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Phosphate Vibrations Probe Local Electric Fields and Hydration in Biomolecules

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Supporting Information

ABSTRACT: The role of electric fields in important biological processes such as binding and catalysis has been studied almost exclusively by computational methods. Experimental measurements of the local electric field in macromolecules are possible using suitably calibrated vibrational probes. Here we demonstrate that the vibrational transitions of phosphate groups are highly sensitive to an electric field and show how that sensitivity can be quantified, allowing electric field measurements to be made in phosphate-containing biological systems without chemical modification.

Phosphates are ubiquitous in biology and play important roles in the function of many biomolecules. Despite the difficulty of observing phosphate group vibrations in IR spectra of biological samples because of band overlap, a number of studies, especially those using caged compounds and/or isotopic labels, have demonstrated the potential for using IR spectroscopy to probe phosphate in the active sites of important enzymes.[1−4] In this communication, we quantify the sensitivity of the symmetric and antisymmetric stretches of phosphate to an applied electric field (the vibrational Stark effect), allowing spectral shifts observed in response to a variety of perturbations to be interpreted in terms of changes in the electric field experienced by the phosphate group (here called the matrix electric field). The proper interpretation of the results and the effects of hydration are analyzed using quantum-chemical calculations.

Vibrational Stark spectroscopy. For a vibrational probe in two environments that differ because of a pH titration, mutation, change in ligation in an enzyme, or other perturbation, the difference in the observed IR frequency, $\Delta\bar{\nu}_{obs}$ (in cm^{-1}), depends on the difference in the electric field of the matrix surrounding the probe, $\Delta\vec{F}_{matrix}$ (in MV/cm), according to the equation $hc\Delta\bar{\nu}_{obs} = -\Delta\vec{\mu}_{probe}\cdot\Delta\vec{F}_{matrix}$, where h is Planck's constant, c is the speed of light, and $\Delta\vec{\mu}_{probe}$ is the linear Stark tuning rate of the probe, whose magnitude $|\Delta\vec{\mu}_{probe}|$ [in $cm^{-1}/(MV/cm)$] can be obtained by measuring the vibrational Stark spectrum of the probe in a frozen glass solvent.[5,6] With $|\Delta\vec{\mu}_{probe}|$ thus calibrated (and assumed to be an intrinsic property of the probe vibration that does not change appreciably in different environments, which can be demonstrated in favorable cases[7,8]), $\Delta\bar{\nu}_{obs}$ can be used to obtain $\Delta\vec{F}_{matrix}$. While the direction of $\Delta\vec{\mu}_{probe}$ is expected and observed to be parallel to the bond axis for a simple oscillator like −C≡N,[9] phosphate vibrations are more complex, and calculations are indispensable for interpreting the results of the Stark experiments.

Phospholipids were chosen as a model for the phosphate group because they are soluble in several glass-forming solvents. The phosphate vibrations occur in a crowded region of the IR spectrum, so we screened several different phospholipid/solvent combinations to identify samples for which the phosphate bands could be clearly resolved [see the Supporting Information (SI) for further discussion]. All of the phospholipids were phosphoethanolamines and differed only in the nature of the fatty acid chains, and the spectra of all of the phospholipid/solvent samples were very similar (Figure S1 in the SI). The best spectra were obtained using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) in 1:3 (v/v) dichloromethane/dichloroethane and 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (DDHEPE) in deuterated toluene for the symmetric and antisymmetric stretches, respectively (Figure 1).

The absorption spectra of phosphate diesters display two bands for the phosphate PO_2^- group at 1080 and 1230 cm^{-1}, corresponding to the symmetric and antisymmetric stretches of this group, respectively.[10−12] It is immediately obvious from the Stark spectra that the Stark effect is much larger for the antisymmetric stretch than for the symmetric stretch. For both stretching vibrations, the Stark spectra are dominated by the linear component of the Stark effect (related to the second derivative of the absorption for an isotropic, immobilized sample; see the SI for details of the Stark analysis). Numerical fitting of the Stark spectra produced $|\Delta\vec{\mu}_{probe}|$ values of 0.54 ± 0.02 and 1.35 ± 0.02 $cm^{-1}/(MV/cm)$ for the symmetric and antisymmetric stretching bands, respectively. These values represent the shift in the absorption that would be observed if an electric field change of 1 MV/cm were projected along the C_2 symmetry axis of the PO_2^- group. Here we assumed that the direction of $\Delta\vec{\mu}_{probe}$ is along the C_2 axis for both modes (see the SI), which was supported by the results of our calculations as described below. We note that these sensitivities to an electric field, especially for the antisymmetric stretch, are large (the value for the antisymmetric stretch is almost twice as large as those for typical nitriles, which have been extensively studied[6,9]), making phosphate an unusually sensitive vibrational probe.

