Delta G_{\text{elec}} \tag{6}
$$

$$
\Delta G_{\text{intr}} = \langle E_{\text{intr}}^P \rangle - \langle E_{\text{intr}}^{P/L} \rangle \tag{7}
$$

where $\langle E_{\text{intr}}^P \rangle$ are the internal energies of the protein–ligand complex (PL), the protein (P) and the ligand (L), respectively and $E_{\text{vdW}}$ and $E_{\text{elec}}$ are the van der Waals and electrostatic interaction energies between the蛋白 and the ligand. They were calculated using the MM force fields. The $\Delta G_{\text{desolv}}$ consists of polar and non-polar contribution and is calculated by solving the generalized Boltzmann (GB) equation and with a linear relation to the solvent accessible surface area (SASA),63 respectively. The nonpolar binding free energy $\Delta G_{\text{gbp}} = A + b$, where $A$ is SASA and $\gamma$ and $b$ are constants with values of 5.0 ± 0.5 kcal mol$^{-1}$ Å$^{-2}$ and 860 ± 100 kcal mol$^{-1}$, respectively.65 The entropic contribution was calculated from a normal mode analysis for every snapshot using vibran.62

The calculated enthalpic and entropic contributions and the overall binding free energies for PhCN to WT, L99G and L99A lysozyme are given in Table 5. For PhCN the ligand binding free energy to L99A is $\Delta G = -3.93$ kcal mol$^{-1}$ whereas the interaction with the WT protein is weak ($-0.52$ kcal mol$^{-1}$) and binding to the L99G mutant is stronger ($\Delta G_{\text{bind}} = -2.59$ kcal mol$^{-1}$) but still somewhat weaker than the L99A mutant. Energy decomposition for the three protein–ligand complexes (see Table 5) shows that the contribution from van der Waals interactions is large and stabilizing (around $-19$ kcal mol$^{-1}$) but differs by less than 1 kcal mol$^{-1}$ between the different protein variants. Contrary to that, the electrostatic contribution is repulsive but differs significantly for the three proteins and largely determines the magnitude of the binding free energy.

No direct comparison with experiment is possible. However, a previous study for benzene binding to the L99A mutant found $K_d \approx 10^{-3} M$ which corresponds to $\Delta G_{\text{bind}} = -4.1$ kcal mol$^{-1}$.64,65

<table>
<thead>
<tr>
<th>Protein</th>
<th>$E_{\text{vdW}}$ (kcal mol$^{-1}$)</th>
<th>$E_{\text{elec}}$ (kcal mol$^{-1}$)</th>
<th>$\Delta G_{\text{desolv}}$ (kcal mol$^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$T \Delta S$ (kcal mol$^{-1}$)</th>
<th>$\Delta G_{\text{bind}}$ (kcal mol$^{-1}$)</th>
<th>$\Delta \omega_{\text{max}}$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L99G</td>
<td>18.82</td>
<td>5.43</td>
<td>-1.00</td>
<td>-14.39</td>
<td>-11.80</td>
<td>-2.59</td>
<td>1</td>
</tr>
<tr>
<td>WT</td>
<td>18.83</td>
<td>9.46</td>
<td>-2.69</td>
<td>-12.05</td>
<td>-11.43</td>
<td>-0.52</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 5** Thermodynamic parameters and the corresponding vibrational Stark shift ($\Delta \omega_{\text{max}}$ compared to $\omega_{\text{max}}$ of PhCN in WT lysozyme) for lysozyme–PhCN binding.
For the same protein, previous free energy perturbation simulations report a value of $\Delta G_{\text{bind}} = -6.0$ kcal mol$^{-1}$. Additional data for benzenoid-related ligands is available. $\text{N}$-Phenyglycino-nitrile binds with $-5.52$ kcal mol$^{-1}$ to L99A whereas the crystal structures of L99A with iodobenzene ($\text{C}_6\text{H}_5\text{I}$) and other halogenated benzenoids suggest that their binding is favourable. For example, isothermal titration calorimetry finds a binding affinity of $\Delta G \approx -3.5$ kcal mol$^{-1}$ for iodobenzene to the L99A mutant. This strongly supports the result that PhCN also interacts favourably with the L99A mutant of T4 lysozyme. On the other hand, the present simulations suggest that binding of PhCN to the WT protein is weak ($-0.52$ kcal mol$^{-1}$) whereas binding to the L99G mutant is stronger ($\Delta G_{\text{bind}} = -2.59$ kcal mol$^{-1}$) but still somewhat weaker than the L99A mutant. The results for binding of PhCN to the WT and the L99A mutant parallel those for benzene which found that the ligand binds to the L99A mutant but not to the WT protein.

