Vibrational Stark Effects Calibrate the Sensitivity of Vibrational Probes for Electric Fields in Proteins†

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Received July 22, 2003; Revised Manuscript Received August 22, 2003

ABSTRACT: Infrared spectroscopy is widely used to probe local environments and dynamics in proteins. The introduction of a unique vibration at a specific site of a protein or more complex assembly offers many advantages over observing the spectra of an unmodified protein. We have previously shown that infrared frequency shifts in proteins can arise from differences in the local electric field at the probe vibration. Thus, vibrational frequencies can be used to map electric fields in proteins at many sites or to measure the change in electric field due to a perturbation. The Stark tuning rate gives the sensitivity of a vibrational frequency to an electric field, and for it to be useful, the Stark tuning rate should be as large as possible. Vibrational Stark effect spectroscopy provides a direct measurement of the Stark tuning rate and allows a quantitative interpretation of frequency shifts. We present vibrational Stark spectra of several bond types, extending our work on nitriles and carbonyls and characterizing four additional bond types (carbon−fluorine, carbon−deuterium, azide, and nitro bonds) that are potential probes for electric fields in proteins. The measured Stark tuning rates, peak positions, and extinction coefficients provide the primary information needed to design amino acid analogues or labels to act as probes of local environments in proteins.

The application of infrared spectroscopy to protein structure and dynamics has largely focused on the intense amide modes of the protein backbone. These modes, the strongest of which have been classified amide I, amide II, and amide III, are sensitive to secondary contacts, have relatively large intensities, and have been used to estimate secondary structure (*1*), monitor protein fluctuations (*2*), and observe conformational changes upon ligand binding (*3*) or during folding (*4*). The main disadvantage of the amide vibrations is that they arise from bonds throughout the protein and provide no information about local environments. It has been shown that ^{13}C labeling of specific amide carbonyls can provide site-specific amide I transitions (*5*), but the modest isotopic frequency shift ($\sim$37 cm^{-1}) means the peak of interest is often partially or completely obscured by the dominant ^{12}C amide I transition. The infrared spectra of amino acid side chains have also been characterized (*6*), and changes in the IR spectra of specific side chains can sometimes be observed and assigned (*7*). However, the assignment of transitions to specific side chains often involves extensive site-directed mutagenesis and isotopic labeling. In addition to vibrations arising from the protein itself, the vibrational spectra of prosthetic groups (e.g., retinal, flavins, hemes, chlorophylls, metal centers, etc.) have been widely exploited in studies of particular proteins.

An attractive alternative to observing infrared transitions contained in the natural amino acids is the site-specific introduction of a unique probe vibration in an unnatural amino acid. An ideal probe would meet several criteria. (1) The vibration's frequency should fall in a clear region of the protein/water/buffer IR spectrum. (2) The vibration's extinction coefficient should be high to allow good signal-to-noise spectra at reasonable concentrations. (3) The sensitivity of the vibration's frequency to changes in local environment should be great. (4) The probe molecule should be stable in buffer solutions. Ideally, this functionality could be built into a small amino acid or label to minimize perturbations to protein structure and incorporated into proteins or peptides using methods such as nonsense suppression (*8*, *9*), auxotrophic *Escherichia coli* strains containing mutated tRNA synthetases (*10*, *11*), or peptide ligation methods (*12*). An alternative to amino acid substitution is site-specific labeling of reactive amino acid side chains, most notably the thiol of cysteine, with a suitable probe (*13*). The environment of enzyme active sites could also be investigated by using inhibitors with high binding affinities and useful infrared spectra. Several inhibitors with useful probe vibrations have been structurally characterized in the active sites of their targets, and these complexes provide one method of investigating active site electrostatics.

As methods for incorporating nonnatural amino acids and labels improve, it is important to determine which bond types are most suitable as infrared probes. Requirements (2) and (3) are intimately related to the vibrational Stark effect (VSE), the sensitivity of the vibrational frequency to an applied electric field. Vibrational Stark spectroscopy provides several valuable bond parameters that can be used to design

† This work was supported by grants from the National Institutes of Health and the National Science Foundation Chemistry Division. The FTIR system used for this work is located at the Stanford FEL Center supported by the Air Force Office of Scientific Research (Grant F49620-00-1-0349).

