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Extended timescale 2D IR probes of proteins: *p*-cyanoselenophenylalanine†

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Abstract

The importance of dynamics to the function of proteins is well appreciated, but the difficulty in their measurement impedes investigation into their precise role(s). 2D IR spectroscopy is a developing approach for the study of dynamics and has motivated efforts to develop spectrally resolved IR probe groups that enable its application for measuring the dynamics at specific sites in a protein. A challenge with this approach is that the timescales accessible are limited by the vibrational lifetimes of the probes. Toward development of better probes for 2D IR spectroscopy of protein dynamics, we report the characterization of *p*-cyano-seleno-phenylalanine (CNSePhe), a derivative of the well established IR probe *p*-cyano-phenylalanine (CNPhe), by FT IR, pump–probe, and 2D IR spectroscopy. The incorporation of the heavy Se atom decouples the CN vibration from the rest in the molecule. Although this leads to a reduction of the transition dipole strength, and thus a reduction in signal intensity, it also dramatically increases the vibrational lifetime, enabling collection of 2D IR spectra for analysis of molecular dynamics on much longer timescales. Interestingly, we also find that the lifetime for CNSePhe shows increased sensitivity to the presence of hydrogen bonding interactions with the CN, suggesting that the probe should be useful for interpretation of CN spectra and possibly for the study of solvation.

Introduction

Significant effort has been directed toward development of infrared (IR) reporter groups with vibrational frequencies (1900–2300 cm⁻¹) that provide spectrally isolated absorptions when incorporated into proteins.¹ The IR spectra of the probes reflect the nature of the specific protein environment at the site of incorporation due to their sensitivity to electrostatic, hydrogen bonding (H-bonding), and/or packing interactions. Even richer information is available from two-dimensional (2D) IR spectroscopy, which enables direct measurement of the temporal evolution of IR frequencies and the correlations among frequencies of one or multiple vibrational modes.^{2,3} 2D IR spectroscopy provides information, for example, about the dynamics of the probe's environment, chemical exchange, energy transfer, and coupling between modes.^{4–6} However, the experimental timescale accessible to 2D IR spectroscopy is limited by the vibrational excited state lifetimes of the IR probe employed. Thus, new IR probes with longer lifetimes are needed to explore dynamics on all of the timescales that may contribute to protein function. Towards

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the identification of probes with longer lifetimes, we report the synthesis and spectroscopic characterization of p-cyano-selenophenylalanine (CNSePhe) (Fig. 1A).

A variety of functional groups, including carbon–deuterium, cyano (CN), isocyano, azido, and metal-carbonyl complexes, have been appended to amino acid side chains and demonstrated or proposed as IR probes of local environments in proteins.^{7–13} In addition to the requirement that the probe's vibrational frequency be spectrally isolated from those of proteins, an optimal probe should provide strong signals over long timescales and be easily incorporated site-specifically in proteins with minimal perturbation to their structure or function. The intensity of linear IR signals varies quadratically with the strength of a probe's transition dipole moment, whereas the third-order signals measured to generate 2D IR spectra show a stronger, quartic dependence, which mandates the use of probes with large transition dipole moments. For example, the small transition dipole strengths of carbon–deuterium bonds, combined with the limited concentrations possible for protein samples, have so far prevented their use as 2D IR probes of proteins, despite that they are ideal in the respect that they are completely non-perturbative upon incorporation. In contrast, azido groups and metal-carbonyl complexes can be appended to amino acid side chains to provide intense 2D IR probes.^{14–17} However, azide lifetimes are very short (B1 ps), which limits their utility for studying dynamics. Metal-carbonyl complexes, on the other hand, have longer lifetimes (B20 ps) but are relatively large. Because of this, their perturbation to protein structure or function must be carefully evaluated, and their incorporation at certain locations, like the packed interior of proteins, may not be tolerated.

Nitriles are popular IR probes of proteins.^{8,9,18–27} Although they are prone to participate in H-bonding interactions, they are relatively small and have been demonstrated in many cases to introduce minimal perturbation to protein structure and function.^{20,23–27} CN groups are weaker probes than azides and metal carbonyl complexes, but unlike carbon–deuterium bonds, provide sufficiently intense signals to be feasible for 2D IR spectroscopy. Aryl nitriles such as p-cyanophenylalanine (CNPhe) typically show more intense absorptions than alkyl nitriles,^{28,29} which makes them preferable for characterization of protein samples at low concentration. Moreover, CNPhe is particularly attractive as it is a good structural mimic of Tyr or Phe, and its site-selective incorporation into proteins is well established via several approaches, including amber codon suppression and protein total or semi-synthesis.^{20,22,30,31} CNPhe has been successfully used as a 2D IR probe of protein dynamics in several studies of the autonomously folding peptide HP35,^{22,24,31} however, its short, ~4 ps, lifetime limits characterization of dynamics to a ~10 ps timescale.

