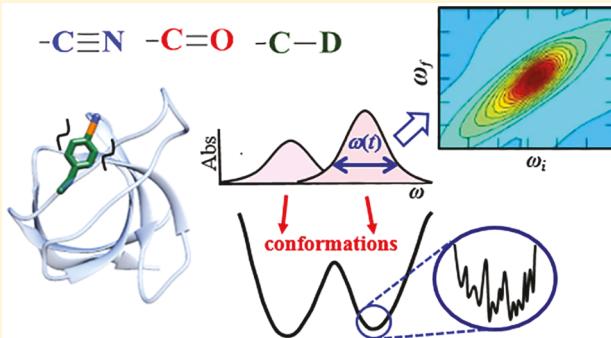


# Site-Specific 1D and 2D IR Spectroscopy to Characterize the Conformations and Dynamics of Protein Molecular Recognition

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**ABSTRACT:** Proteins exist as ensembles of interconverting states on a complex energy landscape. A complete, molecular-level understanding of their function requires knowledge of the populated states and thus the experimental tools to characterize them. Infrared (IR) spectroscopy has an inherently fast time scale that can capture all states and their dynamics with, in principle, bond-specific spatial resolution, and 2D IR methods that provide richer information are becoming more routine. Although application of IR spectroscopy for investigation of proteins is challenged by spectral congestion, the issue can be overcome by site-specific introduction of amino acid side chains that have IR probe groups with frequency-resolved absorptions, which furthermore enables selective characterization of different locations in proteins. Here, we briefly introduce the biophysical methods and summarize the current progress toward the study of proteins. We then describe our efforts to apply site-specific 1D and 2D IR spectroscopy toward elucidation of protein conformations and dynamics to investigate their involvement in protein molecular recognition, in particular mediated by dynamic complexes: plastocyanin and its binding partner cytochrome *f*, cytochrome P450s and substrates or redox partners, and Src homology 3 domains and proline-rich peptide motifs. We highlight the advantages of frequency-resolved probes to characterize specific, local sites in proteins and uncover variation among different locations, as well as the advantage of the fast time scale of IR spectroscopy to detect rapidly interconverting states. In addition, we illustrate the greater insight provided by 2D methods and discuss potential routes for further advancement of the field of biomolecular 2D IR spectroscopy.



## INTRODUCTION

A complete understanding of protein function must go beyond the static structure–function paradigm of biochemistry and include the range of structures adopted in solution and how they interconvert. Proteins are described by hierarchical energy landscapes with states that interconvert on a range of length and time scales, including slower, large-scale motions among conformations but also fast, smaller-scale motions among substates within a conformation, and even faster motions among substates within substates (Figure 1).<sup>1</sup> Further contributing to complexity, proteins are heterogeneous spatially, such that different parts could show different dynamics that variably contribute to function. Thus, an ideal experimental method to study protein dynamics would provide both high spatial and temporal resolution. A range of spectroscopic techniques have been applied, including NMR, visible, and EPR spectroscopy, each with their advantages and limitations. IR spectroscopy is emerging as a powerful alternative approach due to its potential for combined spatial and temporal resolution. IR chromophores can be as small as a bond, and the time scale of IR spectroscopy (ps) is very fast.

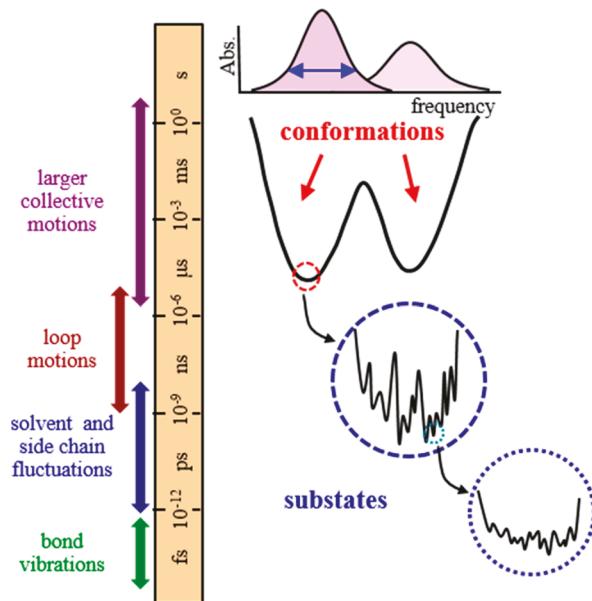
Any spectroscopic approach fundamentally relies on the sensitivity of a chromophore to its environment to serve as a local reporter. The frequency of a vibration, for example, can vary due to electrostatic, hydrogen bonding, or other short-range interactions.<sup>2,3</sup> The observation of multiple distinct

absorption bands for a single vibration provides evidence that it experiences multiple distinct environments or that the protein adopts multiple conformational states (Figure 1). If the relative transition dipole strengths of the bands are known or at least similar, then the relative populations of the states can be determined from the integrated absorbance of the bands. However, a vast number of states make up a protein energy landscape, many of which are associated with subtle structural variations that do not lead to distinct bands but rather a distribution of frequencies, i.e., inhomogeneous broadening. A key advantage of IR spectroscopy is the ability to directly capture the majority of states, even those involving small scale changes that fluctuate on fast time scales. IR spectroscopy also can in principle characterize specific parts of proteins, similarly to NMR spectroscopy, but the difference in time scale makes them complementary techniques. Because NMR dephasing times are slow, NMR resonances have narrow homogeneous line widths, providing high spectral selectivity. IR dephasing times are very fast, leading to broad line widths that impair spectral resolution. However, because the energy scale of NMR spectroscopy is small, the associated time scale is slow, such that the signals from different states that rapidly interconvert

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**Figure 1.** Time scales of protein motion (left) and illustration of example protein energy landscape and relationship to IR spectral features (right). Multiple distinct bands reflect population of multiple conformations, while inhomogeneous broadening of a band reflects the populations of substates at lower tiers in the energy landscape.

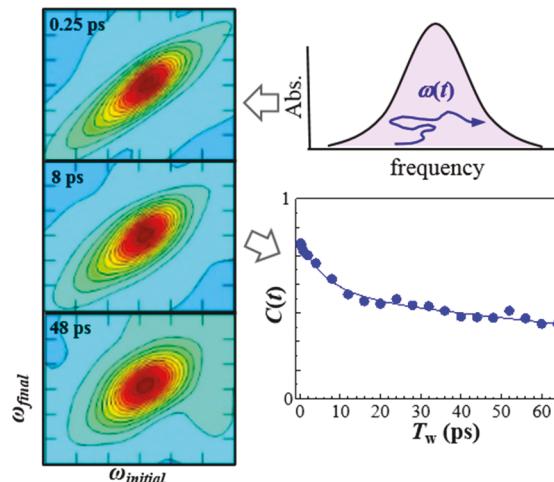
coalesce to their average. In comparison, IR spectroscopy can capture states with faster dynamics. For example, NMR resonances separated in energy by 100 Hz would be averaged if the associated states interconverted faster than an  $\sim 1$  ms time scale, whereas IR bands separated by  $5\text{ cm}^{-1}$  would have to interconvert faster than an  $\sim 1$  ps time scale. Thus, while the broad line widths of IR bands hinder the resolution of distinct species, the line widths also contain information about inhomogeneity, which enables detection of all populated states, including those that rapidly interconvert.

## ■ 2D IR SPECTROSCOPY

While linear spectroscopy cannot provide a rigorous measure of the inhomogeneous broadening, due to its convolution with homogeneous line broadening, nor provide information about the underlying dynamics, the inhomogeneity and associated dynamics can be unmasked via multidimensional techniques, in particular 2D IR spectroscopy. Multidimensional spectroscopy at optical frequencies has recently advanced significantly, and a number of excellent texts covering the theory and methods are now available.<sup>4–6</sup> In short, the techniques generate 2D spectra, where one axis reports the frequencies of vibrations initially labeled, or pumped, while the other axis probes the frequencies of vibrations that are correlated, because they either belong to the same species or are in some manner coupled. The time between the initial pumping and later probing is typically referred to as the waiting time ( $T_w$ ). The bands along the diagonal of a 2D spectrum reflect those found in the linear spectrum, while bands off the diagonal associate the vibrational transitions. For example, coupling between two distinct vibrations leads to cross bands, such as those observed between amide backbone vibrations due to their strong transition dipole coupling.<sup>7–10</sup> Alternatively, two absorption bands at distinct frequencies may correspond to a single probe in two distinct environments or states, for example, those associated with two protein

conformations. In this case, cross bands between the absorptions report that the vibration was pumped at one frequency but later probed at the other, indicating that the interconversion of the states took place in the intervening time period  $T_w$ . The increase in cross band amplitude in 2D IR spectra taken as a function of  $T_w$  directly follows the exchange dynamics.<sup>11</sup> For example, myoglobin conformational states associated with different orientations of a histidine side chain in the binding pocket lead to distinct absorption bands, and the conformational exchange has been directly followed through the appearance of distinct cross bands in  $T_w$ -dependent 2D IR spectra.<sup>12</sup>

