Protein Electrostatics

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# Ester Carbonyl Vibration as a Sensitive Probe of Protein Local Electric Field**

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***Abstract:*** *The ability to quantify the local electrostatic environment of proteins and protein/peptide assemblies is key to gaining a microscopic understanding of many biological interactions and processes. Herein, we show that the ester carbonyl stretching vibration of two non-natural amino acids,* L*-aspartic acid 4-methyl ester and* L*-glutamic acid 5-methyl ester, is a convenient and sensitive probe in this regard, since its frequency correlates linearly with the local electrostatic field for both hydrogen-bonding and non-hydrogen-bonding environments. We expect that the resultant frequency–electric-field map will find use in various applications. Furthermore, we show that, when situated in a non-hydrogen-bonding environment, this probe can also be used to measure the local dielectric constant (ε). For example, its application to amyloid fibrils formed by $A\beta_{16-22}$ revealed that the interior of such β-sheet assemblies has an ε value of approximately 5.6.*

Electrostatic interactions are ubiquitous in biological molecules and, in many cases, play a key role in molecular association and enzymatic reactions.[1] However, quantification of the local electric field or how it changes inside a protein, especially in a site-specific manner and/or as a function of time, still remains a challenging task. One promising method in this regard is vibrational Stark spectroscopy,[2] which capitalizes on the intrinsic dependence of vibrational transitions on the local electrostatic environment. This method is based on the use of an infrared (IR) probe that has a well-defined, localized vibrational mode to sense the amplitude of the local electric field through the frequency response.[3] For example, the vibrational Stark effect has been used to determine the local electric field at protein interfaces and to monitor protein conformational transitions and dynamics.[4] Although the theoretical underpinning of this methodology is straightforward, in practice the application of vibrational Stark spectroscopy to biological systems is currently limited by the availability of suitable vibrational probes. Herein, we show, by the use of linear and nonlinear IR spectroscopic measurements and molecular-dynamics (MD) simulations, that the ester carbonyl vibration in two non-natural amino acids can be used to quantitatively and site-specifically probe the electric fields of proteins, including those arising from hydrogen-bond (H-bond) interactions.

The utility of a vibrational probe to reliably and conveniently measure local electric fields in proteins is evaluated by how well it meets several criteria. First and foremost, its frequency must show a sensitivity to and quantifiable dependence on the local electric field. Also, a chemical or biological method must exist to incorporate the probe into a protein. Furthermore, it must minimally perturb the native chemical and structural environment of interest. Finally, its vibration must be a localized mode with a large cross-section and, ideally, be located in an uncongested region of the IR spectrum of proteins (e.g., 1700–2400 $cm^{-1}$).

For naturally occurring proteins, the amide I vibration arising from backbone amide units offers the largest IR intensity (molar extinction coefficient: ca. 800 $M^{-1} cm^{-1}$) and shows a strong dependence on the local electrostatic environment, such as hydration. As a result, it has been widely used to investigate protein conformational transitions.[5] However, the amide I transition is generally delocalized and also contains contributions from other vibrational modes, thus making its use as a stand-alone probe of local electric field rather challenging. One viable strategy to overcome this limitation is to incorporate a single carbonyl group (C=O) into an amino acid side chain. A computational study by Choi and Cho predicted that the stretching mode of such a carbonyl group is not only localized, but its frequency also varies linearly with the electrostatic field for both H-bonding and non-H-bonding environments,[6] thus making it an ideal candidate for the aforementioned applications. Indeed, Boxer and co-workers[7] recently showed that the C=O stretching frequency of *p*-acetyl-L-phenylalanine (*p*-Ac-Phe) can serve as a reporter of the local electrostatic field of proteins. However, this vibrational transition (at ca. 1673 $cm^{-1}$) overlaps with the protein amide I band, and thus its application requires careful background subtraction by using the wild-type protein. To circumvent this inconvenience, we propose the use of the C=O stretching vibration of an ester moiety as an alternative probe. Previous studies[8] have shown that the ester carbonyl group absorbs in a spectral region (1700–1800 $cm^{-1}$) in which no other protein IR bands are present at a neutral pH value,[9] except those arising from protonated carboxylic groups.[10] Specifically, we tested the utility of two ester-containing non-natural amino acids,