Many studies have demonstrated that incorporation of a heavy atom, such as sulfur (S) or selenium (Se), to decouple a vibrational mode from others in the molecule, can dramatically lengthen vibrational lifetimes.^{32–34} It is possible to take advantage of this effect for the generation of amino acid analogs as potential extended timescale IR probes. For example, a cyanoseleno-(CNSe)-proline analog has been shown to have an extended CN lifetime (170–330 ps),³⁴ which has also been demonstrated recently for a benzyl-substituted CNSe Phe/Tyr analog (295 ps).³³ The increased lifetimes of these CNSe probes is promising, but a potential downside is the attachment of the IR chromophore to an alkyl carbon center, as such chromophores tend to generate weaker IR signals.²⁹ Considering this, we sought to

evaluate a Phe/Tyr analog with CNSe-substitution at an aryl carbon, which we reasoned might provide relatively intense IR signals. In addition, previous spectroscopic characterization of phenyl-selenocyanate found a long, B300 ps, lifetime for the CN vibration, suggesting that derivatization of Phe with CNSe would generate an extended timescale probe.³² The direct attachment of the CNSe to the aryl group also introduces only one additional atom in comparison to CNPhe (Fig. 1A), minimizing the differences from Phe and Tyr, such that it may also be minimally perturbative. Thus, we investigated CNSePhe as a possible substitute for the established 2D IR probe CNPhe.

Results and discussion

CNSePhe was synthesized from commercially available KSeCN and *p*-aminophenylalanine as previously reported,³⁵ and prepared at 50 mM in DMSO or H₂O, a polar aprotic and polar protic solvent, respectively. The UV/visible spectrum of the CNSePhe shows a large absorption at 236 nm characteristic of the CNSePhe chromophore (ESI†).³⁵ It is noted that in the presence of light or oxygen, irrespective of solution pH, CNSePhe was unstable and formed a red precipitate, consistent with previous reports.³⁵ However, when kept in the dark, an anaerobic solution of the chromophore was stable. Due to the possibility of CNSePhe degradation during solution preparation, 65 : 35 methanol : H₂O was used as a solvent for determination of the IR extinction coefficients so that the CN probe concentration could be determined independently by UV/vis spectroscopy with the known extinction coefficient at 236 nm.³⁵

We first characterized solutions of CNSePhe by FT IR spectroscopy. The linear FT IR spectra of CNSePhe show absorption bands associated with the CN stretch at 2160 cm⁻¹ and 2152 cm⁻¹ in H₂O and DMSO, respectively (Fig. 1B). The higher frequency found for the aqueous solution is attributed to H-bonding of the CN to H₂O, as has been well established in previous studies of CNPhe and other nitriles.^{23,26,27,36,37} The extinction coefficients for the CN stretches of CNSePhe and CNPhe were determined to be ~65 M⁻¹ cm⁻¹ and ~180 M⁻¹ cm⁻¹, respectively (65 : 35 methanol : H₂O solvent). The decreased absorption intensity upon incorporation of the heavy Se atom thus suggests that the CN transition dipole strength decreases by about 1.7-fold, anticipating a ~7.7-fold weaker 2D band, which is likely due to the decoupling of the CN vibration from the electron-rich aromatic ring. The CNSePhe is however a stronger probe in comparison to the alkyl cyanate of cyano-alanine (~20 M⁻¹ cm⁻¹ extinction coefficient). To our knowledge, no report of an extinction coefficient for an alkyl CNSe is available for direct comparison.

We then employed frequency-dispersed magic angle pump–probe spectroscopy to characterize the vibrational population dynamics of CNSePhe in the solutions of H₂O or DMSO. The pump–probe spectra show a positive band at the fundamental 0–1 transition frequency of CN, which reflects the pump-induced increase in probe transmission due to ground state bleaching and stimulated emission, and a negative band at the 1–2 transition frequency, which reflects decreased probe transmission due to excited state absorption (Fig. 2). The changes in pump transmission intensity at the 0–1 frequencies with increasing

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pump–probe delay for CNSePhe in H₂O and DMSO, as well as for CNPhe in H₂O, are plotted in Fig. 3. Fits of the pump–probe data to exponential decays give 763 ps and >200 ps for the vibrational lifetime for CNSePhe in the aqueous and DMSO solutions, respectively. Due to limited travel of our delay lines (200 ps), the decay time for the very slow vibrational relaxation of CNSePhe in DMSO was not well defined. The lifetimes measured for CNSePhe reflect 17–30 fold longer vibrational lifetimes relative to those of 4.6 and 6.0 ps for CNPhe in H₂O and DMSO, respectively.

