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Isotope-Labeled Aspartate Sidechain as a Non-Perturbing Infrared Probe: Application to Investigate the Dynamics of a Carboxylate Buried Inside a Protein

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Abstract

Because of their negatively charged carboxylates, aspartate and glutamate are frequently found at the active or binding site of proteins. However, studying a specific carboxylate in proteins that contain multiple aspartates and/or glutamates via infrared spectroscopy is difficult due to spectral overlap. We show, herein, that isotopic-labeling of the aspartate sidechain can overcome this limitation as the resultant ¹³C=O asymmetric stretching vibration resides in a transparent region of the protein IR spectrum. Applicability of this site-specific vibrational probe is demonstrated by using it to assess the dynamics of an aspartate ion buried inside a small protein via two-dimensional infrared spectroscopy.

Graphical abstract

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#Equal contribution

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Supporting Information

Electronic Supplemental Information with additional synthetic details and CD, FTIR and 2D IR spectra is available. See DOI: xxxxx.

1. Introduction

Various linear and nonlinear spectroscopic techniques based on probing the spectral and/or dynamic properties of various vibrational modes present in biological molecules have found a wide range of applications in biochemistry and biophysics [1,2]. For example, vibrational transitions arising from protein backbone units, such as the amide I vibration, have long been exploited to study the structure, dynamics, interactions, folding and aggregation of proteins and peptides [3,4]. Despite their broad utility, the commonly used intrinsic protein vibrational modes are often unable to yield site-specific structural or environmental information, due to vibrational coupling or spectral overlapping. An effective strategy to overcome this limitation is to introduce an ‘isolated’ and distinct vibrational mode via either isotopic labeling of an intrinsic vibrator or incorporation of an extrinsic vibrational probe [5–8]. Examples of the former include replacing a backbone $^{12}\text{C}=^{16}\text{O}$ unit with $^{13}\text{C}=^{16}\text{O}$ or $^{13}\text{C}=^{18}\text{O}$ [9] or substituting a $-\text{CH}_3$ group with $-\text{CD}_3$ [10], while the latter uses unnatural amino acids that exhibit a spectrally-distinguishable and environmentally-sensitive vibrational transition [11]. In the current study, we expand the toolbox of protein vibrational probes by showing that a ^{13}C sidechain-isotope-labeled aspartate can be used as a non-perturbing IR reporter to study, for example, the dynamics of a charged carboxylate buried in a solvent-inaccessible position inside a protein.

The sidechains of aspartic acid (Asp) and glutamic acid (Glu) can exist in either a neutral or an anionic form (i.e., aspartate and glutamate), thus making them versatile and, in many cases, allowing them to play a vital role in protein folding and function. For example, salt-bridges formed between the carboxylate groups of Asp or Glu and basic amino acid sidechains are important to protein structure and stability as well as protein-protein interactions. Additionally, Asp and Glu residues often play important functional roles in proton pumps [12], catalysis [13], and ligand or protein binding [14]. These carboxylate groups of deprotonated Asp and Glu give rise to a strong vibrational band (mainly the COO^- asymmetric stretching vibration) around 1585 cm^{-1} and 1568 cm^{-1} in D_2O , respectively [15]. However, only a few studies [16] have utilized this naturally-occurring vibrational band

as a site-specific IR probe because oftentimes multiple Asp and/or Glu residues are present in any one protein molecule. For a C=O stretching vibration, changing ^{12}C to ^{13}C typically red-shifts the vibrational frequency by $\sim 40\text{ cm}^{-1}$ [17]. Previous studies [18,19] have used a biological approach to incorporate a ^{13}C label into the sidechain of Asp for analysis of the protonation states changes of Asp residues during the photocycle of bacteriorhodopsin. This approach was limited, however, as it also isotopically-labeled the Thr (threonine) and Glu residues in the protein of interest and the labeling efficiency of the Asp residue was low ($\sim 40\%$). Herein, we present a new method to introduce ^{13}C site-specifically into Asp residues (hereafter this isotopically-labeled Asp is referred to as Asp*), and demonstrate that the ionic form of an Asp residue with a $^{13}\text{COO}^-$ group is capable of acting as a reporter of Asp sidechain interactions and dynamics, particularly in proteins which have multiple acidic residues.