In contrast to conformational states that differ by large-scale structural changes, the numerous substates that make up a conformation typically manifest as inhomogeneous broadening that results in the elongation of a band along the diagonal of the 2D spectrum (Figure 2). However, similarly as for distinct

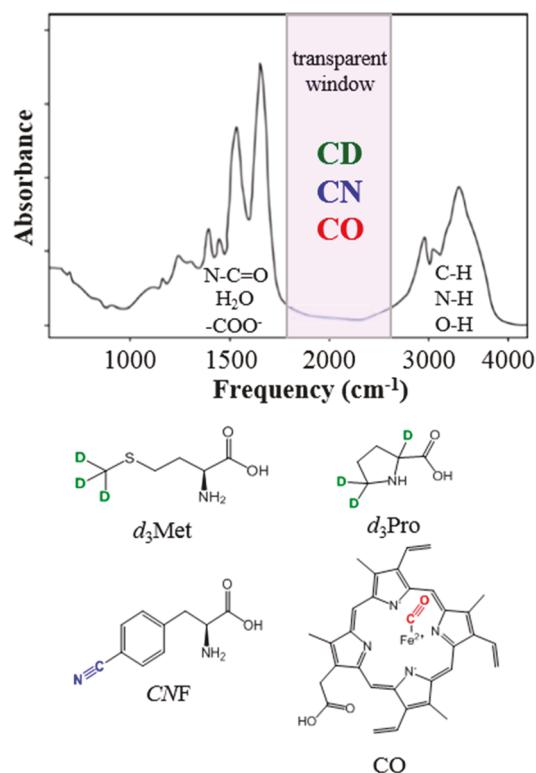


**Figure 2.** Inhomogeneous broadening results in elongation of 2D bands along the diagonal (right). Interconversion among the underlying states with increasing  $T_w$  reduces the elongation. Analysis of the 2D line shapes can yield a frequency–frequency correlation function that describes the spectral diffusion within the inhomogeneous frequency distribution (left).

bands, the amplitude off the diagonal increases with longer  $T_w$  as the substates underlying the inhomogeneous distribution have increasingly more time to interconvert. The interconversion results in 2D spectra that appear less elongated, such that the dynamics among the inhomogeneous distribution (spectral diffusion) can be followed by analysis of the  $T_w$ -dependent 2D band shapes. The spectral line broadening and dynamics can be conveniently described by a frequency–frequency correlation function ( $C(t)$ ), which can be determined from 2D IR data by established methods.<sup>13</sup> The time scales of spectral diffusion reflect the barrier heights among the substates that underlie the inhomogeneous broadening, providing insight into the ruggedness of the local energy landscape. This information is useful for assessing the role of substate heterogeneity and dynamics in protein function but also can provide guidance for assigning distinct bands to the same or different conformational states. Such information from 2D experiments, for example, has been applied for our investigations of the conformational dynamics involved in cytochrome P450 activity (*vide infra*).<sup>14,15</sup>

## ■ IR SPECTROSCOPY OF PROTEINS

IR spectroscopy of amide backbone vibrations has been historically utilized as a routine approach to estimate the secondary structural content of proteins.<sup>16</sup> However, because of the large number of amide vibrations and their delocalized nature, the information provided is global rather than site-specific. Indeed, the lack of spectral selectivity due to the spectral congestion has been a key hindrance in the widespread application of IR spectroscopy for the study of proteins. The issue of spectral congestion can be avoided by use of ligands with vibrations in a “transparent window” of a protein IR spectrum (1900–2500  $\text{cm}^{-1}$ ; Figure 3) as probes of the



**Figure 3.** Representative protein IR spectrum highlighting the transparent frequency window and the structures of frequency-resolved probes used in our studies.

ligands' binding sites in the proteins.<sup>17–19</sup> For example, a carbon monoxide (CO) ligand has long been used to characterize hemeprotein active sites.<sup>18,20</sup> The application of such ligand probes, however, is limited in scope to the specific proteins that bind them and, moreover, to the one location in a protein where the ligand binds.

A more generally applicable approach is the introduction of IR probes with frequencies in the transparent window by appending them to amino acid side chains. The side chains of proteins not only commonly mediate critical interactions in biochemical processes, but they are also the part of proteins directly subjected to manipulation by evolution. A wide variety of such transparent window side chain probes have been developed and applied toward the study of protein function, and several excellent reviews are available.<sup>19,21,22</sup> When choosing a probe, one must balance the desire for strong signals, small size to minimize perturbation, sensitive solvatochromism, and practical feasibility of site-specific incorporation. The first demonstration of the use of

frequency-resolved probes of protein side chains utilized carbon–deuterium (C–D) bonds specifically incorporated into cytochrome *c*.<sup>23</sup> C–D bonds can in principle provide nonperturbative probes of any side chain.<sup>19,24–27</sup> Another nonperturbative probe with a vibrational frequency around 2550  $\text{cm}^{-1}$ , at the edge of the transparent window, is the thiol group of native cysteine residues.<sup>28,29</sup> While ideal as probes of the native protein, the transition dipole strengths of both are relatively small, which makes their absorptions challenging to discern, even in the absence of other allowed absorptions. Unnatural probes can provide more intense absorptions. For this reason, cyano- and azido-functionalized side chains have been utilized widely.<sup>30–38</sup> Strong carbonyl probes can be generated by functionalization of side chains with metal complexes, ketones, or esters, although incorporation is more prone to be perturbative due to their relatively large size.<sup>39–44</sup> Alkynes are also under development as probes with vibrations in the transparent window.<sup>45</sup> Both alkyne and C–D labeling of lipids and proteins also have been utilized for vibrational imaging.<sup>46,47</sup> Rather than their use as probes of specific locations of proteins, this application uses the spectroscopic handle for detecting the spatial locations of the biomolecules in a cell.

A consideration in addition to the spectroscopic properties of an IR probe is the practical need to selectively introduce it at a specific location in a protein. Solid phase peptide synthesis (SPPS) can be used to directly incorporate virtually any labeled amino acid anywhere in a sequence of up to ~50 residues, enabling access to specific labeling of peptides and some small folded protein domains.<sup>48,49</sup> Larger labeled proteins can be constructed via native chemical ligation or other conformationally assisted ligation reactions from multiple synthesized peptides or protein fragments generated via recombinant expression or expressed protein ligation.<sup>19,49–53</sup> Residue-selective labeling with deuterated amino acids or analogues similar to natural amino acids, such as the methionine analogue azidohomoalanine (Aha), can be achieved through expression in defined media in an auxotrophic host or one that cannot efficiently biosynthesize the amino acid.<sup>24,27,34,54</sup> This approach is most useful for labeling rare amino acids, as they may be present at only one or a few positions, or in proteins that can tolerate the mutational removal of all but one instance of an amino acid. Unique residues also can be functionalized to generate site-specific IR probes through chemical post-translational modification.<sup>31,39,41,42,55</sup> For example, chemical modification of unique cysteine residues to introduce thiocyanate groups is becoming a popular route for introducing IR probes.<sup>31,35–37,55</sup> Alternatively, cell-free methods to isotopically label any residue in a protein are now available, but production of quantities needed for biophysical studies remains challenging.<sup>56,57</sup> An approach for the facile introduction of different unnatural amino acids at virtually any specific site in any protein is nonsense (amber) suppression, in which an unnatural amino acid is incorporated during ribosomal protein synthesis in response to an amber stop codon introduced into the gene sequence at the desired site of labeling.<sup>58,59</sup> The scope of this approach is limited by the availability of tRNA and aminoacyl tRNA synthetases that are laboratory-evolved for incorporation of the desired unnatural amino acid within the expression host. Nonetheless, the approach is established for selective introduction of many IR probes, including C–D bonds, nitriles, and azides.<sup>25,33,60</sup>

While IR spectroscopy is becoming a popular approach for the study of proteins, 2D IR spectroscopy of proteins remains limited. As with linear IR spectroscopy, 2D IR spectroscopy was first directed at the vibrations of diatomic ligands of heme proteins and the amide backbone of peptides.<sup>7,61</sup> A carbon monoxide ligand of a heme generates what is among the strongest signals possible for characterization of a protein and thus has been leveraged to study the active site of a number of heme proteins by our group and other groups.<sup>14,15,61–63</sup> A variety of ligands with frequency-resolved vibrations, ranging from polyatomic ions to small molecule inhibitors and modified peptides, have been exploited as IR probes for the characterization of their binding sites.<sup>64–69</sup> Unlike such ligand probes, amide backbone vibrations can be characterized for all proteins. A particular advantage of 2D compared to linear spectroscopy of amide vibrations is the fourth power rather than square dependence on the transition dipole strength, which accentuates the signals of amide and other vibrations with transition dipole strengths greater than water.<sup>6</sup> In addition, the spreading of the spectra along the diagonal in 2D plots facilitates spectral deconvolution. Nonetheless, spectral congestion of the amide frequency region typically prevents site-specific interpretation. Isotopic labeling of specific residues or protein segments shifts the amide frequencies; however, except for some impressive examples, its utility remains limited to peptides and small proteins.<sup>10,70–75</sup> In a few cases, native side chains of proteins have been utilized as probes for 2D IR spectroscopy, such as the thiol of cysteine and carboxylate side chains.<sup>29,76</sup> As native vibrations, these are attractive probes, although their weak transition dipole strengths or spectral congestion, respectively, could present challenges for their widespread use in larger proteins.