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L-aspartic acid 4-methyl ester (hereafter referred to as $D_M$) and L-glutamic acid 5-methyl ester (hereafter referred to as $E_M$). Although, to the best of our knowledge, $D_M$ and $E_M$ have not previously been introduced in proteins, we expected that it would not be a challenging task, as other ester-containing side chains have been successfully incorporated into proteins by genetic methods.[11]

As a quantitative assessment of the electric-field dependence of the ester carbonyl stretching vibrations of $D_M$ and $E_M$, we first performed detailed vibrational solvatochromism studies on the respective side-chain mimics, methyl acetate (MA) and methyl propionate (MP). We chose MA and MP in these experiments, instead of the non-natural amino acids, because the model compounds are soluble in a wide range of polar and nonpolar solvents. The ester carbonyl stretching frequencies of MA and MP exhibited a strong dependence on the chosen solvent, ranging from hexane, an aprotic solvent with a very low dielectric constant (1.89 at 20 °C), to water (Figure 1; see also Table S1 in the Supporting Information).

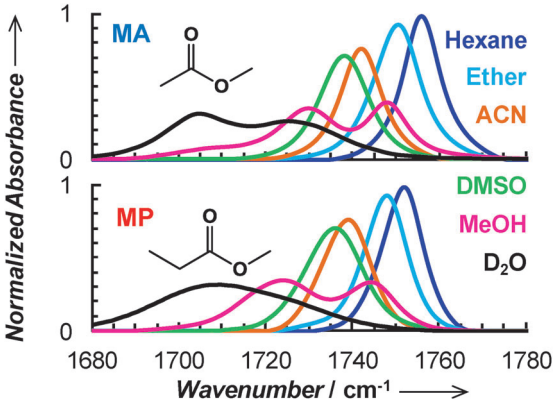


***Figure 1.*** Normalized FTIR spectra of MA and MP in different solvents, as indicated. The concentration of the solute in each case was 20 mM, and normalization is based on the integrated area of the band observed in hexane (i.e., the spectra collected in other solvents were scaled so that their integrated areas were equal to that observed in hexane). For MA in hexane, the peak absorbance was measured to be 0.0715, which corresponds to a molar extinction coefficient of 650 $M^{-1}cm^{-1}$.

For example, when the solvent was changed from hexane to DMSO, the center frequency of MA was redshifted by 18.1 $cm^{-1}$, as compared to the 14.4 $cm^{-1}$ shift observed for *p*-Ac-Phe.[7] Thus, these results substantiate the utility of these ester carbonyl stretching vibrations as sensitive probes of the local electric field, provided that a quantitative relationship between the electric field and frequency can be determined.

Interestingly, in aprotic solvents the ester carbonyl stretching vibration results in a single absorption band, whereas in protic solvents, in which H-bonding between the vibrator and solvent is possible, the linear IR spectra contained more than one resolvable feature, thus suggesting that differently solvated or H-bonded species are present. Such spectral features have also been observed for nitrile and amide modes in protic solvents, such as methanol.[12] For example, in $D_2O$ the IR spectrum of MA contains to two distinct peaks, centered at 1703.6 and 1727.0 $cm^{-1}$, which is consistent with the two-dimensional IR spectroscopic study of Righini and co-workers.[13] In agreement with the study of Tominaga and co-workers,[14] the ester carbonyl stretching band of MA in methanol consists of three resolvable spectral features, centered at 1748.1, 1729.6, and 1708.1 $cm^{-1}$. For MP, however, the spectrum obtained in $D_2O$ is broad and almost featureless. To be able to better discern the underlying spectral contributions, we further carried out 2D IR spectroscopic measurements on MP in $D_2O$ and methanol. The 2D IR spectrum indicates that under the linear IR profile of MP in $D_2O$, two peaks, at 1703.1 and 1721.1 $cm^{-1}$, are present and represent two distinct species (see Figure S1 in the Supporting Information).