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10.1021/bi0352926 CCC: $25.00
Published on Web 09/26/2003

molecules with sensitive infrared transitions (*14*). Although the VSE is measured in an external electric field, it can be used to calibrate the sensitivity of any vibrational frequency to the internal electric field in an organized system. In proteins, local electric fields are predicted by electrostatics calculations to be large and vary tremendously in different locations. Variations in the protein electric field by as much as plus or minus tens of megavolts per centimeter are often predicted; however, quantitative values are rarely available from direct measurements. A primary goal of this work is to provide quantitative information about the sensitivities of vibrational frequencies to electric fields to optimize the choice of vibrational probes.

Until recently, there were almost no measurements of vibrational Stark effects. Substantial improvements to methods of data acquisition and sample preparation in our lab have now made such measurements quite routine, allowing for fairly rapid acquisition of vibrational Stark spectra. In most cases, the primary effect of an electric field on a molecular vibration is the interaction of the field with the change in dipole moment of the vibration, $\Delta\vec{\mu}$ (*15*, *16*). For these cases, the change in frequency is a linear function of the applied field, $\vec{F}_{ext}$, and $\Delta\vec{\mu}$, leading to a frequency shift $\Delta\bar{\nu}$ (in cm^{-1}):

$$hc\Delta\bar{\nu} = -\Delta\vec{\mu}\cdot\vec{F}_{ext} \quad (1)$$

where h is Planck's constant and c is the speed of light. $\Delta\vec{\mu}$ can be expressed either in units of debyes or as a Stark tuning rate in units of cm^{-1}/(MV/cm), that is the frequency shift per unit of applied field. For an isotropic, immobilized sample, the application of the field leads to band broadening, and the change in absorption ΔA [$=A(F_{ext}) - A(F=0)$] or Stark spectrum has the shape of the second derivative of the absorption band (*17*). In general, one expects that the angle between $\Delta\vec{\mu}$ and the transition dipole moment direction, $\vec{m}$, often denoted ζ in the literature on Stark effects, should be zero for an isolated vibrator, and this has been confirmed in several cases (*14*). Since $\vec{m}$ is typically parallel to the bond axis for an isolated vibrator, $\Delta\vec{\mu}$ is a directional probe of electric fields and its direction is known as accurately as the direction of the bond axis. If it is assumed that $\zeta = 0°$, it is straightforward to obtain $|\Delta\mu|$ from the magnitude of the observed Stark effect (*17*).

Once $|\Delta\mu|$ has been measured, either in a simple model compound or in a complex environment such as a protein, observed variations in vibrational frequencies, $\Delta\bar{\nu}_{obs}$ (in cm^{-1}), can be interpreted as arising from variations in the magnitude and direction of the protein electric field, $\Delta\vec{F}_{protein}$, projected onto $\Delta\vec{\mu}$:

$$\Delta E = hc\Delta\bar{\nu}_{obs} = -\Delta\vec{\mu}\cdot\Delta\vec{F}_{protein} \quad (2)$$

Note that because there is a well-defined relationship between $\Delta\vec{F}_{protein}$ and $\Delta\vec{\mu}$ to the extent that the protein is well-structured, the internal Stark effect due to the protein electric field gives a linear shift in the vibrational frequency. Thus, there is a direct correlation between observed frequency shifts $\Delta\bar{\nu}_{obs}$ for an oscillator in different environments and the electric field associated with that environment, so long as $|\Delta\mu|$ can be measured. Because calculated variations of electric fields in proteins can be as large as ±10 MV/cm and Stark tuning rates of possible probe vibrations are found in the following to range from 0.4 to 2.9 cm^{-1}/(MV/cm), variations of up to ±60 cm^{-1} are expected depending on the location and orientation of the probe oscillator in the protein's electric field. An example of this concept has been presented for the special case of CO or NO bound to the heme iron in myoglobin (*18*, *19*). The Stark tuning rates for bound CO and NO were found to be approximately 2−2.5 cm^{-1}/(MV/cm), leading to vibrational frequency shifts of ±tens of cm^{-1} for mutations in the heme pocket near the bound ligand. These frequency shifts were interpreted as arising from variations in $\Delta\vec{F}_{protein}$ due to these mutations. In principle, this concept can be extended to more general probes, allowing the quantitative mapping of a protein's electric field by inserting the probe oscillator at many sites of the protein or to measure the response of a fixed probe to changes in its environment. In the following, we present vibrational Stark data for several bond types, including peak frequencies, extinction coefficients, and values of $|\Delta\mu|$ to guide the choice of probes.