To develop an approach that is generally applicable for characterization of any location in any protein, our and other groups have been pursuing 2D IR spectroscopy of side chains with probe vibrations in the transparent frequency window.<sup>40,41,77–82</sup> Hochstrasser and co-workers first demonstrated 2D IR spectroscopy of two cyanophenylalanine (CNF) probes incorporated by SPPS into the 35-residue peptide HP35 that folds into a small domain.<sup>77</sup> The HP35 peptide was concurrently investigated by Fayer and co-workers, who used single CNF probes to characterize folding of the wild-type and a mutant peptide,<sup>78,83</sup> and later, this system was further studied by Gai and co-workers.<sup>84</sup> Fayer and co-workers first extended site-specific 2D IR spectroscopy to full sized proteins through use of azidophenylalanine specifically introduced into myoglobin via amber suppression.<sup>79</sup> Thiocyanate groups introduced through cyanylation of native cysteine residues have been employed to characterize the dynamics of dihydrofolate reductase by Cheatum and co-workers and hemoglobin by Brendenbeck and co-workers.<sup>80,85</sup> Aha specifically incorporated into both a PDZ domain, and its peptide ligand has been employed in several studies by the Hamm group directed at protein folding and allostery of binding.<sup>68,81,82,86</sup> Aha is attractive as a 2D IR probe due to its strong absorbance and convenient incorporation as a methionine analogue via auxotrophic expression. Particularly intense 2D IR probes have been generated by the covalent attachment of metal carbonyl complexes to unique amino acid side chains.<sup>40–42</sup> These probes might be too large and perturbative to place in a protein core but are useful for

characterization of protein surface properties, such as surface hydration, as reported by Kubarych and co-workers.<sup>40</sup>

Although 2D IR spectroscopy with transparent window probes is actively under development, it remains in a relatively early stage. Thus far, the approach has almost exclusively been applied to demonstrate 2D IR spectroscopy of a single probe in a protein, and most studies have not yet taken advantage of its intrinsic power to investigate the spatial heterogeneity of proteins by characterization of different sites to elucidate how specific parts contribute to function. Toward expanding the approach in this direction, a recent study by the Hamm group employed multiple Aha probes to investigate allostery of a Pdz domain.<sup>81</sup> They acquired difference 2D IR spectra induced by photoisomerization of a covalently attached azobenzene linker, which was used to model the conformational change associated with ligand binding. Unexpectedly, the 2D IR difference spectra could be detected for only one of six labeled sites. Nonetheless, the study demonstrated differential 2D IR spectral responses among sites within a protein domain. Recently, we applied  $T_w$ -dependent 2D IR spectroscopy with multiple CNF probes to obtain site-specific information about the heterogeneity and dynamics throughout a Src homology 3 (SH3) domain to investigate their involvement in molecular recognition,<sup>87</sup> which is described in more detail below.

## ■ OUR CONTRIBUTIONS TO THE FIELD OF SITE-SPECIFIC IR SPECTROSCOPY OF PROTEINS

Our research program has aimed to take advantage of site-specific IR spectroscopy toward advancing our understanding of protein molecular recognition. In particular, we have focused on proteins that form dynamic complexes that are challenging to investigate with the more conventional biophysical techniques: plastocyanin (Pc) and its binding partner cytochrome *f* (cyt *f*), cytochrome P450s (P450s) and substrates or redox partners, and SH3 domains and proline-rich (PR) peptide motifs. These proteins play critical roles in diverse cellular processes, including photosynthesis, metabolism, and signaling; however, in each case, their biological function is mediated via the formation of relatively low affinity and dynamic complexes.<sup>88–90</sup> The complexes are thought to involve a heterogeneity of states, including those that rapidly interconvert. Through the use of IR spectroscopy with site-specific labeling, we have aimed to characterize the proteins and their complexes with high spatial resolution and capture the heterogeneity of states and fluctuations with high temporal resolution, to gain new information for generating a better molecular-level understanding of dynamic protein recognition.

While investigating protein molecular recognition, we also have aimed to push forward the experimental method of site-specific IR spectroscopy as a general approach to investigate protein biophysics. With this consideration, we have explored probes with frequencies in the transparent frequency window that are anticipated to be minimally perturbative while still providing sufficiently intense absorptions to make feasible the characterization of samples at low mM concentrations (Figure 3). C–D bonds are not only the least perturbative probes, but they also provide intrinsic probes of the protein itself.<sup>19,23–27</sup> While in many ways they are our ideal probes, their small transition dipole strengths make their absorptions difficult to discern, and their use as 2D IR probes is not yet feasible. Nonetheless, we believe that their appeal makes their development worthy of further exploration. For 2D IR studies of the heme protein P450, we have leveraged the intense signals

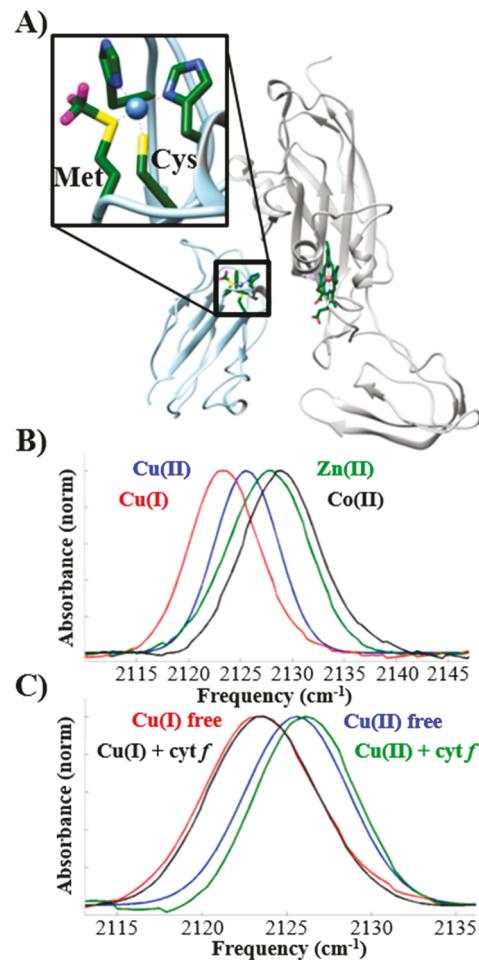
from a CO ligated to the heme.<sup>14,15</sup> For our efforts to develop site-specific 2D IR spectroscopy as a tool to investigate any part of any protein, we have focused on the cyano (CN) group of CNF as a 2D IR probe. The CN group is extrinsic to the protein but relatively small in comparison to other options and, importantly, is a sufficiently strong chromophore to make possible 2D IR spectroscopy of singly labeled proteins.<sup>87</sup> Furthermore, the CN probe has other attractive spectroscopic properties. Fermi resonances do not typically complicate spectral interpretation, and the vibrational lifetime, which determines the temporal decay of the 2D bands, is sufficiently long to enable the characterization of spectral diffusion.<sup>78</sup> Finally, CNF can easily be site-specifically incorporated via amber suppression.<sup>60</sup>

In the following, we highlight some of our efforts to use the transparent window probes in combination with linear and 2D IR spectroscopy to gain insight into the molecular recognition underlying the biological function of the three protein systems mentioned above. First, we describe our application of C–D probes with linear IR spectroscopy to detect subtle changes in ligand–metal interactions in the blue copper protein Pc induced by binding to its electron transfer partner cyt *f*, providing new information to suggest a molecular mechanism for the associated decrease in Pc midpoint potential.<sup>27</sup> We then describe 2D IR spectroscopy with a CO ligand as a probe of the active site of a P450 to uncover how conformational dynamics contribute to the regioselective activity with different substrates and to the effector role played by redox partner binding.<sup>14,15</sup> These studies provide evidence for the importance of fast dynamics in protein function and illustrate the richer information provided by 2D over linear methods. Finally, we describe our studies of SH3-mediated molecular recognition of PR motifs from the perspective of both protein domain and ligand.<sup>26,87</sup> We first describe how linear spectroscopy of C–D probes introduced in the PR ligand can capture states that are in rapid exchange on the NMR time scale. We then describe our use of 2D IR spectroscopy with CNF probes to characterize the heterogeneity and dynamics at different sites in the SH3 domain and uncover variation in how the sites are involved in the molecular recognition of the PR ligand.