With this information at hand, we then tried to determine how the ester C=O stretching frequencies of MA and MP vary with the local electric field. The commonly used Onsager reaction field model[15] described fairly well the trend observed with aprotic solvents (see Figure S2), consistent with the study of Asbury and co-workers,[16] but failed to predict the frequency shifts induced by protic solvents (see Figure S3).

Therefore, following the work of Boxer and co-workers,[17] we used MD simulations to directly quantify the electric field experienced by the ester carbonyl vibration and to help assign the two C=O stretching bands observed in protic solvents (see details and Figures S4 and S5 in the Supporting Information). Briefly, for aprotic solvents the electric field was directly calculated by averaging the values obtained from about 20000 frames from each MD simulation, whereas for protic solvents, because of the possibility of different H-bonding patterns, we first divided the frames from each MD simulation into different clusters, according to a set of geometric criteria for H-bond formation (see details in the Supporting Information), and then calculated the average electric field for each cluster.

For MA in water, the majority of the carbonyl groups form either one (51%) or two H-bonds (44%) to water (see Figure S5). Thus, we propose that the two peaks observed in the linear IR spectrum arise predominantly from these two species. Because the H-bonding of water to a carbonyl group induces a redshift in the carbonyl stretching vibration, we attribute the lower-frequency component to the doubly H-bonded species and the higher-frequency peak to the singly H-bonded species. Furthermore, the percentages of the lower- and higher-frequency components of the C=O stretching vibration of MA in $D_2O$ (Figure 1), as calculated on the basis of the integrated peak areas, are 55 and 45%. This result provides further evidence in support of the above assignment. MD simulations revealed the existence of less-populated but differently H-bonded species, which were assumed to contribute to the broad width of the spectrum. A similar observation and assignment was made for MP in water. For both compounds in methanol, the majority of the carbonyl groups were found to be either non-H-bonded or singly H-bonded, with relative percentages in agreement with the ratio of the integrated areas of the two IR peaks. Thus, we assigned the two major IR bands to these species, with the H-bonded carbonyl groups vibrating at a lower frequency. For MA,

  

a minor band at approximately 1710 $cm^{-1}$ is clearly observable. On the basis of MD simulations, this band was attributed to doubly H-bonded carbonyl groups. Finally, the width of the calculated electric-field distribution for each differently solvated species shows a correlation with the corresponding spectral width of the C=O stretching vibration (see Figure S6), thus suggesting that the MD simulations are able to exhaustively sample the heterogeneous electrostatic environments of the probe.

The center frequencies of the ester carbonyl stretching vibrations of MA and MP show a linear dependence on the calculated electric field for both protic and aprotic solvents (Figure 2), thus indicating that an ester moiety, such as that in $D_M$ and $E_M$, could be used to quantitatively determine the local electrostatic field of proteins by the use of this frequency–field map.

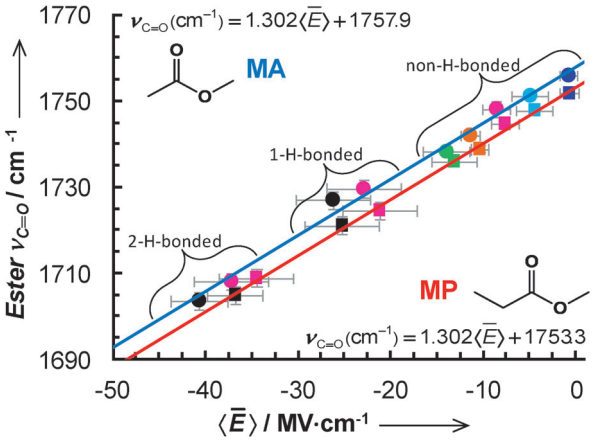


***Figure 2.*** Center frequencies of the carbonyl stretching vibrations of MA (circles) and MP (squares) versus the calculated local electric field for different solvents (represented by the same colors as those used in Figure 1). The solid lines are the best fits of these data to the linear equations indicated in the figure.