Effects of hydration. Substantial amounts of water can be solubilized by phospholipid/toluene mixtures, in which the water becomes encapsulated within reverse micelles. In these partially

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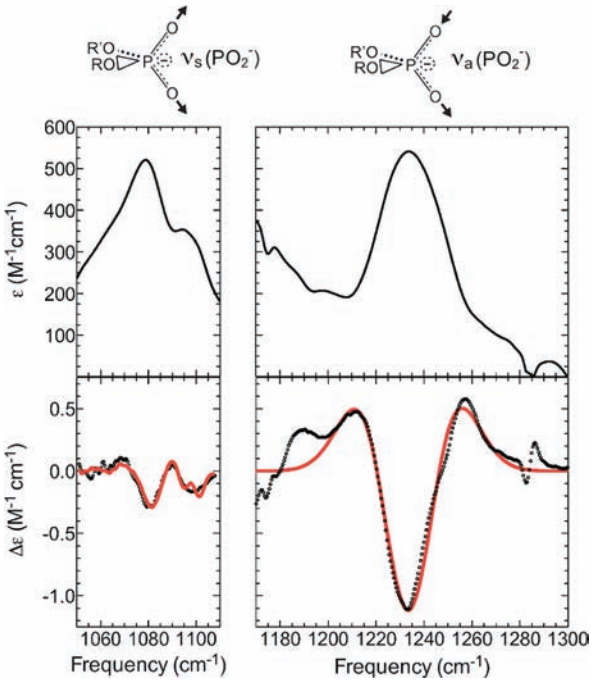

Figure 1. (top) Absorption and (bottom) Stark spectra of the phosphate region of phospholipid/toluene samples at 77 K. The spectra of the phospholipid symmetric stretch (left) and the antisymmetric stretch (right) were scaled to M^{-1} cm^{-1}, and the corresponding Stark spectra were additionally scaled to an applied field of 1 MV/cm. Experimental Stark spectra are shown as dots and numerical fits as solid red lines.

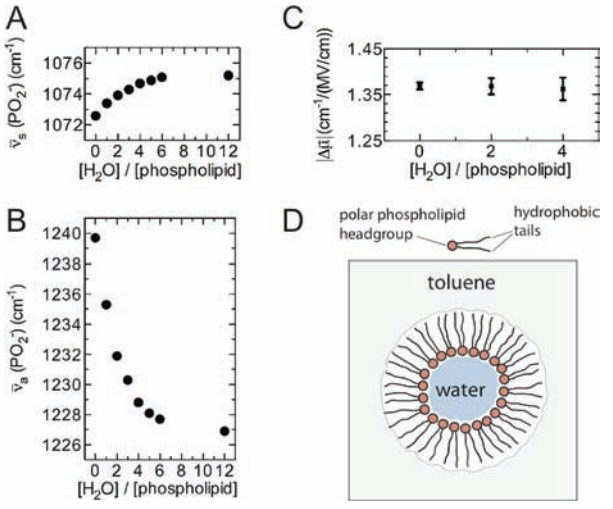

Figure 2. Effect of hydration on the phosphate vibrational frequency and Stark tuning rate. (A, B) Frequencies of the (A) symmetric and (B) antisymmetric stretches of PO_2^- in room-temperature DOPE/toluene as functions of the amount of water added. (C) Stark tuning rate measured for the antisymmetric stretch of DDHEPE/toluene as a function of the molar ratio of water to phospholipid. (D) Schematic representation of the reverse micelles used in these experiments.

hydrated samples the environment of the phospholipid headgroups is similar to the environment experienced in phospholipid membranes, allowing us to study the phosphate vibrations under more physiological conditions. While the addition of water to phospholipid/toluene samples had only a modest effect on the PO_2^- symmetric stretch (~3 cm^{-1}; Figure 2A), it resulted in a pronounced shift in the peak maximum of the antisymmetric stretch to lower frequency (~13 cm^{-1}; Figure 2B) as well as a significant broadening of the band. These changes reached a plateau at ~10 mol equiv of water relative to phospholipid, suggesting that hydration of the phospholipid headgroups is largely complete by that point. The red shift of the antisymmetric stretch in response to hydration has been well-documented for a wide range of phosphate diesters and has been attributed to the formation of hydrogen bonds between the phosphate groups and water molecules.[11,13−15] The question remains whether the peak shifts are due to the linear Stark effect and whether the hydrogen bonds affect the Stark tuning rates of the phosphate vibrations.