## BINDING-INDUCED CHANGE IN THE COPPER SITE OF Pc INVESTIGATED BY IR SPECTROSCOPY

Unlike electron transfer between small molecules, biological electron transfer occurs between redox centers embedded within environments that are structurally and electrostatically heterogeneous and undergo fluctuations over a wide range of time scales.<sup>91</sup> Furthermore, interprotein electron transfer typically involves transient protein–protein interactions in highly dynamic complexes.<sup>89,92</sup> An example of central importance in photosynthesis is the complex formed to mediate electron transfer between the blue copper protein Pc and cyt *f* (Figure 4A).<sup>89,93</sup>

The reactivity of the metal site in Pc and other proteins is well-known to be modulated by the protein environment.<sup>94</sup> In Pc, the metal coordination geometry is thought to arise from a weak and elongated bond between Cu and an axial methionine (Met97) ligand, which is compensated by a strong and shortened bond with a cysteine ligand (Cys89) (Figure 4A).<sup>95</sup> Comparison of the metal sites among blue copper proteins suggests that the strength of the bonding with the axial ligand can modulate reactivity, for instance, by affecting the midpoint potentials or reorganization energies.<sup>96</sup> While Pc has been



**Figure 4.** (A) Structural model of the *Pc*–*cyt f* complex (PDB ID: 1TU2). The inset provides an expanded view of the Cu site of *Pc* with the introduced C–D probes at Met97 highlighted in purple. (B) FT IR spectra of  $d_3$ Met97 of Cu(I)Pc (red), Cu(II)Pc (blue), Zn(II)Pc (green), and Co(II)Pc (black). (C) FT IR spectra of  $d_3$ Met97 of free Cu(I)Pc (red) and Cu(II)Pc (blue) and *cyt f*-bound Cu(I)Pc (black) and Cu(II)Pc (green). Reproduced with permission from ref 27. Copyright 2016 American Chemical Society.

extensively studied, how the axial Met determines the geometry and bonding of the Cu site and, moreover, how formation of the complex with *cyt f* might impact the metal site reactivity is not well understood. For example, complexation with *cyt f* was shown long ago to decrease the midpoint potential of *Pc*,<sup>97</sup> but the mechanism is unclear. One challenge is that the redox reaction of course involves both the reduced Cu(I) and oxidized Cu(II) states of *Pc*, but most spectroscopic methods are not able to characterize the reduced state due to its filled  $d^{10}$  orbital configuration. In contrast, IR spectroscopy is applicable to both redox states. To enable the use of IR spectroscopy to selectively investigate the metal site of *Pc*, we incorporated C–D probes at the methyl group of the axial Met97 ligand ( $d_3$ Met97).<sup>27</sup>

The C–D vibrations of  $d_3$ Met97 were highly sensitive to the interaction of the adjacent sulfur-based orbitals with the positively charged metal center, making them useful reporters of the Cu–Met97 bond (Figure 4B). Specifically, the symmetric stretch of  $d_3$ Met97 showed a shift to higher frequency upon oxidation of Cu(I)Pc to Cu(II)Pc. Then, a series of *Pc* variants were compared to assign the source of the

frequency change to either the ionic interaction of Met97 with the metal center or covalent mixing of the unoccupied d orbitals of Cu(II) with the protein ligand orbitals. Briefly, comparison of Cu(I) Pc to Zn(II)-substituted Pc and a state of Cu(I) Pc with a shorter Cu–Met bond formed at low pH was used to assess the effect on the C–D frequency of increasing the ionic interaction with a filled  $d^{10}$  metal center, where bonding is exclusively ionic. Then, Zn(II) Pc was compared to the Cu(II) or Co(II) Pc to assess the effect due to the Cys89 or Met97 ligand interaction with the unoccupied  $d_{x^2-y^2}$  or  $d_{z^2}$  metal orbitals, respectively, within the context of a constant ionic interaction with metal centers of the same nominal charge. Altogether, this analysis revealed that the increase in the C–D frequency upon oxidation of Cu(I) to Cu(II) Pc resulted from a stronger ionic interaction with the formally doubled metal charge, but the effect was reduced by mixing of the Cys89 ligand orbitals with the unoccupied  $d_{x^2-y^2}$  orbital of Cu(II).

After calibrating the C–D probes at  $d_3$ Met97, IR spectroscopy was applied to characterize the effects of binding to cyt f (Figure 4C). Association led to an increase in the C–D frequency, consistent with a stronger interaction between Met97 and the Cu ion in the complex. A stronger interaction between the Cu ion and the axial ligand is expected to decrease the midpoint potential of Pc,<sup>95</sup> as is known to occur upon complexation with cyt f.<sup>97</sup> The IR data suggest that the functional change in Pc results from an interaction with cyt f that induces a slight contraction of the Met97–Cu bond. Altogether, the study illustrates how IR spectroscopy of C–D probes can provide molecular-level information about how the metal sites of metalloproteins are controlled by protein–protein interactions and demonstrates the utility of the high structural resolution afforded by IR spectroscopy of C–D probes for the nonperturbative characterization of localized changes in proteins.

## ■ FUNCTIONAL DYNAMICS OF CYTOCHROME P450s

P450s are hemeprotein oxidases involved in a wide variety of metabolic and catabolic reactions in organisms throughout all kingdoms of life.<sup>98,99</sup> The ability of these enzymes to insert oxygen atoms at inactivated carbon centers with high regio- and enantioselectivity makes them a subject of considerable interest to synthetic chemists and for biotechnology. In addition, P450s play central roles in drug metabolism, and their activities are key determinants of drug pharmacokinetics.<sup>100</sup> Most P450s share a common multistep catalytic cycle, which includes substrate binding, two reduction steps, uptake of two protons, and formation of an oxo-ferryl species called compound I, a highly reactive intermediate that initiates substrate oxidation.<sup>101</sup> Despite extensive study, many aspects of P450 activity remain poorly understood. A missing factor in our current picture is the role of dynamics. For instance, P450 homologues can vary greatly in their substrate specificity as well as in the regio- and enantioselectivity of their activity on different substrates.<sup>98,99</sup> Because P450s have similar structures and catalytic mechanisms, protein flexibility is often evoked to explain the variation in activity;<sup>102–104</sup> however, we lack a rigorous molecular-level understanding of how conformational dynamics are involved. In addition, conformational changes are known to be induced upon the binding of substrates,<sup>105,106</sup> but whether dynamics participate in other steps in the catalytic cycle is still unknown. Toward addressing these outstanding

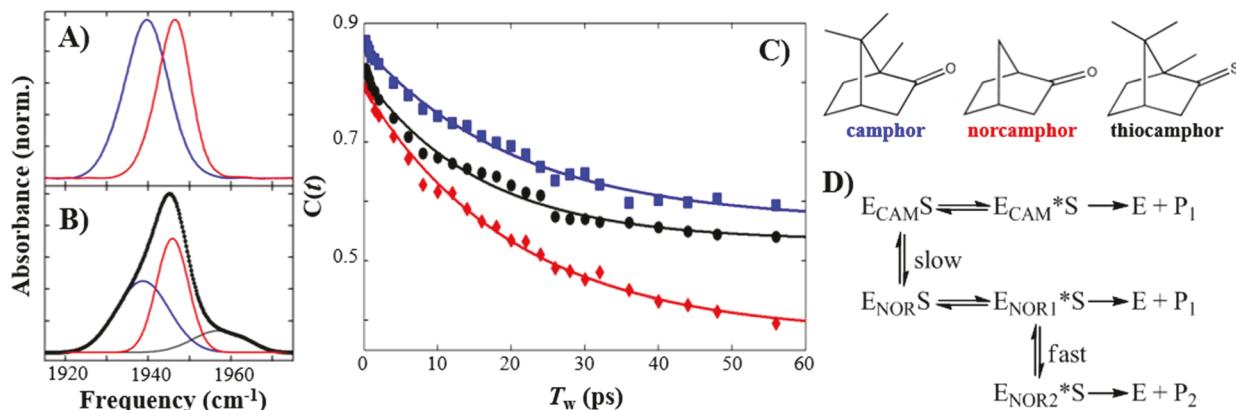
questions, we have applied 2D IR spectroscopy to measure P450 conformations and dynamics and unravel their contributions to function.