To demonstrate the utility of this ester vibrational mode in biological applications, we first used $D_M$ and $E_M$ to probe the local electrostatic and/or hydration environment of two short peptides, Ac-YD$_M$K-NH$_2$ (hereafter referred to as $D_M$-P) and Ac-YE$_M$K-NH$_2$ (hereafter referred to as $E_M$-P). The ester carbonyl stretching bands of these peptides in $D_2O$ indicate, when compared to those of MA and MP, that the population of the doubly H-bonded species (i.e., the spectral intensity at ca. 1705 $cm^{-1}$) is significantly decreased (Figure 3). This result is not surprising as, in comparison to their respective model compounds, the side chains of $D_M$ and $E_M$ are expected to be situated in a more crowded environment, thus limiting the accessibility of water molecules to the ester carbonyl group and hence decreasing the probability of the formation of two H-bonds. Furthermore, and perhaps more convincingly, $E_M$-P, the ester carbonyl group of which is expected to be further extended into the solvent than that of $D_M$-P, shows a smaller decrease in this regard. Thus, these results provide further validation of the sensitivity of the C=O stretching vibration of the ester moiety to its local electrostatic environment. In

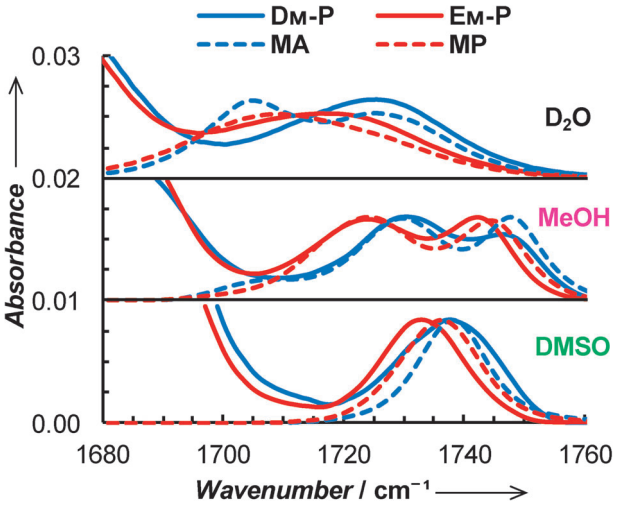


***Figure 3.*** Offset FTIR spectra in the ester C=O stretching region of $D_M$-P, MA, $E_M$-P, and MP in different solvents, as indicated. The peptide concentration was 2 mM, and in each case, the spectrum of the model compound has been normalized with respect to that of the corresponding peptide.

support of this notion, Xie and co-workers have shown that the native structural analogues of $D_M$ and $E_M$, that is, the protonated carboxylic acid side chains of Asp and Glu, can be used to sense H-bond formation in proteins.[10] The spectra of these two peptides in DMSO and methanol also support this notion. For example, in DMSO the center frequency of the ester C=O stretching band of $E_M$-P is redshifted by 3.4 $cm^{-1}$ from that of MP, whereas that of $D_M$-P is similar to that of MA. This redshift results from the addition of the peptide environment around the vibrational probe. Besides the solvent-induced electric field, the ester carbonyl group will also experience electrostatic forces arising from the peptide backbone and other amino acid side chains, thus making the vibrational frequency dependent on the position of the amino acid in the peptide. In other words, the redshift observed for $E_M$-P is most likely due to the closer (as compared to $D_M$-P) proximity between the ester carbonyl group and the polar amine group of the lysine side chain. The observed non-hydrogen-bonded peaks of these peptides in methanol also corroborate this picture (Figure 3). Thus, these results demonstrate the ability of the ester carbonyl stretching vibration to sense minimal changes in its local electrostatic environment.