MATERIALS AND METHODS

All vibrational Stark spectra were obtained using methods described previously (*14*). In short, samples were dissolved in appropriate solvents or solvent mixtures that form glasses at 74 K [most commonly 2-methyltetrahydrofuran (2-MeTHF)]. For most cases, solutions were made to yield absorption maxima between 0.1 and 0.2. Sample cells were formed from two sapphire or CaF_2 windows coated with semitransparent nickel electrodes. These windows were separated by Teflon spacers, and the electrodes were connected to a high-voltage power supply through copper wires. The sample thickness was determined by measuring interference fringes between 600 and 1000 nm and was typically in the range of 26−30 μm. Once filled, Stark cells were immediately submersed in a liquid nitrogen immersion cryostat (*20*), and the resulting solvent glass ensured that an isotropic distribution of molecules was retained when the electric field was applied. Spectra were obtained using a Bruker IFS 66V/S FTIR system with a nitrogen-cooled indium antimonide detector (for frequencies above 1700 cm^{-1}), or nitrogen-cooled MCT (HgCdTe) detector (for frequencies below 1700 cm^{-1}). Stark spectra were obtained by collecting a minimum of 256 field-on and 256 field-off interferograms at 1 cm^{-1} resolution, using the DC method described previously (*21*). Voltages as high as 1.75 kV were applied across the sample, yielding external fields of as high as 0.65 MV/cm. For all samples, spectra were collected for at least three different applied fields and the Stark signal was observed to scale quadratically with field as expected (*17*). Measured path lengths were used to convert spectra to units of extinction coefficient and all Stark spectra were scaled to a field strength of 1.0 MV/cm. In all experiments, an unpolarized infrared source was used with the electric field applied perpendicular the infrared electric field.

Stark spectra are fit to a sum of derivatives of the absorption spectra. Because high-frequency fringes caused by the sample cell contribute significantly to absorption derivatives, these fringes were removed by first calculating a power spectrum and then filtering out the high-frequency components. All Stark spectra were fit to a sum of the zero, first, and second derivatives of the absorption spectrum, and for all single-mode transitions, the second derivative dominated