## ■ FAST DYNAMICS PLAYS A CRITICAL ROLE IN THE REGIOSELECTIVITY

Our studies have focused on the archetypical P450, P450cam (CYP101A1), from *Pseudomonas putida*, which hydroxylates camphor in the metabolism of the compound as a carbon source.<sup>99</sup> P450cam hydroxylation of camphor shows complete regioselectivity to produce 100% 5-hydroxylcamphor.<sup>107</sup> P450cam is also active with the structurally related substrates norcamphor and thiocamphor but with low and intermediate regioselectivity (45 and 63% 5-hydroxy product), respectively.<sup>107,108</sup> The product-determining step in the P450cam catalytic cycle is the reaction of the activated oxygen of the compound I intermediate with a particular carbon center of the substrate. Therefore, what determines the regioselectivity of P450cam on the substrates is either (1) the intrinsic reactivity of the substrate or (2) how the enzyme binds the substrate within the binding site to control its mobility during the limited lifetime of the reactive compound I.<sup>109</sup> Previous calculations have indicated that the difference in the intrinsic reactivity of the carbon centers of camphor, thiocamphor, and norcamphor does not explain the observed product distributions.<sup>110</sup> To investigate how the active site environment and dynamics might contribute to the regioselectivity, we applied 2D IR spectroscopy to characterize the complexes with each of the three substrates. As P450cam is a hemeprotein, CO could be ligated to the heme center for use as a vibrational probe of the active site.

The linear spectra of the CO probe for the camphor and norcamphor complexes showed symmetric, Gaussian absorption bands, suggestive of environments of a single conformational state (Figure 5A). However, the absorptions were shifted from each other, implying some difference in the states. In contrast, the linear spectra of the CO for the thiocamphor complex were more complex, and adequate fitting required a superposition of three Gaussian bands, indicative of three underlying absorptions due to three distinct states (Figure 5B). Interestingly, the two dominant bands showed the same frequency and line width as the two bands individually measured for the camphor and norcamphor complexes. These results indicate that the camphor and norcamphor complexes adopt distinct conformational states, associated with high and low regioselectivity, respectively, whereas the thiocamphor complex populates both states, with the intermediate regioselectivity likely resulting from a combination of the activity associated with each.

To further investigate the origins of the high or low regioselectivity in each conformation,  $T_w$ -dependent 2D IR spectra were acquired and analyzed to determine the dynamics within the inhomogeneous distribution of frequencies of each absorption band (Figure 5C), providing insight into the local energy landscape within each associated conformation. Interestingly, the dynamics reported by the CO at the active site of the substrate complexes were correlated with regioselectivity. Specifically, the complexes with camphor, thiocamphor, and norcamphor, which are hydroxylated with high, intermediate, and low regioselectivity, showed slow, intermediate, and fast dynamics, respectively. As found for the linear spectrum, the 2D data for the thiocamphor complex could be well modeled by a superposition of two states with



**Figure 5.** (A) IR spectra of the P450cam–CO complex with camphor (blue) and norcamphor (red). (B) IR spectrum of the thiocamphor complex (black) and multicomponent fit (blue, red, and gray). (C) Frequency–frequency correlation functions determined for the P450cam–CO complex with camphor (blue), norcamphor (red), and thiocamphor (black). (D) Model for conformational dynamics underlying P450cam regioselectivity. See text for details. Structures of substrates are shown above the mechanism. Reproduced with permission from ref 14. Copyright 2015 American Chemical Society.

the dynamics of the camphor and norcamphor complexes. Altogether, the spectral data indicate that substrate complexes of P450cam adopt two major conformational states and that a particular substrate can selectively stabilize one or the other. However, what appears to be the key factor that determines the regioselectivity on a substrate is the dynamics among the substates within the populated conformation. A model consistent with the data is shown in Figure 5D. When P450cam adopts the norcamphor-stabilized conformation ( $E_{NOR}S$ ), dynamics within the active site on time scales faster than the lifetime of compound I allow for multiple carbon centers to approach and react with the activated oxygen, leading to low regioselectivity. In contrast, in the camphor-stabilized conformation ( $E_{CAM}S$ ), the dynamics within the active site are slower, and the substrate is held more rigidly such that only the S-carbon can approach the reactive compound I species and be hydroxylated. In the thiocamphor complex, both of the conformations are populated but do not interconvert during the lifetime of compound I; thus, the reactions proceed among both populations in parallel, leading to the intermediate distribution of products. 2D IR spectroscopy thus provides a model for the mechanism of regioselectivity in which the fast dynamics within a conformation play a central role.

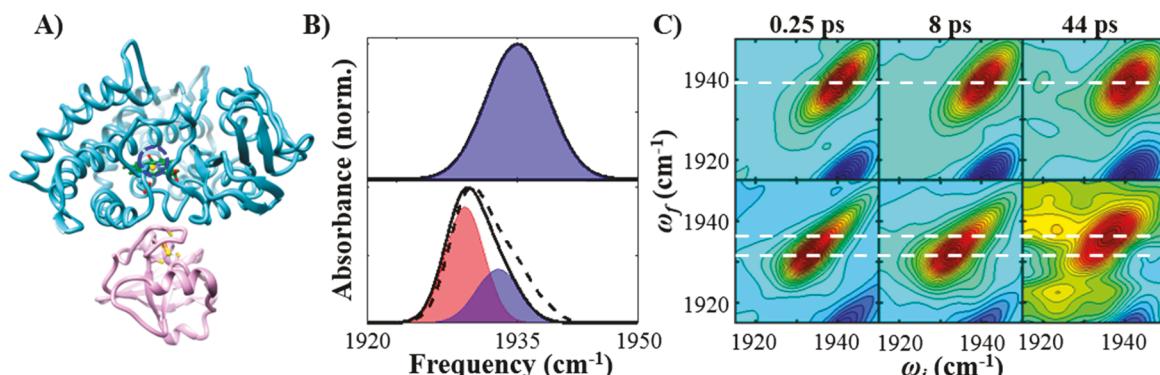
## ■ DETECTION OF A CONFORMATIONAL CHANGE IN P450cam INDUCED BY REDOX PARTNER BINDING

A second aspect of P450cam activity illuminated by 2D IR spectroscopy is the mechanism of the effector role of the redox partner, putidaredoxin (Pdx) (Figure 6A). Pdx provides both electrons in the two reduction steps in the catalytic cycle of P450cam. Whereas the first reduction can be mediated by any reductant with appropriate potential, the second reduction requires Pdx.<sup>111</sup> The reason why is a long-standing question. The complex formed by P450cam and Pdx, like most electron transfer partners, is low affinity and highly dynamic,<sup>90</sup> which challenges investigation by conventional biophysical methods. Although it is well-known that a conformational change is induced by substrate binding,<sup>105,106</sup> whether a similar or different conformational change occurs upon binding Pdx is currently under intense investigation.<sup>112–114</sup> We again took

advantage of heme-ligated CO as a vibrational probe of the P450cam active site to characterize how binding of Pdx affects the conformations and dynamics.<sup>15</sup>

The CO-ligated ferric state of the camphor complex of P450cam, a model for the ferric, oxygen-ligated state of the enzyme involved in the second reduction step, was characterized in the absence and presence of Pdx (Figure 6B). As observed previously, the absorption for the camphor complex was well fit by a single Gaussian band,<sup>14</sup> suggesting that the CO experiences a single environment. In contrast, the absorption in the Pdx complex was shifted to lower frequency and slightly asymmetrical. Whereas this asymmetry hinted at the presence of two underlying bands, they were more obvious from comparison of the linear FT IR spectrum and the diagonal of the 2D spectrum. The band at higher frequency is accentuated in the 2D diagonal, indicating that the two CO vibrations have different transition dipole strengths. Analysis of the component bands from fitting the linear and 2D spectra indicated that the transition dipole strength of the vibration at higher frequency was about 1.3-fold greater, information that enabled accurate quantification of the relative populations of the states from the relative band areas.

Another observation from analysis of the  $T_w$ -dependent 2D spectra was that the two CO vibrations have different lifetimes. Because the lifetime of the vibration at lower frequency was shorter, its 2D band decayed more rapidly than the band at higher frequency. The result was that at sufficiently long  $T_w$ , when the dominant band at lower frequency was mostly decayed, the presence of the second minor band at higher frequency was unequivocally revealed (Figure 6C). Furthermore, analysis of the  $T_w$ -dependent 2D shapes found differences in the spectral diffusion within the bands, indicating distinct local energy landscapes within the associated conformations, providing evidence that the bands reflect two distinct conformational states. The data suggest that the Pdx complex populates two states, which we attribute to a loosely bound encounter state and more tightly associated state, according to the prevalent model for complexes of electron transfer partners.<sup>92</sup> The minor band (25%) showed similar average frequency and spectral dynamics as the free enzyme, and thus was interpreted as the encounter state, whereas the major band (75%) found at lower frequency with distinct



**Figure 6.** (A) Structural model (PDB ID: 4JWU) of the complex of P450cam (blue) and Pdx (pink). The CO probe is circled in blue. (B) FT IR spectra (black lines) and fits (shaded bands) of heme-ligated CO for the camphor complex of P450cam in the absence of Pdx (top) and in complex with Pdx (bottom). The black dotted line is the diagonal slice of the 2D spectrum obtained for  $T_w = 0.25$  ps. (C)  $T_w$ -dependent 2D IR spectra of the CO probe for the camphor complex of P450cam in the absence of Pdx (top) and in the complex with Pdx (bottom). The white dotted lines represent the center frequencies of each spectral component. Adapted with permission from ref 15. Copyright 2018 Ramos, Basom, Thielges.

dynamics was attributed to a more tightly engaged state. In agreement with our interpretation, NMR studies of the P450cam–Pdx complex are consistent with an ensemble of states,<sup>90</sup> but their rapid exchange on the NMR time scale complicates analysis of the populations. 2D IR spectroscopy provided clear evidence that Pdx binding induces the partial population of a new conformation of P450cam, which could likely underlie the effector role of Pdx in reduction. Moreover, 2D IR enabled quantification of the distinct populations observed.