Table 1. Calculated Frequencies and Frequency Shifts for Symmetric (s) and Antisymmetric (a) Vibrations of Dimethyl Phosphate and Dimethyl Phosphate−Water Complexes[a]

		harmonic	anharmonic	point charge[b]
dimethyl phosphate	a	1248.0	1234.5	–
frequencies	s	1059.0	1053.5	–
ADD frequency shift	a	7.4	9.3	4.6
	s	−2.6	2.7	−0.8
SDD frequency shift	a	−35.9	−34.8	−29.3
	s	0.3	−2.3	−1.8

[a] All values in cm^{-1}. [b] Frequency shifts calculated with H_2O molecules modeled as point charges.

The vibrational Stark spectra of phospholipid/toluene samples with differing amounts of water were very similar in appearance (Figure S2) despite the substantial spectral shifts. Numerical fitting of these Stark spectra showed no evidence of a change in the linear Stark tuning rate due to hydration (Figure 2C), indicating that the formation of hydrogen bonds between the phosphate groups and water molecules does not significantly affect the sensitivity of the phosphate antisymmetric stretch to electric fields.

Quantum-chemical analysis. We next turned to calculations to better understand the effects of electric field and hydration on the phosphate vibrations. We used dimethyl phosphate as model system, which is amenable to density functional theory (DFT) calculations with reasonably large basis sets. The room-temperature absorption spectrum of 1 M dimethyl phosphate in water at pH 7.0 displays narrow symmetric and broader antisymmetric phosphate bands at 1080 and 1210 cm^{-1}, respectively (Figure S3), similar to what is observed for the phospholipid samples. The geometry of the dimethyl phosphate molecule was optimized in the gas-phase using DFT with the B3LYP functional[16−18] and the 6-311++G(d,p) basis set as implemented in Gaussian 03.[19] Harmonic vibrational analysis predicted symmetric and antisymmetric phosphate stretch vibrations at 1059.0 and 1248.0 cm^{-1}, respectively (Table 1). Anharmonic vibrational frequencies were computed for the PO_2^- group using methodology described in the SI. For dimethyl phosphate in the gas phase, this procedure yielded symmetric and antisymmetric stretch frequencies of 1053.5 cm^{-1} and 1234.5 cm^{-1}, respectively (Table 1).

Anharmonic symmetric and antisymmetric stretch frequencies were then computed for dimethyl phosphate in the gas phase with electric fields between 0 and ±2.057 MV/cm (±0.0004 au) aligned along the C_2 axis of the phosphate group (Figure 3, main

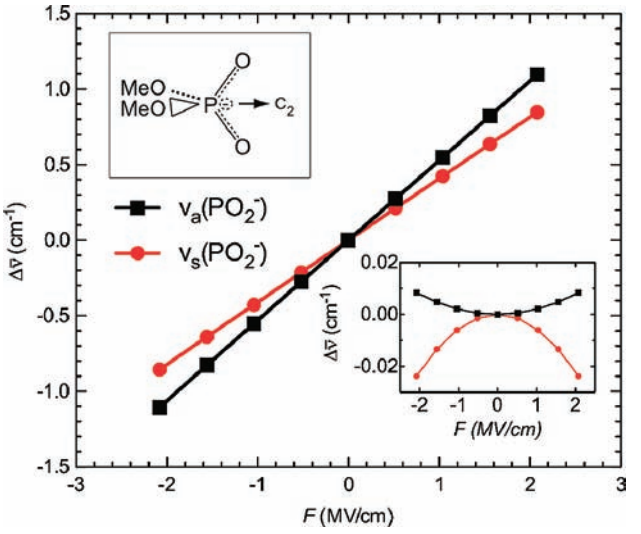

Figure 3. Field-induced frequency shifts in the phosphate vibrations calculated using DFT. Shifts in the anharmonic symmetric (red) and antisymmetric (black) stretch vibrational frequencies of dimethyl phosphate ($\Delta\bar{\nu}$) as functions of the applied electric field (F) are shown. The main panel shows the shifts calculated for a field applied along the C_2 axis of the PO_2^- group (depicted in the upper-left inset); the lower-right inset shows the shifts for a field applied along the O−O axis of PO_2^-.