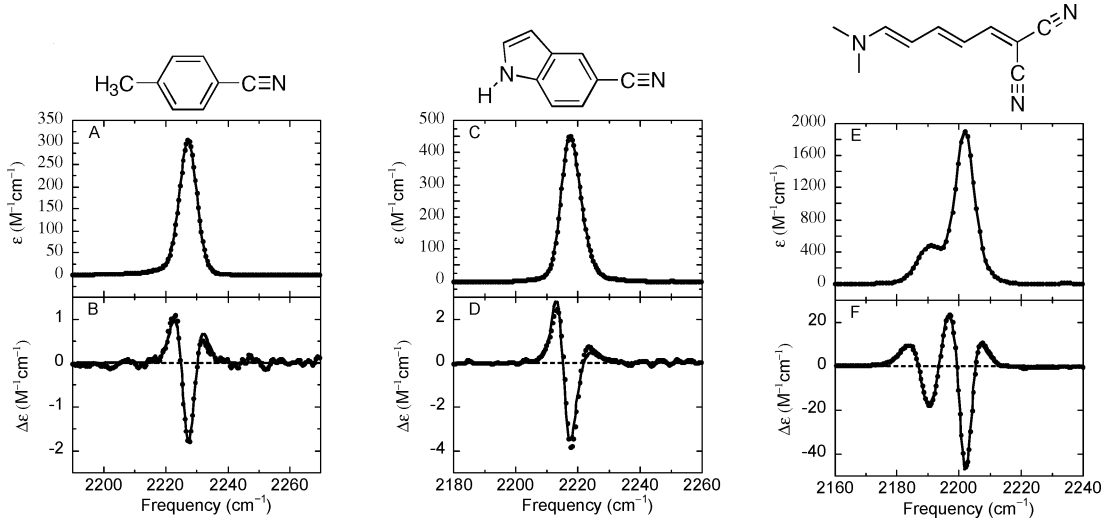

FIGURE 1: IR extinction and vibrational Stark spectra in the C−N stretch region of *p*-toluidine (A and B), 5-cyanoindole (C and D), and dicyanomethylene polyene 1 (E and F; see Figure 4 for the structure). Top panels are extinction coefficient spectra; bottom panels are Stark spectra scaled to a field strength of 1 MV/cm (dots) with a fit to the sum of the zero, first, and second derivatives of the absorption spectrum (solid line).

this fit. The second-derivative contribution to the Stark spectrum fit is a function of $|\Delta\mu|$, χ, and ζ. If we assume that $\zeta = 0°$ and with the experimental angle χ set at 90°, it is straightforward to extract $|\Delta\mu|$ [see the work of Bublitz and Boxer (*17*) for more details].

RESULTS

Nitriles. The vibrational Stark effect of nitriles has already been extensively studied (*14*, *15*). In general, the value of $|\Delta\mu|$ for aromatic nitriles is greater than that for aliphatic nitriles, and within the aromatic nitriles increases with the strength of substituent donors, following a Hammett relationship. It is expected that the values of $|\Delta\mu|$ measured for *p*-toluidine [0.71 cm^{-1}/(MV/cm); see Figure 1A,B] and acetonitrile [0.44 cm^{-1}/(MV/cm) (*14*)] provide reasonable estimates of $|\Delta\mu|$ for the side chains of the commercially available amino acids 4-cyanophenylalanine and cyanoalanine, respectively (the low solubility of these amino acids in glass-forming solvents prevented us from obtaining high-quality Stark data for their C−N stretches). This estimate of the value of $|\Delta\mu|$ for 4-cyanophenylalanine may help explain the frequency shifts observed by Getahun and co-workers when binding a 4-CN-Phe-containing peptide to calmodulin (*22*).

Table 1 lists results for eight additional single-mode nitriles. These compounds were chosen as simple examples that might be built into ligands, amino acid analogues, or labels. Of particular interest are the results for 5-cyanoindole and 4,4′-dithiobis(benzonitrile). 5-Cyanoindole provides a good estimate of bond parameters for a cyano-substituted tryptophan. The electron donating ability of the indole nitrogen increases both the extinction coefficient and the value of $|\Delta\mu|$ relative to those of *p*-toluidine and suggests that a cyano-substituted tryptophan would be a more sensitive probe than cyanophenylalanine (Table 1 and Figure 1C,D). 4,4′-Dithiobis(benzonitrile) is a model for the product of cysteine labeling with an aromatic CN-containing disulfide. The spectra of this compound show that the disulfide is an overall donor and that aromatic nitriles incorporated via cysteine labeling should exhibit a sensitivity to the local environment that is as good as or better than that of cyanophenylalanine. The nitrile group can also be bound directly to electron donors, without a bridging π-system. The result is a dramatic weakening of the C−N bond and a corresponding decrease in the stretching frequency. This allows for the possibility of incorporating two nitrile probes in two different locations of the same protein and sampling both environments without spectral overlap. Methyl thiocyanate and phenyl selenocyanate are two examples for which the peak position is shifted ∼70 cm^{-1} relative to that of aromatic nitriles but which retain comparable sensitivity. While investigating donor−acceptor polyenes that had been used in a series of electronic Stark effect experiments (*23*), we discovered that the popular dicyanomethylene acceptor exhibits intense C−N vibrations with very large values of $|\Delta\mu|$ (Table 1 and Figure 1E,F). The dicyanomethylene