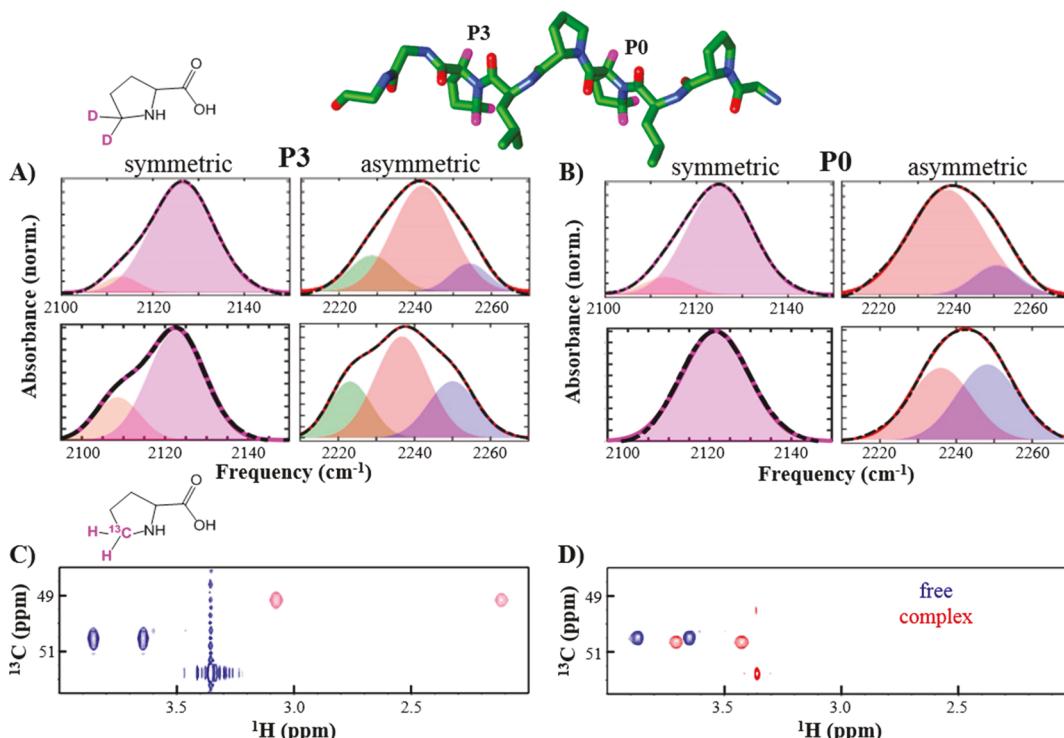
#### ■ Src HOMOLOGY 3 DOMAIN-MEDIATED MOLECULARrecognition OF COGNATE PROLINE-RICH MOTIFS

To mediate rapid responses via cellular signaling, molecular recognition within protein interaction networks often involves low affinity, highly dynamic protein–protein complexes. Such complexes, for example, are formed by SH3 domains and PR linear recognition motifs. SH3 domains are among the most abundant domains in human and other eukaryotic proteomes, and their recognition of PR motifs underlies a range of diverse signaling cascades.<sup>88</sup> Moreover, they have become an archetypical system for investigating biological molecular recognition. SH3 domains recognize a PR motif that contains a core P<sub>xx</sub>P sequence, where  $x$  is typically proline or a hydrophobic amino acid. Crystal structures of the complexes of SH3 domains and peptides consisting of the PR recognition motif show that the PR ligand adopts a polyproline II secondary structure in which the proline residues pack within grooves on the SH3 surface formed by three conserved Tyr residues. As these models indicate that binding is mediated by hydrophobic interactions, the change in entropy is expected to be favorable due to the release of water molecules, yet most studies have found SH3–PR peptide complex formation is entropically unfavorable. The unfavorable entropy changes must arise from restriction in the conformational freedom of the SH3 domain, PR sequence, or water. While the system has been extensively studied via a wide range of experimental techniques, the role of conformational heterogeneity and dynamics in their molecular recognition is still not well understood.

#### ■ SITE-SPECIFIC IR SPECTROSCOPY CAN RESOLVE RAPIDLY INTERCONVERTING STATES OF THE PR LIGAND

We have used IR spectroscopy with site-selectively introduced transparent window probes to gain molecular insight into how SH3 domains recognize cognate ligands.<sup>26</sup> Our initial studies have focused on the yeast Sho1 Src homology 3 (SH3<sup>Sho1</sup>) domain-mediated recognition of a PR peptide from its physiological target, the protein Pbs2 (pPbs2). Interaction of SH3<sup>Sho1</sup> and pPbs2 was shown to be specific within the yeast proteome,<sup>115</sup> so the system provides a case in which the binding of the proteins, rather than spatial or temporal regulation of their expression alone, is known to be crucial for their function. We introduced C–D bonds as transparent window probes by incorporation of (C<sub>α</sub>D<sub>1</sub>, C<sub>δ</sub>D<sub>2</sub>)proline during synthesis of pPbs2 and then used the probes to site-specifically characterize the changes along the peptide induced by binding to SH3<sup>Sho1</sup>. The symmetric and asymmetric stretching vibrations of the C<sub>δ</sub>D<sub>2</sub> group showed reasonably intense absorptions that facilitated analysis. As anticipated, the spectra of the peptides labeled at the proline residues of the recognition motif were the most sensitive to binding. A less expected observation was that the absorption bands of single vibrational modes showed asymmetry (Figure 7A,B). In addition, second derivative analysis and band fitting indicated that the spectra consisted of multiple overlapping bands. The observation of multiple bands for single vibrational modes provides evidence that the C–D probes experience multiple, distinct environments.

For comparison, we prepared the pPbs2 peptide labeled at the same residues with the <sup>13</sup>C-proline for characterization by NMR spectroscopy (Figure 7C,D). The <sup>1</sup>H/<sup>13</sup>C HSQC NMR spectra showed single resonances for the <sup>1</sup>H/<sup>13</sup>C nuclei associated with C–D bonds that showed multiple IR absorptions. Considering that interactions sufficient to alter the IR frequencies of the bonds are likely to also affect the NMR resonances given the relative energy scales of the techniques, the presence of only single NMR resonances indicates that the states uncovered by IR spectroscopy are in rapid exchange on the NMR time scale. These results illustrate the value of the high inherent temporal resolution of IR spectroscopy to ensure the detection of all protein heterogeneity that potentially contributes to function. We have



**Figure 7.** IR and NMR spectra of specifically labeled pPbs2. Shown is a structural model of pPbs2 with locations of introduced C–D probes at motif residues P0 and P3 highlighted (pink, top). The IR absorptions for the symmetric (left plots) and asymmetric stretches (right plots) of the C<sub>δ</sub>D<sub>2</sub> probes for the free pPbs2 (top plots) and complex with SH3<sup>Sho1</sup> (bottom plots) are shown for (A) P3 and (B) P0. <sup>13</sup>C–<sup>1</sup>H HSQC (800 MHz) spectral region with resonances for the equivalent nuclei are shown for (C) P3 and (D) P0 for free pPbs2 (blue) and SH3<sup>Sho1</sup> complex (red). Adapted from ref 26 with permission. Copyright 2016 American Chemical Society.

extended these studies by performing a more systematic investigation of a set of PR peptides, combined with support from molecular dynamics and density functional theory calculations, and have assigned the multiple absorptions found for the asymmetric stretch of the C<sub>δ</sub>D<sub>2</sub> group of proline to distinct backbone conformations.<sup>116</sup> Currently, we are working to elucidate how the binding-induced changes in these backbone conformations along the PR ligand contribute to the unfavorable entropy changes characteristic of SH3-PR motif recognition.