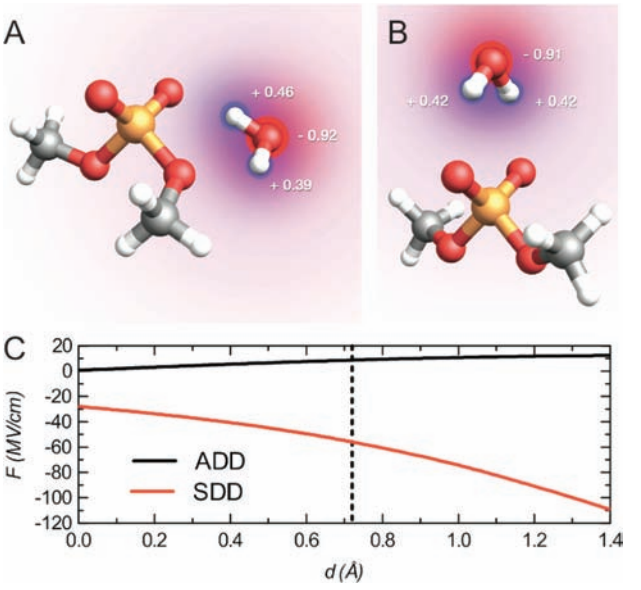

Figure 4. (A) Antisymmetric double donor (ADD) geometry of a single water molecule complexed to dimethyl phosphate. (B) Symmetric double donor (SDD) geometry of water complexed to dimethyl phosphate. The areas of the red and blue circles are proportional to the calculated atomic charges of water, which are indicated as fractions of e. (C) Projection of the electric field (F) along the PO_2^- symmetry axis as function of the distance d from the phosphate atom for SDD (red) and ADD (black). The vertical dotted line represents the value of d for the intersection of the PO_2^- C_2 axis and the O−O axis.

panel). The shifts in the phosphate vibrational frequencies were found to be large and to be nearly perfectly linear functions of the applied electric field. We then repeated the analysis for electric fields applied along the O−O axis of the phosphate group (Figure 3, lower-right inset). In this case, the shifts in the vibrational frequencies were nearly 2 orders of magnitude smaller than for the field applied along the C_2 axis, and the field dependence was quadratic. A similar result was obtained for an electric field applied in a direction orthogonal to both the C_2 and O−O axes. This indicates that along these axes a small quadratic Stark effect is the only response to the electric field and that the direction of $\Delta\vec{\mu}_{\text{probe}}$ is along the C_2 axis, as assumed above.

For the field applied along the C_2 axis, the slopes (which represent the vibrational Stark tuning rates in the gas phase) were found to be 0.40 and 0.53 $cm^{-1}/(MV/cm)$ for the symmetric and antisymmetric stretch frequencies, respectively. While the calculations underestimate the value of $|\Delta\vec{\mu}_{\text{probe}}|$ for the antisymmetric stretch, they do support the experimental observation that the antisymmetric stretch is intrinsically more sensitive to electric fields than the symmetric stretch. The linear Stark tuning rate is known to arise in large part from bond anharmonicity,[20] and the observation that the antisymmetric stretch is more than twice as anharmonic as the symmetric stretch (a shift of -13.5 cm^{-1} relative to the harmonic calculation for the antisymmetric stretch vs a shift of -5.8 cm^{-1} for the symmetric stretch) partly explains its greater sensitivity to the electric field.

These calculations complement the experiments nicely by verifying that nonlinear Stark effects are negligible, that the antisymmetric stretch is more sensitive than the symmetric stretch, and that the direction of $\Delta\vec{\mu}_{\text{probe}}$ is along the C_2 axis. We conclude that phosphate groups have a surprisingly simple response to an electric field that depends linearly on the projection of the field along a single axis, as observed with diatomic vibrational probes such as −C≡N.

We next wished to understand the physical origin of the peak shifts observed in response to hydration. The red shift of the antisymmetric stretch (Figure 2B) could be due to favorable electrostatic interactions through the linear Stark effect, but the blue shift of the symmetric stretch (Figure 2A) is difficult to reconcile with this model, as demonstrated by implicit solvent calculations, which indicated that both stretching modes were red-shifted in a high-dielectric medium (Figure S4).