Table 1: Vibrational Stark Effect Data for Nitriles

compound	peak position (cm^{-1})	fwhm (cm^{-1})	ϵ_{max} (M^{-1} cm^{-1})	$\lvert\Delta\mu\rvert f^a$ [cm^{-1}/(MV/cm)]
single mode				
3-cyanopyridine[b]	2231.1	8.6	130	0.55
4-cyanopyridine[b]	2237.2	6.1	51	0.42
p-toluidine[b]	2227.2	6.5	300	0.71
5-cyanoindole[b]	2217.3	7.5	450	0.86
4,4′-dithiobis(benzonitrile)[b]	2227.9	6.1	420	0.74
methyl thiocyanate[b]	2153.6	7.1	300	0.71
phenyl selenocyanate[b]	2151.7	8.2	100	0.77
selenocyanate[c]	2088.9	7.5	950	1.1
dicyanomethylene acceptors				
dicyanomethylene polyene 1[b]	2191.2	9.3	470	2.9
	2202.1	7.4	1900	1.4
dicyanomethylene polyene 2[b]	2205.8	8.1	50	2.0
	2218.0	11.3	200	1.2
dicyanomethylene polyene 3[d]	2175.5	10.7	410	1.4
	2185.9	11.5	260	
	2203.7	8.7	1500	1.1
dicyanomethylene polyene 4[b]	2209.2	7.8	100	1.8
	2221.8	7.1	1700	0.92

[a] Because the applied field is enhanced by a local field correction factor *f*, we report Stark tuning rates in all tables as $|\Delta\mu|f$ (*17*). Although the value of *f* is not known precisely, it is predicted to be close to 1 and to be constant for a series of compounds in the same solvent. [b] 2-Methyltetrahydrofuran. [c] Ethanol. [d] In a 1:3 CH_2Cl_2/CH_2ClCH_2Cl mixture.

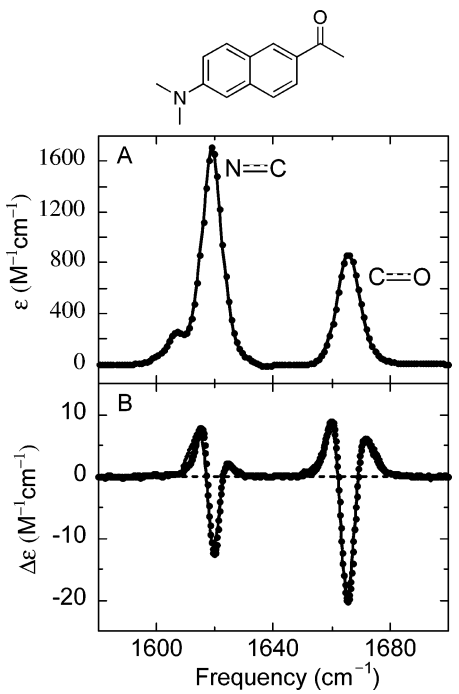

FIGURE 2: IR extinction and vibrational Stark spectra of 6-propionyl-2-(dimethylamino)naphthalene (acedan). (A) Extinction spectrum showing the C−N stretch (1619.1 cm^{-1}, $\epsilon_{max} = 1700$ M^{-1} cm^{-1}) and the C−O stretch (1665.7 cm^{-1}, $\epsilon_{max} = 850$ M^{-1} cm^{-1}). (B) Stark spectrum (dots) with a fit (solid line). For the 1619.1 cm^{-1} band, $|\Delta\mu|f = 0.86$ cm^{-1}/(MV/cm), while for the 1665.7 cm^{-1} band, $|\Delta\mu|f = 1.8$ cm^{-1}/(MV/cm).

acceptors used in the dyes synthesized by Twieg also show these strong infrared transitions, and the ease of installing this group (*24*) should allow the synthesis of reasonably small amino acid side chains or labels.