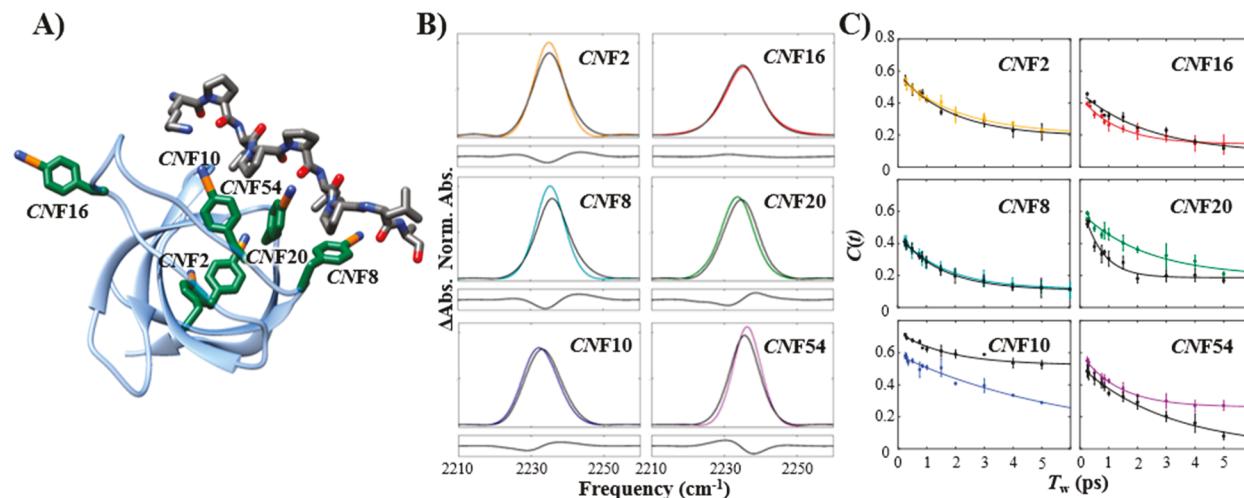
## ■ SITE-SPECIFIC 2D IR SPECTROSCOPY UNCOVERS VARYING ENGAGEMENT OF THE SH3 DOMAIN WITH THE PR LIGAND

We have also investigated the molecular recognition mediated by SH3 domains from the perspective of the protein.<sup>87</sup> So that we could use 2D IR spectroscopy to site-specifically measure the frequency inhomogeneity and dynamics, we utilized CNF as a probe. CNF was introduced via amber suppression at six different sites in the SH3<sup>Sho1</sup> (Figure 8A). CNF substituted each of the three conserved Tyr residues, Tyr8, Tyr10, and Tyr54, that form the binding surface (CNF8, CNF10, and CNF54), Tyr16 within the so-called RT loop (CNF16), Phe20 in a more buried location (CNF20), or Tyr2 that is distant from the ligand binding surface (CNF2). We then applied linear and 2D IR spectroscopy to characterize their microenvironments and how their nature and heterogeneity change upon binding the pPbs2 ligand.

The variation in the spectra of the CNF probes and how they are affected by pPbs2 binding reveal distinct environments throughout the SH3 domain and provide information

with which to build a picture of the dynamic complex in solution (Figure 8). The absorptions of residue CNF2, the site distant from the recognition surface, as anticipated, showed no change either in its average frequency or spectral dynamics. Similarly, CNF16 was not highly sensitive to pPbs2 binding. In contrast, association with pPbs2 impacted all of the other probes, indicating that their local environments were changed upon complex formation.

The linear absorptions of the three probes placed along the binding surface, CNF8, CNF10, and CNF54, and the most buried probe, CNF20, shifted in frequency upon binding to pPbs2, indicative of perturbation to the average environment, but the changes were rather small (Figure 8B). In comparison, 2D IR spectroscopy uncovered relatively dramatic effects to the inhomogeneous broadening and the spectral diffusion, as reflected by the C(t) shown in Figure 8C. These results indicate that the heterogeneity of the environments of the probes and the rates of interconversion among the underlying states are impacted by complexation. With assistance from molecular dynamics (MD) simulations, the 2D data support a molecular picture for how the different probes' environments are affected by complex formation. At CNF20, no change in inhomogeneity occurred upon ligand binding but the dynamics became faster. Correspondingly, MD simulations indicated that CNF20 does not undergo substantial changes in its interactions with its surrounding environment nor does it contact the ligand. Nonetheless, the perturbed dynamics suggest that the binding event is sensed at CNF20, although the residue is not part of the interaction surface. In comparison to CNF20 and all other sites, CNF10 showed the largest increase in inhomogeneity associated with states that slowly



**Figure 8.** (A) Structural model of the complex of SH3<sup>Sho1</sup> and pPbs2 (PDB ID: 2VKN) showing locations of CNF incorporation. (B) FT IR spectra of the unligated state (colored lines) and the pPbs2 complex (black lines); difference spectra are displayed in black below each set of spectra. (C) Correlation functions (points) and fits (lines) for unligated SH3<sup>Sho1</sup> (colored) and the pPbs2 complex (black). Reproduced from ref 87 with permission. Copyright PCCP Owner Societies.

interconvert, suggestive of greater exposure to slower protein motions in the complex. In MD simulations, CNF10 experiences a heterogeneous protein environment where it interacts with numerous different residues, many of which are impacted by ligand binding. In contrast, CNF54 showed the opposite behavior, as the association with pPbs2 induced a decrease in inhomogeneity associated with slowly interconverting states, indicative of exposure to a more homogeneous environment with slowly fluctuating interactions. MD simulations suggested that this change results from the packing of CNF54 between the side chains of the two motif prolines of the ligand, where it experiences a relatively homogeneous environment. Surprisingly, CNF8 showed no change in inhomogeneity or dynamics upon ligand binding, despite that it replaced a conserved Tyr residue that packs against the ligand in the crystal structure. The insensitivity suggests that in solution the complex of SH3<sup>Sho1</sup> and pPbs2 might not be as tightly engaged near the location of CNF8.

2D IR spectroscopy revealed site-specific heterogeneity and dynamics that depend significantly on ligand binding, including at the probes placed at the adjacent conserved Tyr residues that form the binding surface. As side chain dynamics play a key role in entropy changes of molecular recognition by other proteins,<sup>117,118</sup> we speculate that the site-specific changes in the heterogeneity and dynamics uncovered in our study of pPbs2 recognition by SH3<sup>Sho1</sup> may make important contributions to binding thermodynamics. For example, the results indicate that part of the recognition surface, specifically the location of CNF54, could contribute more substantially to the unfavorable binding entropy than other locations in the protein, such as those probed by CNF10, and particularly CNF8. Information about binding-induced changes to heterogeneity and dynamics should help address the long-standing question about the molecular origins of the large unfavorable entropy contributions typical of SH3-mediated molecular recognition.

Altogether, the data underscore how generating a complete molecular description of protein recognition and other aspects of function requires the ability to measure conformational heterogeneity and dynamics with residue-specific precision. This study illustrates site-specific 2D IR spectroscopy as an

experimental approach with sufficient spatial and temporal resolution to tackle the complexity of proteins. In addition to the SH3 domain, we have applied the approach to uncover site-dependent involvement of residues in the molecular recognition of P<sub>c</sub> and cyt f.<sup>119</sup> Indeed, the intrinsic power of IR spectroscopy lies in its ability to measure rapidly interconverting states at multiple specific locations in a protein with high spatial detail to test if and how each contributes to function. The elucidation of differences in heterogeneity and dynamics throughout a protein, and how they change in a biologically relevant process, is an essential step toward the longer-term aspiration of using biomolecular 2D IR spectroscopy as a routine approach to study protein biophysics.

## FUTURE DIRECTIONS

Site-specific incorporation of frequency-resolved probes has extended the applicability of IR spectroscopy from the characterization of global secondary structure to the detailed characterization of local side chain environments at any specific location. In addition to the desired properties of IR probes already discussed, another characteristic of importance for 2D IR spectroscopy is the probe's vibrational lifetime. The lifetime determines the rate of decay of 2D bands with  $T_w$ , so it ultimately limits the time window over which 2D IR spectroscopy can directly follow vibrational dynamics. As probes with longer lifetimes would enable characterization of dynamics over longer time scales, their development is actively being pursued.<sup>120–124</sup> A common strategy is to decouple the probe vibration from others within the molecule to disrupt intramolecular energy relaxation, for instance, by attachment of the probe group to a heavy atom. This rationale has motivated the characterization of a number of thio- and selenocyanate functionalized amino acids.<sup>120,121,124</sup> Our group for example has synthesized and evaluated selenocyanophenylalanine (CNSeF) as a potential probe with a significantly extended lifetime.<sup>124</sup> However, this and most reported extended lifetime probes have not yet been incorporated into a protein or utilized in practice to study function. For CNSeF, issues with its instability during incorporation still need to be addressed. An accompanying challenge is that probes with longer lifetimes

tend to also show weaker absorptions. Thus far, the frequency-resolved IR probe with the longest lifetime ( $\sim 30$  ps) previously utilized for study of a protein is the thiocyanate group generated from derivatization of cysteine,<sup>80,85</sup> but its small transition dipole strength challenges application for 2D studies of dynamics at single sites.