The structural analysis of the trajectories reveals that PhCN adopts two conformations (see Fig. 1) in L99A which are populated for 45% and 30% of the simulation time. These two binding orientations are stable approximately for 3 and 2 ns, respectively. These results are in line with the crystallographic data of iodobenzene in L99A for which two different binding modes with similar orientations were found. Contrary to that, PhCN is relatively free to move in L99G and WT and no preferential orientation could be identified.

The relative stability of the two binding modes of PhCN in L99A was further analyzed. The structures show that $N_{\text{phen}}$–H–C interactions between the N of PhCN and the side-chain of the amino acids around the cavity (Tyr88, Ala99, Leu91, Val87, Leu84, Ile89 for the structure with 45% occupation and Leu133, Leu121 and Met102 for the other orientation) are present. The strongest interaction as judged from the distance between N and H was found with the H–C of the aromatic side chain of Tyr88. The N–H–C interaction between the negatively charged atom (N, O, F) and the positively charged H–C of the aromatic ring in liquid PhCN is well known in the literature. On the other hand, for PhCN in WT and in L99G, initially the CN group is oriented towards the solvent or is orthogonal to the side chains which hinders the PhCN to engage in stabilizing interactions. N–H–C is not a strong interaction, e.g. compared to a hydrogen bond, the accumulation of several such contacts is sufficient to stabilize a particular orientation.

It is found (see Table 5) that the binding strength ($\Delta G_{\text{bind}}$) correlates with the red shift encountered by the –CN group. This can also be qualitatively related to the presence of only two binding orientations for L99A compared to a rather unspecific positioning of the ligand near the binding site. It is further noted that similar to the red shifts, no particular energy contribution can be unequivocally correlated with the binding strength.

4 Conclusion

In this work, the vibrational Stark shift and time scale of spectral diffusion of a nitrile probe (PhCN) in a protein environment (lysozyme) were calculated using validated force fields for the spectroscopic (–CN) probe. The nitrile vibration was described as a Morse oscillator and a fluctuating point charge model was used to take the bond polarization of the nitrile probe into account. The results from two different point charge models were compared. The computational methods were validated by comparing with experiments for PhCN in water.

The simulations confirm that depending on the electrostatic environment in the protein the peak frequency in the linear absorption spectrum shifts. The shift approximately correlates with the relative binding free energy, see Table 5: the stronger the binding, the larger the red shift. This is a useful basis for the proposed strategy to locate ligand-binding sites through a combination of experiment and computation. However, it should be pointed out that the present work does not only correlate experimental FTIR spectra with computed electrostatic fields for the protein in implicit solvent as was done in ref. 2. Instead, the conformationally averaged 1D- and 2D-IR spectra for the –CN probe in the protein environment are explicitly determined from atomistic simulations in solution, which can be compared directly with future experiments.

On the other hand, using 2D-IR spectroscopy two observations are made: (a) there is a pronounced static inhomogeneous component for PhCN in the protein which is absent for PhCN in water. However, the magnitude of $\Delta G^2$ does not appear to be related to the binding free energy depending on the protein variant considered and (b) the long time scale $\tau_2$ is largest (2.0 ps) for the L99A mutant to which PhCN binds most strongly. Given that in state-of-the-art experiments a relaxation time can be determined to be within 40%, WT and L99G show a similar $\tau_2$ and the binding of PhCN to these two protein variants is weaker. Hence, strong protein–ligand binding correlates with long decay times in the FFCF.

The current simulations quantitatively reproduce the 1D and 2D spectroscopy of PhCN in water. Furthermore, they find – in agreement with experiments on related systems – that the FFCF for a CN probe in a protein exhibits a pronounced static inhomogeneous component. The magnitude of this contribution is in good quantitative agreement with experiments. Finally, similar to the experimental finding that benzene does not bind to WT lysozyme, the present simulations find a small stabilization of $-0.5$ kcal mol$^{-1}$ for PhCN binding to WT lysozyme. Contrary to that, binding to L99A and L99G mutants is considerably stronger which, again, has been found in previous experiments for benzene in L99A. Hence, the current simulations agree with all important, available experiments and it is expected that the approach outlined in the present work will be a successful strategy for future characterizations of protein–ligand binding using suitable spectroscopic probes.

Acknowledgements

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