Acedan. 6-Propionyl-2-(dimethylamino)naphthalene (acedan) is the core structure of the fluorescent dyes Prodan and Badan that have been widely used to label proteins, and the artificial amino acid Aladan that was incorporated synthetically into the B1 domain of protein G (*25*). We were interested in whether the C−O and C−N vibrations of this bridged amide could be used to probe electrostatics in the same sites used to investigate dynamics with time-dependent fluorescent measurements. As was the case with nitriles, the addition of a π−system-bridged donor increases the sensitivity of the carbonyl vibration to an electric field (Figure 2A,B). While the value of $|\Delta\mu|$ is larger than for other carbonyls (*16*) (see the legend of Figure 2), the overlap with amide vibrations will make it difficult to obtain good infrared spectra with this probe in proteins. Note, however, that since Stark measurements are field-on minus field-off, it is sometimes possible to observe the Stark spectrum for a transition even when the transition itself is obscured by a much larger background absorption, as long as the Stark spectrum of the background absorption is small as demonstrated for NO bound to the heme iron in myoglobin (*19*).

C−F Stretches. Carbon−fluorine vibrations are potentially attractive probes because of the minimal steric perturbation incurred when replacing hydrogen with fluorine. Although the stretching frequency of the C−F bond is in a cluttered region of the protein/buffer spectrum, the large Stark effect of the vibration should provide a method of determining peak frequencies. Figure 3A shows an extinction spectrum of

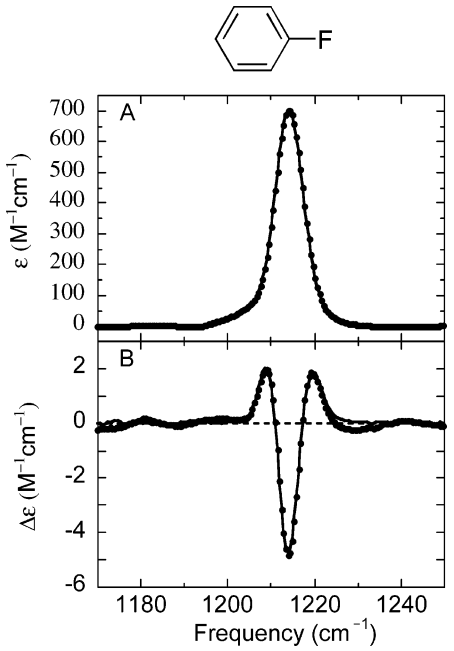

FIGURE 3: IR extinction (A) and vibrational Stark spectra (B; dots for the spectrum and solid line for the fit) of fluorobenzene.

fluorobenzene where solvent bands have been subtracted. The Stark spectrum of fluorobenzene in 2-methyltetrahydrofuran (Figure 3B) shows a single signal corresponding to the C−F vibration with no signal arising from the solvent vibrations. Because the magnitudes of Stark spectra scale quadratically with $|\Delta\mu|$, the vibrational Stark effect can be used as a sensitive difference spectroscopy to identify peaks with large values of $|\Delta\mu|$ in the background of intense transitions with small values of $|\Delta\mu|$. The use of difference spectroscopy to identify the peak position of a vibration is particularly attractive for labeled proteins where the intensity of the label's IR spectrum is weak and where obtaining good baselines is difficult. Table 2 contains results for a series of substituted fluorobenzenes. Unlike those of aromatic nitriles (*14*), the values of $|\Delta\mu|$ for these substituted fluorobenzenes exhibit little dependence on the electron donating ability of the substituent. Overall, aromatic carbon−fluorine bonds give transitions with large extinction coefficients and large values of $|\Delta\mu|$.

C−D Stretches. Carbon−deuterium vibrations would be ideal probes. Replacing nonexchangeable hydrogens with deuteriums causes no steric or electrostatic perturbation to the protein structure, and the large isotopic shift results in absorption bands in a clear region of the protein's IR spectrum (*26*). In addition, deuterated analogues could be synthesized for any of the 20 amino acids (including glycine) and are taken up by cells and incorporated into proteins by unmodified biosynthetic machinery. The disadvantages of C−D stretches are their small transition dipoles, leading to small extinction coefficients. The infrared spectra of d_3-methionine in cytochrome *c* (*26*) highlight the difficulty of observing C−D vibrations in proteins, with estimated extinction coefficients below 10 M^{-1} cm^{-1} [the data presented in Table 2 for 2,2,2-trifluoroethanol suggest that trifluoromethionine, which can be incorporated using auxotrophic strains of *E. coli* (*27*), might be an interesting alternative]. At the root of the small transition dipole for C−D vibrations is the small difference in electronegativity between carbon and