While the vibrational lifetime of a probe limits the measurement of equilibrium dynamics directly via analysis of  $T_w$ -dependent 2D IR cross bands, interrogation of kinetic processes on longer time scales is possible via nonequilibrium 2D IR spectroscopy.<sup>125,126</sup> Nonequilibrium experiments involve triggering a process and the collection of 2D IR difference spectra at successive time intervals to characterize the subsequent dynamics. A key advantage of nonequilibrium experiments is the better detection limits possible via acquisition of difference compared to absolute spectra as a result of the more accurate background subtraction. For example, measurement of absolute 2D IR spectra for transparent window probes typically requires 1 mM sample concentrations,<sup>79,80,85,87</sup> whereas a difference 2D IR spectrum of an Aha probe at 10-fold lower concentration of 0.1 mM has been obtained.<sup>86</sup> However, nonequilibrium experiments require greater instrument complexity and the ability to repetitively trigger a biologically relevant process with high efficiency. In the first nonequilibrium 2D IR study reported by Hamm and co-workers, a UV pulse was used to trigger a reversible azobenzene photoswitch placed in the backbone of a cyclic octapeptide.<sup>127</sup> Similar photoswitches have since been employed by the group to trigger nonequilibrium studies of peptide folding and protein–ligand binding.<sup>72,81,82,86,125</sup> Tokmakoff and co-workers have developed a 2D IR spectrometer capable of reaction triggering via temperature jump and have applied the approach to study the kinetics of protein folding and protein–protein binding.<sup>128–130</sup> However, not all samples are amenable to repetitive triggering to average the temporal response over many experiments. To overcome this issue for the study of peptide aggregation kinetics, Zanni and co-workers have developed a mid-infrared pulse shaper with “rapid-scan” capability to track peptide aggregation in real time.<sup>131,132</sup> Although still relatively underutilized, nonequilibrium 2D IR spectroscopy has potential for investigation of a range of kinetic processes and to further improve sensitivity of 2D IR spectroscopy.

Another exciting avenue for improving sensitivity is surface-enhanced vibrational spectroscopy, an approach based on the large enhancement of signal strengths of molecules near the surface of noble metals due to excitation of local plasmon resonances.<sup>133–135</sup> The technique has been extensively developed for linear spectroscopy and could provide even greater benefits for 2D methods due to expected squared greater signal enhancement. Surface enhancement of 2D IR signals was first demonstrated by Hamm and co-workers using capped colloidal gold nanoparticles and later at the surface of metal-coated ATR prisms.<sup>136,137</sup> Even stronger signal enhancements can be achieved using optical nanoantennas with dimensions fabricated to tune plasmonic transitions to be resonant with the molecular frequencies.<sup>138–141</sup> Signal enhancements as large as 7 orders of magnitude have been reported.<sup>141</sup> The strong enhancements occur only for molecules near the metal surface, typically within a few nm, making possible the selective characterization of proteins or other biomolecules confined within a monolayer. We envision the approach will be particularly powerful for the study of

monolayers of membrane proteins within lipid bilayers. Linear surface enhanced IR spectroscopy has previously been applied to the study of membrane proteins, such as the electron transfer and protein–protein interactions of cytochrome *c* oxidase and photosystem I.<sup>142,143</sup> Because of the greater signal enhancement possible for nonlinear spectroscopic signals, 2D IR studies of membrane proteins and other biochemical systems that are hindered by low solubility could be achieved by surface enhancement.

Low sensitivity due to the weak signals and limited sample concentrations is a principle challenge of using 2D IR spectroscopy for the characterization of proteins. Spectral selectivity is another major issue but can be alleviated through use of frequency-resolved probes. The field is beginning to tackle these challenges,<sup>144</sup> and a number of studies have demonstrated site-specific 2D IR spectroscopy of single side chain probes in proteins.<sup>40,41,79,81,82</sup> An obvious extension of the approach is the measurement of the interaction of two probes to generate an IR experiment analogous to the well-established biophysical techniques of FRET, NOE, or DEER spectroscopy. As the strength of the coupling of two IR probes depends on the distance and orientation of the two probes, the amplitude and polarization dependence of cross bands can provide constraints for modeling the structure of proteins. For example, Hochstrasser and co-workers have shown the capability for structure determination of small peptides through measurement of couplings between amide vibrations.<sup>145</sup> Coupling between two side chain probes has been detected via the observation of 2D cross bands for a trimer peptide<sup>146</sup> but not yet for larger peptides or proteins. Specific introduction, into a protein, of two probes with distinct frequencies increases the challenges of sample preparation but is nonetheless feasible. Multiple unnatural amino acids have been introduced into proteins via amber suppression, albeit typically with lower efficiency and yield.<sup>147</sup> Alternately, two probes could be introduced by SPBS into peptides and larger labeled proteins then generated via native chemical ligation. Measurement of the coupling between two probes in different proteins is another possibility. Along these lines, a recent study showed the possibility to measure vibrational energy transfer between multiple unnatural azido-labeled amino acids selectively introduced in a PDZ domain and peptide ligand, demonstrating a potential method for determination of distances between sites within or between proteins.<sup>148</sup>

While unnatural amino acids with vibrations that provide strong, frequency-resolved signals are currently the most promising candidates as 2D IR probes of proteins, our long-term aspiration is to utilize C–D bonds introduced at native amino acid residues to directly interrogate the protein and eliminate concerns about perturbation. At this time, application of C–D bonds as probes even via linear spectroscopy is challenging and methodological improvement is needed to facilitate widespread adoption. Endeavors to apply 2D IR spectroscopy with C–D probes will benefit from selection of deuterated amino acids, such as ( $d_{10}$ )leucine, that have vibrations with transition dipole strengths larger than water, such that the fourth power enhancement of the 2D will facilitate their detection in aqueous protein samples.<sup>149,150</sup> Another possibility for enabling detection of C–D vibrations is to take advantage of coupling to stronger transitions, potentially those of the amide backbone, and detect the C–D vibrations via cross bands.<sup>150,151</sup> Because the amplitude of cross bands depends on the transition dipole strengths of both

vibrations, the cross bands arising from C–D bonds coupled to strong transitions could be more intense than the fundamental bands on the diagonal. Pursuit of these experiments, and moreover general progress in the development of biomolecular 2D IR spectroscopy, will require the combination of new biochemical methods for labeling proteins alongside recent instrumental developments, including dual frequency or broad band implementations of 2D IR experiments.

## ■ SUMMARY AND CONCLUSIONS

IR spectroscopy with frequency-resolved probes is emerging as a powerful approach to characterize specific parts of proteins while ensuring detection of all conformational heterogeneity, including states that undergo rapid interconversion. Notably, we detected multiple IR absorptions but single NMR resonances for the atoms associated with the same bonds in a peptide, thus uncovering multiple populated states by IR that are in fast exchange on the NMR time scale. In addition, we illustrated the potential rich insight provided by 2D IR spectroscopy. For example, 2D IR spectroscopy unequivocally revealed the presence of two underlying bands that provided evidence for a conformational change of P450cam. Comparison of linear and 2D diagonal spectra yielded the relative transition dipole strengths of the probe vibration for each state to enable rigorous quantification of the populations. Our studies also revealed how the measurement of spectral diffusion within different bands is useful for assigning them to the same or distinct conformational states, as well as providing insight into the dynamics among the underlying substates. Notably, the spectral diffusion measured for substrate complexes of P450cam suggested that the dynamics among substates within a populated conformation are central to the regioselectivity of catalysis, supporting the controversial idea that fast dynamics can play an important role in protein function.

The heterogeneity and dynamics at any specific location in a protein can be measured by 2D IR spectroscopy of frequency-resolved side chain probes, motivating our and others' efforts to develop better 2D IR probes. While 2D IR spectroscopy of unique sites in proteins has been demonstrated for a number of probes, further extension of the approach to multiple, distinct sites is needed to take full advantage of the method's potential for spatial resolution. Although the field of 2D IR spectroscopy has advanced in the past decades, the applications to proteins and other biomolecules remain relatively undeveloped. Further advances in methodology and application to additional systems and questions should generate a powerful, routine biophysical method that provides site-specific information with high temporal resolution. The new experimental tools of 2D IR spectroscopy open up the investigation of potentially unappreciated contributions to the functional biophysics of proteins.

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### Notes

The authors declare no competing financial interest.

## Biographies

Sashary Ramos completed her B.S. in chemistry at San Diego State University in 2015. She is currently a 4th year analytical chemistry student at Indiana University in Megan Thielges' group. Her doctoral research has focused on the development and application of multidimensional infrared spectroscopy for characterization of protein conformational dynamics in diverse biological systems.

Megan C. Thielges received her Ph.D. in biophysics at The Scripps Research Institute (La Jolla, CA) in 2009 and completed postdoctoral studies in multidimensional IR spectroscopy at Stanford University. In 2012, she joined the faculty at Indiana University and became an associate professor in 2018. Her primary research interests center around elucidating the conformations and dynamics of proteins and understanding how they are involved in function. In support of these goals, her research program aims to advance experimental methods of site-specific linear and multidimensional infrared spectroscopy for characterizing protein dynamics with high spatial and temporal detail.

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