In order to show the utility of this site-specific IR probe, we employ it to probe the dynamics of a charged residue located in the interior of a small protein, psbd41, which corresponds to a truncated version of the peripheral subunit-binding domain of dihydrolipoamide acetyltransferase (E2) from the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus* [20]. This small protein consists of 41 amino acids, including three Asp and two Glu residues. In particular, a charged Asp residue (i.e., Asp34), although buried in the interior of the protein, has been shown to be critical to the folding and stability of psbd41 [21]. Although bringing a charged moiety into a protein's interior, which is generally hydrophobic, is energetically unfavorable, it is not uncommon to find buried charges in proteins due to structural and/or functional purposes [22]. In such scenarios, the mechanism by which nature oftentimes chooses to overcome this energetic penalty is by forming various favorable inter-residue electrostatic interactions, such as hydrogen-bonds (H-bonds) and salt-bridges [23]. In addition, the presence of water molecules near a buried charge can also help significantly reduce the associated energetic penalty [24]. According to an NMR structure of the peripheral subunit-binding domain of the E2 chain (Figure 1), the otherwise unfavorable burial of the charged sidechain of Asp34 is alleviated by multiple hydrogen-bonding (H-bonding) interactions formed between its carboxylate ion and the backbone amides of Gly23, Thr24, Gly25 and Leu31 as well as the sidechain of Thr24 [20]. However, it is not clear whether water is also present near Asp34 because the NMR structure suggests that this site is inaccessible by solvent [20]. Thus, in order to verify the spectroscopic utility of Asp*, as well as provide insight into the dynamics of the interactions that stabilize a charged group inside a protein, a topic important for protein electrostatics and energetics [25], we chose to replace Asp34 with Asp* in psbd41 and, in turn, use two-dimensional infrared (2D IR) spectroscopy [26,27] to probe, site-specifically, the spectral diffusion dynamics of the $^{13}\text{COO}^-$ asymmetric stretching vibration arising from Asp*34. The spectral diffusion dynamics of an inhomogeneously broadened molecular vibration would report on the time evolution of microscopic states contributing to the vibrational bandwidth and thus reveal information about the environmental fluctuations of the infrared (IR) reporter in question [28]. For example, spectral diffusion dynamics measured in aqueous solution typically contain a component on the 1–2 ps timescale, due to water dynamics [29]. Interestingly, our results not only show that Asp* can be used as a site-

specific IR probe in the presence of other carboxylates, but also provide evidence suggesting that water may exist near the Asp34 site in psbd41.

2. Experimental

2.1. Preparation of Cap-Asp and Cap-Asp*

In order to synthesize N-terminal acetylated and C-terminal N-methyl amidated, or capped, Asp and Asp* (hereafter referred to as Cap-Asp and Cap-Asp*), Fmoc-*N*-methyl indole resin (0.12 mmol) was first placed in a peptide synthesis vessel and swelled in dichloromethane (CH₂Cl₂) (10 mL) for 1 h. The solvent was drained and the resin was washed with dimethylformamide (DMF) (3 × 6 mL) and then treated with 20% piperidine/DMF (2 × 6 mL), allowing the solution to contact the resin for 10 minutes. The resin was washed with DMF (5 × 6 mL) and a pre-mixed solution of Fmoc-amino acid (0.1 mmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (40 mg, 0.105 mmol, 1.05 equiv), oxyma (15 mg, 0.105 mmol, 1.05 equiv) and *N,N*-diisopropylethylamine (DIPEA) (35 μL, 0.2 mmol, 2 equiv) dissolved in DMF (1 mL) was added to the resin. The contents were rocked gently for 1 h, then drained and the resin was washed with DMF (3 × 6 mL).