To further understand the effect of water, we performed calculations on a single water molecule hydrogen-bound to the dimethyl phosphate molecule. At the B3LYP/6-311++G(d,p) level of theory, two stable geometries of dimethyl phosphate and one water molecule that differ in energy by 0.54 kcal mol^{-1} were identified (Figure 4A,B). In the lower-energy structure (Figure 4A), the water molecule donates a hydrogen bond to both an O of the PO_2^- group and an O of the P−O−CH_3 connection. In the higher-energy geometry, the water forms a symmetric pair of hydrogen bonds with the two O atoms of PO_2^-. We will refer to the structures in Figure 4A,B as the asymmetric double donor (ADD) and the symmetric double donor (SDD), respectively. Table 1 shows the shifts in the calculated harmonic and anharmonic phosphate stretching frequencies for the ADD and SDD geometries relative to those for dimethyl phosphate.

To understand whether the shifts due to the water molecule can be attributed to electrostatics, we calculated the atomic charges on the water molecule using the CHelpG algorithm.[21] The calculated charges are indicated in Figure 4A,B. The charges do not sum identically to zero because there is a small amount of charge transfer from the water molecule to the dimethyl phosphate molecule. We next repeated the anharmonic frequency calculations with the water molecules modeled as these point

charges (Table 1). The resulting shifts in the antisymmetric phosphate stretches (+4.6 and −29.3 cm^{-1} for the ADD and SDD complexes, respectively) show the same trend observed when the water was modeled explicitly (+9.3 and −34.8 cm^{-1}), implying that the shifts are mostly due to electrostatics. To see whether the linear Stark effect could account for these shifts, we computed the projection of the electric field along the C_2 axis of the PO_2^- group due to the water molecule's atomic charges as a function of the distance from the P atom (Figure 4C). The signs of the electric field projections were qualitatively different for the ADD and SDD complexes, consistent with the observed red shift in the phosphate stretch frequencies for the SDD complex (negative field) and blue shift for the ADD complex (positive field). Moreover, when the magnitudes of the fields at the point where the PO_2^- C_2 axis intersects the O−O axis (+8.8 MV/cm for ADD and −34.8 MV/cm for SDD) were multiplied by the calculated Stark tuning rate of 0.53 cm^{-1}/(MV/cm), frequency shifts of +4.6 and −29.7 cm^{-1}, respectively, were obtained for ADD and SDD, in remarkable agreement with the shifts of +4.6 and −29.3 cm^{-1} computed by DFT using the water partial charges.

These results indicate that the shifts in the antisymmetric stretch resulting from hydrogen bonding are mostly due to the linear Stark effect, with the small discrepancy between the explicit water and partial charge calculations arising from either secondary quantum-chemical effects or slight inaccuracies in the partial charges. A similar analysis of the symmetric stretch frequency showed that in contrast, the shifts are not reproduced by the partial charge calculations and are much smaller than would be expected from the linear Stark effect. This supports the experimental observation that the response of the symmetric stretch to hydrogen bonding is inconsistent with the linear Stark effect and suggests that the shifts are dominated by quantum-chemical effects.

The calculations show that interactions with water molecules can produce large electric fields, and we conclude that the dramatic red shift of the phosphate antisymmetric stretch observed experimentally in response to hydration (Figure 2B) can be attributed to these electric fields. This situation is in marked contrast to what is observed with other vibrational probes such as the nitrile group, where hydrogen bonds produce confounding peak shifts of a nonelectrostatic origin.[22,23] Phosphate groups are therefore superior probes for partially solvated environments such as the active sites of enzymes. In pioneering work by Gerwert and colleagues,[1,24,25] caged guanine nucleotides were used to monitor the time-dependent changes in the phosphate bands during GTP hydrolysis by the small GTPase Ras. This work underscored the importance of difference spectra for visualizing the phosphate transitions in biological samples, and a full assignment of the phosphate bands was possible using isotopic labeling. Shifts of ∼30 cm^{-1} for the phosphate antisymmetric stretching bands were observed during nucleotide binding, indicating large electric field changes of ∼20 MV/cm that could be expected to have profound effects on binding and catalysis. In addition to studies of electrostatics in phospholipid bilayers and DNA, the large number of enzymes that bind phosphate-containing molecules provide a wealth of systems where active-site electrostatics can be studied using phosphate as a vibrational probe.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Complete description of methods, spectra of all phospholipid/solvent samples, and complete ref 19. This material is available free of charge via the Internet at http://pubs.acs.org.

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