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Research paper

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 $^{13}COO^-$ 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.

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 $^{12}C{=}^{16}O$ unit with $^{13}C{=}^{16}O$ or $^{13}C{=}^{18}O$ [9] or substituting a $-CH_3$ group with $-CD_3$ [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 ~40 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 state 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

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of the Asp residue was low (~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 $^{13}COO^-$ 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 *Bactillus 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 (Fig. 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 $^{13}COO^-$ 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.

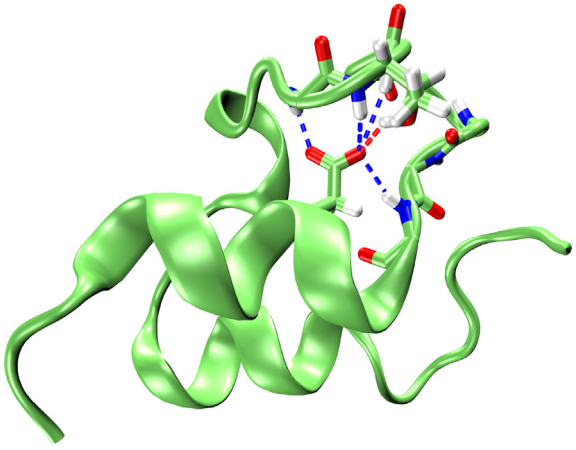

Fig. 1. NMR structure of pdsd41 (PDB code: 2PDD), showing the sidechain of Asp34 as well as the various hydrogen-bonding interactions.

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 (CH_2Cl_2) (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 min. 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 min. The resin was washed with DMF (5 × 6 mL), and then a pre-mixed 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 CH_2Cl_2 (3 × 6 mL).

The resin-bound peptide (~0.1 mmol) was pre-swelled in CH_2Cl_2 for 30 min and then treated with a cleavage cocktail of CH_2Cl_2, trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water (70:25: 2.5: 2.5; 5 mL) and stirred for 1 h. 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 min) to give the desired product.

2.2. Peptide synthesis and purification

The Asp*34-psbd41 peptide (sequence: AMPSVRKY ARE-KGVDIRL 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 0.01 M DCl in D_2O 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