The resin-bound Fmoc-amino acid (0.1 mmol) was washed with DMF (3 × 5 mL) and then treated with a solution of 20% piperidine/DMF (2 × 6 mL) allowing each treatment to contact the resin for 5 minutes. The resin was washed with DMF (5 × 6 mL), and then a premixed solution of acetic anhydride (95 μL, 1.0 mmol, 10 equiv) and DIPEA (348 μL, 2.0 mmol, 20 equiv) dissolved in DMF (2 mL) was added to the resin. The contents were rocked gently for 1 h, then drained and the resin was washed with DMF (3 × 6 mL) and CH₂Cl₂ (3 × 6 mL).

The resin-bound peptide (~0.1 mmol) was pre-swelled in CH₂Cl₂ for 30 minutes and then treated with a cleavage cocktail of CH₂Cl₂, trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water (70:25: 2.5: 2.5; 5 mL) and stirred for 1 hour. The filtrate was collected and an additional cleavage cocktail (3 × 1 mL) was used to wash the resin. The pooled filtrates were evaporated to dryness using a stream of air. The residue was dissolved in water/acetonitrile (MeCN) (4:1, 1 mL) and purified by reverse-phase high-pressure liquid chromatography (5 – 30% organic over 10 minutes) to give the desired product.

2.2. Peptide Synthesis and Purification

The Asp*34-psbd41 peptide (sequence: AMPSVRKY AREKGVDIRL VQGTGKNGRV LKE-Asp*-IDAFLA GGA) was synthesized on a Liberty Blue microwave peptide synthesizer (CEM, NC) using the synthesized Fmoc-Asp* described below and standard Fmoc-protocols. The peptide was cleaved from the rink amide resin using a TFA cleavage cocktail and purified by reverse-phase high performance liquid chromatography using a C18 column. This peptide was then identified by matrix assisted laser desorption ionization (MALDI) mass spectrometry. Peptide samples were exchanged with 0.01M DCl in D₂O two times to remove residual TFA from purification and titrated to either pH 1.5 or pH 8 with DCl or NaOD prior to dissolving in the respective buffer for analysis.

2.3. Fourier Transform Infrared (FTIR) Measurements

FTIR spectra were collected on an iS50 FT-IR spectrometer (Nicolet, WI) with 1 cm^{-1} resolution. A two-compartment CaF_2 sample cell with a $56\text{ }\mu\text{m}$ Teflon spacer was used to allow back-to-back acquisition of the single-beam spectra of the protein sample and the D_2O buffer via a home-made device [30]. All samples used in the FTIR measurements had concentrations of $\sim 5\text{ mM}$ in either a 50 mM deuterated sodium phosphate buffer (pH 8) or a 50 mM DCl-KCl D_2O solution (pH 1.5).

2.4. 2DIR Measurements

The details of the 2D IR instrument have been described elsewhere [31]. Briefly, three ultrafast mid-IR pulses were used to produce a third-order response (i.e., a photon echo signal) in the sample of interest, which was collected in the phase-matched direction following heterodyning with a local oscillator pulse. The resultant signal was dispersed by a monochromator and detected by a 64-element MCT array detector (InfraRed Associates). The final 2D spectra were obtained from three Fourier transform operations performed on the spectra collected in the time domain. The sample was held in a CaF_2 cell with either a $56\text{ }\mu\text{m}$ spacer for Asp*34-psbd41 ($\sim 11\text{ mM}$) or a $25\text{ }\mu\text{m}$ spacer for Cap-Asp* ($\sim 23\text{ mM}$), both of which were prepared in 50 mM deuterated sodium phosphate buffer (pH 8.0).