In a second study, we used $D_M$ to quantify the electrostatic environment in amyloid fibrils formed by a short segment of the β-amyloid peptide KLVFFAE (i.e., $A\beta_{16-22}$) characteristic of Alzheimer's disease. Although it is generally assumed that the interior of amyloid has a low dielectric constant, to the best of our knowledge, no experiments have been attempted to directly measure the electrostatic properties of such β-sheet assemblies. In this study, we mutated the leucine residue of $A\beta_{16-22}$ to $D_M$ (the resultant peptide is referred to as Aβ-$D_M$), since the side chains are similar in size. Prior to full onset of peptide aggregation (as judged by the amide I band at 1625 $cm^{-1}$), the ester band had a peak at approximately 1725 $cm^{-1}$ (Figure 4), thus indicating that the $D_M$ side chain

  

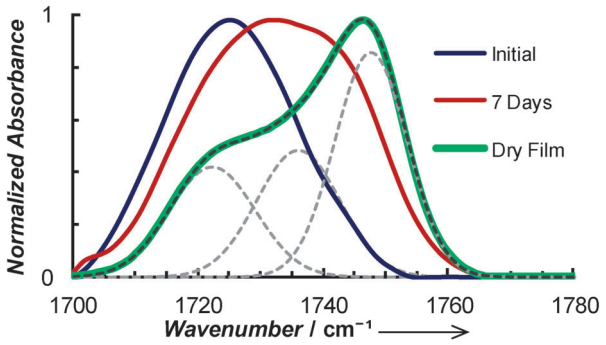


***Figure 4.*** Normalized stretching vibrational bands of the ester carbonyl group of Aβ-$D_M$, as observed immediately after the preparation of a 15 mM sample of Aβ-$D_M$, after incubation of the sample for 7 days in $D_2O$, and in a dry film of Aβ-$D_M$ (dried under a flow of nitrogen for 7 days), as indicated. The band observed for the dry film can be decomposed into three Gaussian functions (gray dashed lines; the center frequencies are given in the main text). It should be noted that in each case a baseline that contains contributions from the amide I′ band has been subtracted.

was mostly hydrated and formed one H-bond with water, as expected. Upon further aggregation, the ester band became broader and was blueshifted, thus indicating that the population of the H-bonded ester carbonyl groups decreased. Further analysis indicated that this band can be decomposed into two Gaussian functions with center frequencies at 1727.2 and 1743.7 $cm^{-1}$ (see Figure S8). This result is consistent with the formation of β-sheet fibrils, which, according to the $A\beta_{16-22}$ fibrillar structures determined by Eisenberg and co-workers,[18] would lead to the creation of two distinct environments for the $D_M$ side chain: one in which the side chain is sequestered in a dehydrated interface, and the other in which it is exposed to solvent (see Figure S7). However, as revealed by AFM measurements (see Figure S9), the fibrils/aggregates thus formed are rather heterogeneous. This heterogeneity not only leads to a broad ester C=O stretching band, but also prevents a more quantitative assessment of the structural features of the fibrils on the basis of their IR signals.

The structural model of Eisenberg and co-workers[18] indicates that the β-strands stack in an antiparallel fashion, with water molecules confined in the core of the fibrils. To probe these water molecules, and also to form more homogeneous fibrils, we placed an aliquot of the above-mentioned aggregated Aβ-$D_M$ sample on the surface of a germanium crystal of an attenuated total reflectance (ATR) unit and allowed it to dry under a gentle flow of $N_2$ for 7 days. This drying procedure should remove most, if not all, of the bulk water. Ester C=O stretching vibrations were observed as three well-resolved peaks at 1722.3, 1736.0, and 1747.6 $cm^{-1}$ in the IR spectrum of the resulting film(Figure 4). The lowest-frequency peak (at 1722.3 $cm^{-1}$) coincides with that due to singly H-bonded ester carbonyl groups (Figure 1), thus indicating that water is indeed present in the amyloid fibrils. On the other hand, the peak at 1747.6 $cm^{-1}$ must arise from non-H-bonded ester carbonyl groups, or those situated at the aforementioned dry interfaces (see Figures S7 and S8), whereas the peak at 1736.0 $cm^{-1}$ most likely corresponds to outward-facing $D_M$ side chains that are H-bonded with water prior to sample drying, and its frequency reflects the local electrostatic environment of the dry air/fibril interfaces. In support of these assignments, the relative percentages of these peaks, as determined from their integrated areas, are 28 (1722.3 $cm^{-1}$), 31 (1736.0 $cm^{-1}$), and 41% (1747.6 $cm^{-1}$), which are similar to those (25, 25, and 50%) calculated on the basis of the structural model of Eisenberg and co-workers (see Figure S7).[18]