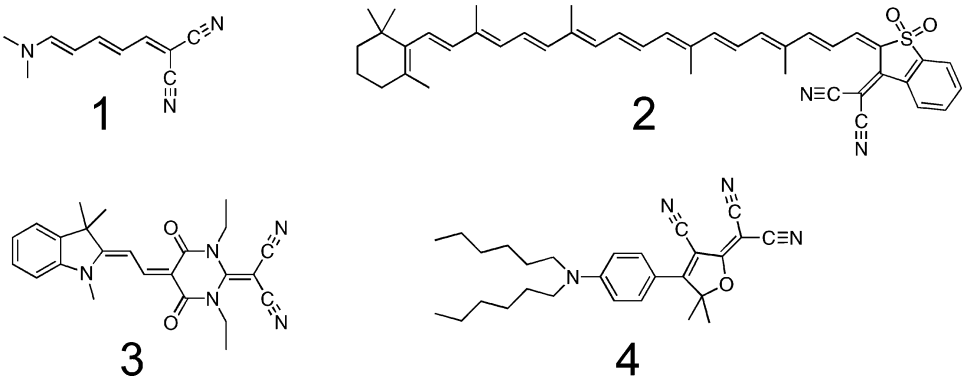

FIGURE 4: Chemical structures of dicyanomethylene polyenes 1 (*30*), 2 (*31*), 3 (*32*), and 4 (*24*) in Table 1.

Table 2: Vibrational Stark Effect Data for C−F Stretches

compound	peak position (cm^{-1})	fwhm (cm^{-1})	ϵ_{max} (M^{-1} cm^{-1})	$\lvert\Delta\mu\rvert f$ [cm^{-1}/(MV/cm)]
fluorobenzene[a]	1214.2	7.6	710	0.84
4-fluorobenzenethiol[a]	1219.5	7.9	820	0.89
1-chloro-4-fluorobenzene[a]	1222.8	7.1	540	0.82
1-fluoro-4-nitrobenzene[a]	1229.2	7.1	650	0.84
3-fluoropyridine[a]	1223.6	7.8	510	0.40
2,2,2-trifluoroethanol[b]	1278.0	18	560	0.77

[a] 2-Methyltetrahydrofuran. [b] Toluene.

Table 3: Vibrational Stark Effect Data for C−D, Si−H, Azide, and Isocyanate Stretches

compound	peak position (cm^{-1})	fwhm (cm^{-1})	ϵ_{max} (M^{-1} cm^{-1})	$\lvert\Delta\mu\rvert f$ [cm^{-1}/(MV/cm)]
C−D stretches				
d_2-glycine[a]	2166.0	17.3	13	
d-benzene[b]	2266.3	13.8	6	
d-phenylacetylene[b]	1962.2	7.9	120	
d_2-dichloromethane[b]	2192.6	10.6	18	0.42
	2304.4	12.0	49	0.47
d-chloroform[b]	2227.9	18.2	120	1.5
Si−H stretches				
triphenylsilane[b]	2124.7	34.2	140	2.3
azide stretches				
azidotrimethylsilane[b]	2152.2	8.9	4200	0.54
phenethyl azide[b]	2099.6	26.8	1300	
p-azidophenylalanine[a]	2124.5	40.0	680	
isocyanate stretches				
phenyl isocyanate[b]	2256.1	19.0	2800	
4-methoxyphenyl isocyanate[b]	2271.3	33.1	3100	

[a] In a 50:50 water/glycerol mixture. [b] 2-Methyltetrahydrofuran.

deuterium. The carbon−deuterium bond can be polarized by changing either the hybridization of the carbon or the electronegativity of groups attached to the carbon. Table 3 provides data for several compounds with C−D vibrations. *d*-Chloroform is the most polarized case with a reasonable extinction coefficient and a surprisingly large value of $|\Delta\mu|$. The vibrational Stark spectrum of triphenylsilane was also obtained, where the bond dipole has been reversed and the weakness of the Si−H bond lowers the frequency dramatically relative to that of a C−H bond. This vibration also had a very large value of $|\Delta\mu|$ but may not be stable enough in aqueous environments to be useful in proteins.