3. Results and Discussion

3.1. Synthesis of Fmoc-Asp*

The synthesis scheme of Fmoc-*L*-Aspartic-4- ^{13}C -Acid (β -tert-butyl ester) is shown in Figure 2. Specifically, the reaction was initiated from O'Donnell's benzophenone imine of glycine **1** [32–34], which, upon a trimethylaluminum mediated acylation with Oppozler sultam auxiliary [35], was converted to **2** [36]. Treatment of **2** with *n*-butyllithium formed the enolate which was combined with **3** [37]. After further reaction at room temperature for three days, two molar equivalents of hydrochloric acid were used to hydrolyze the imine, yielding **4** with good yield after purification. Removal of the sultam from **4** was accomplished by the classical lithium hydroxide saponification. While a side reaction involving cleavage of the sultam ring occurred, as previously encountered in similar reactions [36,38], the desired reaction was achieved. The sultam was extracted from the solution containing the amino acid, and the solution pH was adjusted to 8. Lastly, the final protection was conducted with Fmoc-*N*-hydroxysuccinimide ester in acetone to produce the desired substrate, **5**, after chromatography.

3.2. Linear IR results

In order to determine the effect of isotopic-labeling on the COO^- asymmetric stretching vibrational frequency of the carboxylate group of Asp, we measured the FTIR spectra of the two model compounds, Cap-Asp and Cap-Asp*. As expected (Figure 3), the FTIR spectrum of Cap-Asp at pH 8, which has a deprotonated and hence a charged sidechain, consists of two peaks, one at $\sim 1637\text{ cm}^{-1}$ arising from the amide carbonyls and one at $\sim 1581\text{ cm}^{-1}$ arising from the carboxylate [15]. In comparison, the lower-frequency band of Cap-Asp* at pH 8 is shifted to $\sim 1538\text{ cm}^{-1}$ (Figure 3), indicating that isotopic labeling of the C atom in

the carboxylate group of Asp results in a 44 cm^{-1} red-shift in the corresponding COO^- asymmetric stretching vibrational frequency. However, at pH 1.5 where the sidechain of Asp* becomes protonated, the IR band arising from the sidechain is shifted to a spectral region that is overlapped with the amide I' band (Figure S1 in Supporting Information). Thus, taken together these results indicate that the deprotonated form of Asp* can be used as a site-specific IR probe of proteins, as none of the naturally-occurring protein vibrations reside in the frequency region of $1520\text{--}1550\text{ cm}^{-1}$. For example, it can be used to probe the protonation status of a specific Asp residue as well as its H-bonding dynamics. As the $\text{p}K_a$ of an acidic amino acid buried inside a protein can sample a large range [39], $4.5\text{--}9.4$, the ability of Asp* to be useful as an IR probe in a buried position then depends on the system of interest. However, the $\text{p}K_a$ of a solvent-exposed Asp residue is ~ 4 . Therefore, Asp* can be used to study proteins with solvent-exposed Asp residues in solutions with a pH of 4 or larger.

To demonstrate the potential utility of Asp*, we employed it to probe protein H-bonding dynamics in a site-specific manner. Specifically, we replaced Asp34 in psbd41 with Asp* (hereafter the isotopically labeled psbd41 is referred to as Asp*34-psbd41), as the sidechain of this amino acid is not only engaged in multiple H-bonding interactions, but is also buried in the interior of the protein [20]. As expected, this mutation is non-perturbing as circular dichroism (CD) measurements (Figures S2 and S3 in the Supporting Information) revealed that the melting temperature of Asp*34-psbd41 is within uncertainty the same as that reported for the wild-type protein by Raleigh and coworkers [21]. In addition, their study showed that the folded structure was maintained and stable between pH 5.3 and pH 10. As shown (Figure 4), the FTIR spectrum of Asp*34-psbd41 at pH 8 consists of four resolvable spectral features between $1500\text{--}1700\text{ cm}^{-1}$, with peak frequencies at approximately 1516 , 1540 , 1584 , and 1643 cm^{-1} , respectively. Besides the apparent amide I' band at 1643 cm^{-1} , the 1516 cm^{-1} band can be assigned to a C-C ring mode of the single tyrosine (Tyr) in psbd41, whereas the relatively broad feature between $1550\text{--}1600\text{ cm}^{-1}$ can be attributed to the other four non-labeled carboxylates (i.e., two from Asp and two from Glu) in the protein. Thus, the 1540 cm^{-1} band must arise from Asp*34. This assignment is consistent not only with the result obtained with Cap-Asp* (Figure 3) but also with previous studies demonstrating that the Asp34 is deprotonated at neutral pH [20]. Moreover, in dimethyl sulfoxide (DMSO), a polar but aprotic solvent, the carboxylate IR band of Cap-Asp* is blue-shifted to 1545 cm^{-1} (Figure S4 in Supporting Information). Thus, the above assignment is also consistent with the notion that the carboxylate group of Asp34 is involved in H-bonding interactions. Furthermore, and perhaps most importantly, the FTIR spectrum of Asp*34-psbd41 provides convincing evidence that Asp* can be used to provide site-specific spectroscopic information, even for proteins that contain multiple Asp and Glu residues.