By using the frequency–field relationship obtained for MA (Figure 2), we determined that the three peaks (in order of increasing frequency) give rise to the following local electric fields: −26.3, −16.2, and −8.0 MV $cm^{-1}$. More importantly, we could use the experimentally determined frequency–Onsager field relationship for this probe (see the Supporting Information) to estimate a dielectric constant of 5.6 for the dry interior of the well-packed fibrils. Whereas it is well-known that a low-dielectric-constant environment would increase the strength of H-bonding and other types of electrostatic interactions, it is challenging to quantitatively assess the dielectric constants of proteins and peptides, especially in a site-specific manner. Thus, it is our belief that the methodology demonstrated herein will be useful for relevant biological studies.

In conclusion, we have established that the ester carbonyl stretching frequencies of two non-natural amino acids, L-aspartic acid 4-methyl ester ($D_M$) and L-glutamic acid 5-methyl ester ($E_M$), show a linear dependence on the local electric field and, thus, can be used to quantify, in a site-specific manner, the local electrostatic environment of proteins. In comparison to commonly used nitrile-, azide-, and CD-based IR probes,[19] the ester carbonyl stretching vibration offers one distinct advantage: its large dynamic range makes it more useful for probing small changes in the local electric field. For example, it is sensitive enough to probe the difference in the electric fields between two points in a peptide environment that are separated by a single methylene unit. Furthermore, the size of $D_M$ is similar to that of asparagine, aspartic acid, and leucine, whereas the size of $E_M$ is similar to that of glutamine and glutamic acid. Taken together, these attributes of $D_M$ and $E_M$ suggest that they are two of the most promising local electrostatic IR probes of proteins. We have also devised a new method that enables the determination of the local dielectric constant of proteins. The application of this method to amyloid fibrils formed by an Aβ-peptide fragment indicates that their interiors have a dielectric constant of $\varepsilon = 5.6 \pm 1.5$. Because the C=O stretching vibration of esters is also Raman-active,[20] we expect that the frequency–field relationships devised herein can also be used to study relevant biochemical and biophysical problems in conjunction with techniques based on Raman spectroscopy.[21]

## Experimental Section

Methyl acetate (acetic acid methyl ester, MA) and methyl propionate (propanoic acid methyl ester, MP) were purchased from Acros Organics (Fair Lawn, NJ) and used as received. Fmoc-L-Asp(Me)-OH was purchased from Chem-Impex International Inc. (Wood Dale, IL) and Fmoc-L-Glu(Me)-OH was purchased from Santa Cruz Biotechnology Inc. (Fmoc = 9-fluorenylmethoxycarbonyl). Peptides

  

were synthesized by the use of standard Fmoc solid-phase methods on a PS3 peptide synthesizer from Protein Technologies (Tucson, AZ). All linear IR spectra, except that for the Aβ-$D_M$ dry film, which was obtained by using a Horizon ATR unit from Harrick (Pleasantville, NY), were collected on a Nicolet Magna-IR 860 FTIR spectrometer at 1 $cm^{-1}$ resolution with a home-made $CaF_2$ sample holder. More details about the linear and 2D IR instrumentation can be found elsewhere[22] or in the Supporting Information. AFM images were acquired on a Bruker Dimension Icon AFM instrument (Santa Barbara, CA). The details of MD simulations and electric-field calculations are given in the Supporting Information.

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