Azides and Isocyanates. These two classes of compounds were investigated because of their intense infrared transitions and ideal frequencies (see Table 3). The azide ion has already been used in infrared studies as a ligand bound to the heme iron of myoglobin (*28*). The successful incorporation of organic azides into proteins by the Tirrell lab (*29*) suggests that this vibration might be used as a general probe in proteins. As a test molecule, we obtained spectra of phenethyl azide. This compound gave an intense but complicated absorption spectrum, and a Stark spectrum which was an order of magnitude smaller than those obtained for nitrile or C−F stretches. The absorption spectrum of *p*-azidophenylalanine in a water/glycerol solution gave a similarly structured broad band. The Stark spectrum of azidotrimethylsilane was larger, but despite the very large extinction coefficient of this transition, the value of $|\Delta\mu|$ was relatively small. These results can be explained by considering the azide ion. Like carbon dioxide, this ion is predicted to have an intense antisymmetric transition, and a zero intensity symmetric transition. However, $|\Delta\mu|$ is predicted to be zero for all centrosymmetric compounds. To the extent that an organic azide breaks the symmetry of the azide ion, the "symmetric" mode should gain intensity and the $|\Delta\mu|$ values of both modes should increase. For isocyanates, the difference in electronegativity between oxygen and nitrogen breaks the symmetry of both modes. We had hoped the large extinction coefficients of these transitions would be accompanied by a great sensitivity to electric field. Unfortunately, for both compounds that were investigated, the absorption bands were broad and complicated and the Stark effect was very small.

Nitro Group. The nitro functionality is a polar uncharged group that could substitute for polar amino acids and is expected to have a large change in dipole moment. This was verified with 1-fluoro-4-nitrobenzene which gave an intense transition at 1530 cm^{-1} with a change in dipole moment of 1.26 cm^{-1}/(MV/cm). The overlap of this transition with the amide II band makes it a poor candidate for a vibrational probe, but isotopic substitution might shift it to a sufficiently low frequency to make it useful.

DISCUSSION

As techniques for incorporating nonnatural amino acids improve, the diversity of bond types that can be successfully engineered into proteins should also increase. In this report, we set out to investigate which bond types are most attractive as vibrational probes of electric fields in proteins by using vibrational Stark spectroscopy. To date, the nitrile functionality is the most promising with its ideal frequency, relatively large extinction coefficient, and high sensitivity to electric fields. We have also shown that the properties of the nitrile bond can be tuned dramatically by the bonding arrangement

of the molecule. Of particular interest are nitriles bonded directly to electron donors for their isolated frequencies, and the dicyanomethylene functionality for its large value of $|\Delta\mu|$. Carbon−fluorine bonds are also promising, and the best way to observe these vibrations in the cluttered low-energy region of a protein's IR spectrum may be by vibrational Stark spectroscopy. Deuterium-substituted amino acids are attractive because of their minimal perturbation to protein structure, but we have found that carbon−deuterium bonds are relatively insensitive to electric fields. It is possible to increase this sensitivity by polarizing the C−D bond. The trends presented here for several bond types should guide synthetic efforts toward developing amino acid analogues with intense vibrations and frequencies that are as sensitive as possible to local protein environments.

ACKNOWLEDGMENT

We thank Drs. Steven Andrews and Eun Sun Park for continuing discussions about these experiments. We also thank the labs of Seth Marder (for synthesis of dicyanomethylene polyenes 1−3), Robert Twieg (for synthesis of dicyanomethylene polyene 4), Carolyn Bertozzi (for synthesis of phenethyl azide), and Peter Schultz (for synthesis of *p*-azidophenylalanine).

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BI0352926