3.3. 2D IR measurements

To demonstrate further the utility of Asp*, next we employed 2D IR spectroscopy to measure dynamics underlying the spectral bandwidth of the carboxylate asymmetric stretching transition of Asp*34 in Asp*34-psbd41. For an inhomogeneously broadened vibrational transition, the corresponding 2D IR spectra are often elongated along the diagonal at early waiting times and subsequently become more circular as time proceeds due

to a redistribution of vibrational frequencies. Interestingly, the 2D IR spectrum of Asp*34-psbd41 obtained at an early waiting time (i.e., T), as shown (Figure 5), only exhibits a modest elongation along the diagonal. That is, the CLS^{-1} at waiting time $T = 0$ fs is ~ 0.2 , which is relatively small compared to that of other inhomogeneously broadened molecular vibrations. For example, Hochstrasser and coworkers found that 2D IR experiments on an isotopically-labeled backbone carbonyl in the transmembrane region of the M2 proton channel produced a CLS^{-1} at waiting time $T = 0$ fs of 0.74 [40]. Additionally, Chung *et al.* showed that the CLS^{-1} for the CN stretching vibration of cyanophenylalanine mutants of HP35 were all ~ 0.6 at time $T = 0$ fs [41]. Therefore, there must be a very fast dynamic event, which contributes to the Lorentzian bandwidth ($\sim 17 \text{ cm}^{-1}$) of Asp*34 but evades detection due to our experimental time resolution. Since the 2D IR spectrum of Cap-Asp* displays a similar behavior (Figure S5 in Supporting Information), this result suggests that this phenomenon is intrinsic to the vibrator and is not caused by an environmental factor. One potential interpretation is that this phenomenon is caused by the rapid electronic equilibration dynamics between the two resonant structures of this carboxylate ion, leading to an ultrafast dephasing time of the vibration of interest; however, any rapid electronic equilibration between two states would lead to this ultrafast dephasing. Further analysis of the 2D IR spectra of Asp*34-psbd41 collected at different T values using the commonly used center line slope (CLS) method [42] permitted us to determine the spectral diffusion dynamics occurring on the picoseconds (ps) timescale that contribute partially to the inhomogeneous bandwidth of the $^{13}\text{COO}^-$ asymmetric stretching vibration of Asp*34. As indicated (Figure 6), the value of CLS^{-1} decays exponentially with T with a time constant of 1.1 ± 0.2 ps. Since the carboxylate of Asp*34 is H-bonded with multiple sites, the straightforward interpretation of this result is that it manifests the underlying dynamics of these H-bonds.

However, most existing examples in the literature have attributed such ultrafast spectral diffusion dynamics to mobile water molecules [40,43–49]. For instance, the study of Hochstrasser and coworkers [44] found that the spectral diffusion dynamics of the amide I vibration of certain isotopically-labeled amide units in an amyloid β fibril occur on a 1–2 ps timescale, which was interpreted as the motion of nearby water molecules. Similar studies on other biological systems [40,48,49] revealed that only when mobile or bulk-like water molecules are present the spectral diffusion of the vibrational probe occurs on such an ultrafast timescale. On the other hand, spectral diffusion of an H-bonded vibrator arising from protein backbone motions typically occurs on a slower time scale, as observed by Hamm and coworkers [50]. Based on these previous studies, we tentatively assign the ~ 1.1 ps spectral diffusion component of Asp*34 to mobile water near the carboxylate group rather than from intra-molecular H-bond interactions. Given the small size of psbd41, it is not unreasonable to assume that water can transiently penetrate into its interior. In order to provide further evidence in support of this notion, an ideal control experiment would be to measure the spectral diffusion dynamics of the IR probe in the unfolded state of Asp*34-psbd41, wherein Asp*34 is expected to be solvent exposed. However, both urea and guanidinium chloride, which can denature psbd41 at high concentrations, have a strong absorbance near the IR band of Asp*34, making it difficult, if not impossible, to perform the required 2D IR measurements when these denaturants are present. While psbd41 can also be

denatured by lowering the pH to approximately 2.5 [21], we cannot use this strategy because Asp*34 will become protonated under such acidic pH condition. For these reasons, we simply measured the spectral diffusion dynamics of the $^{13}\text{COO}^-$ asymmetric stretching vibration of Cap-Asp* in water at pH 8, where the sidechain of Asp* is expected to be fully hydrated. As indicated (Figure S6 in Supporting Information), the corresponding CLS $^{-1}$ vs. T curve can be described by a single-exponential function with a time constant of ~ 1.4 ps. This control experiment therefore corroborates the above conclusion that Asp34 in psbd41 is solvated by water.

4. Conclusion

Achieving broad site-specificity in the IR study of protein structure, dynamics and function necessitates the development of a wide array of not only environmentally-sensitive but also structurally-diverse vibrational probes that can be incorporated into proteins. The availability of a large set of such probes is important, as different applications may require substitution of different amino acids in order to avoid or minimize structural perturbation and to obtain certain structural or dynamic information. Despite many previous efforts, until this study we have lacked the ability to target and examine the underlying vibrational properties of a specific carboxylate ion in a protein system. Given the fact that the carboxylates of Asp and Glu are often involved in electrostatic interactions that are crucial for protein folding and function, the development of a method that allows selective interrogation of the structural and environmental properties of any individual acidic residues in a protein would be highly valuable. In this study, we have demonstrated that a $^{13}\text{COO}^-$ isotope-labeled aspartate now provides a convenient, non-perturbing means to achieve this goal. Additionally, using this site-specific IR probe and 2D IR spectroscopy, we are able to provide evidence that water may exist in the interior of the small protein, psbd41.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We demonstrate a new protein vibrational probe.
- This probe is especially useful in studying electrostatic interactions involving an aspartate or glutamate in a site-specific manner.
- Using this probe and 2D IR spectroscopy we show that water may exist in the interior of a small protein, psbd41.

Figure 1.
NMR structure of pdsd41 (PDB code: 2PDD), showing the sidechain of Asp34 as well as the various hydrogen-bonding interactions.

Figure 2.
Scheme of the synthesis of Fmoc-Asp*.

Figure 3. FTIR spectra of Cap-Asp and Cap-Asp* in a 50 mM sodium phosphate D₂O buffer (pH 8), as indicated. In both cases, the bands at ~1637 cm⁻¹ corresponds to the amide I' transition of the backbone carbonyl, whereas the bands centered at 1581 and 1537 cm⁻¹ arise from the sidechains.

Figure 4. FTIR spectrum of Asp*34-psbd41 in a 50 mM sodium phosphate D₂O buffer (pH 8). The arrows indicate the C=O asymmetric stretching band of the Asp*34 sidechain at 1540 cm⁻¹. The inset is a zoom of the spectrum in the region of 1500–1600 cm⁻¹.

Figure 5. Representative 2D IR spectra of Asp*34-psbd41 in a 50 mM sodium phosphate D₂O buffer (pH 8).

Figure 6. CLS⁻¹ versus T plot of the 2D IR spectra of Asp*34-psbd41. The smooth line is the fit of these data to a single-exponential function with a time constant of 1.1 ps.