The vibrational lifetimes of the CN chromophore can depend on intramolecular vibrational relaxation to other lower frequency modes of the CNPhe and CNSePhe molecules, with or without involvement of solvent modes, and/or intermolecular energy transfer to the solvent. The dramatic increase in vibrational lifetime of CNSePhe compared to CNPhe is attributed to the well-known heavy atom effect.^{38,39} Insertion of the massive Se atom is expected to reduce the anharmonic coupling between the CN mode and others in the molecule, thereby disrupting intramolecular vibrational relaxation pathways present for CNPhe. This effect has been observed with several nitriles. For example, a previous comparison of phenyl nitriles, which reflect the side chain functional group of the substituted Phe amino acids investigated here, found vibrational lifetimes of ~5.5, 84, and 282 ps for phenyl cyanate, thiocyanate, and selenocyanate, respectively, in deuterated chloroform solution.³² These lifetimes are comparable to our measured values for CNPhe and CNSePhe in DMSO. The heavy atom effect has been similarly demonstrated to lead to very long lifetimes for alkyl nitriles, including CNS- and CNSe-functionalized proline derivatives and benzyl CNS- and CNSe-functionalized analogs of Phe.^{33,34}

Previous efforts by our and other groups to develop extended lifetime probes have considered isotopomers of CNPhe.^{40,41} In H₂O, the CN lifetime for ¹³C¹⁵NPhe (8.7–10.5 ps) is ~2.0–2.5 fold longer than CNPhe (4.0–4.6 ps), whereas those for ¹³CNPhe (3.4–5.0 ps) and C¹⁵NPhe (0.90–2.2 ps) are roughly the same or even shorter. These are fairly small changes in lifetime and do not correlate with the small changes in mass, which indicates that the increase in mass from isotopic substitution does not substantially decouple the intramolecular vibrational relaxation pathways. In addition, the isotopomers are isoelectronic, so their Coulombic coupling to solvent is not expected to substantially vary. Rather, the differences in lifetimes have been attributed to the effect of isotopic substitution in tuning the CN frequency among the density spectrum of intramolecular acceptor modes.⁴⁰ This mechanism could also contribute to the difference in the CN lifetime for CNSePhe, which has a similar CN frequency as ¹³C¹⁵NPhe but different spectral density of acceptor modes (calculated spectra shown in ESI†). Resonance tuning however is not likely the dominant cause of the extremely long CN lifetime for CNSePhe. Whereas isotopic substitution only increases the lifetime by at most ~2.5-fold, Se incorporation increases the lifetime by more than an order of magnitude, despite that the two probes have similar CN frequencies and a large density of low frequency modes.

The solvent also influences the CN lifetime more with CNSePhe than with CNPhe, which could be due to its involvement in intramolecular energy redistribution to lower frequency modes and/or to direct intermolecular energy transfer from the CN to the solvent. For CNSePhe, the CN lifetime is significantly shorter in H₂O (70 ps) than DMSO (>200 ps). We

attribute this to the ability of H₂O, but not DMSO, to participate in an H-bonding interaction with the CN moiety, which is consistent with the 8 cm⁻¹ higher frequency of the absorption band for CNSePhe in H₂O compared to DMSO. A similar correlation between increased vibrational relaxation and the strength of H-bonding to solvent has been observed with other molecules.^{12,16,42–44} CNPhe can likewise interact via an H-bond with H₂O, but in contrast to CNSePhe, the CN lifetime for CNPhe shows only weak solvent dependence (4.6 and 6.0 ps in H₂O and DMSO, respectively). A likely explanation is that intramolecular relaxation in CNPhe is so efficient that intermolecular relaxation does not substantially compete. The small solvent dependence of the CN lifetime for CNPhe could be due to the solvent's influence on intramolecular relaxation *via* its involvement in the coupling of the CN to accepting modes. In contrast, the decoupling of the CN to the rest of the molecule by the Se heavy atom of CNSePhe disrupts the efficient intramolecular relaxation pathways present in CNPhe, such that the contribution of intermolecular relaxation mediated by the H-bond becomes significant. Consistent with this, the lifetime for the ion SeCN⁻, a rough model for the limit of a CN completely decoupled from the aryl moiety of CNSePhe, similarly shows large solvent dependence (3.8 ps and 112 ps in H₂O and DMSO, respectively).⁴²

The sensitivity of the lifetime of CNSePhe to H-bonding interactions could be exploited for assessing their presence in proteins. A well-known challenge in the interpretation of spectral changes of CN probes is deconvoluting the effects of long-range electrostatic interactions and specific local interactions like H-bonding. Several approaches have been proposed to aid in spectral interpretation, including characterization of the temperature-dependence,²⁶ comparison with NMR chemical shifts,⁴⁵ and a range of approaches based on theoretical modelling.^{27,46–48} However, these approaches are all challenging with many proteins due to protein size or stability. The data presented here suggest that CNSePhe could provide a less ambiguous probe of solvation, with the observation of short lifetimes indicative of H-bonding, and conversely, longer lifetimes indicative of the absence of H-bonds. The presence of solvent-assisted vibrational relaxation due to H-bonding has been similarly proposed as an indicator of H-bonding interactions with metal carbonyl-based protein probes.^{16,49}

The longer lifetime of CNSePhe should also make it useful as an extended timescale probe for 2D IR spectroscopy. 2D IR experiments involve application of three, temporally controlled pulses to the sample to generate 2D correlation spectra.² The 2D spectra associate the initial vibrational frequencies (ω_1) of the ensemble during the time interval between the first two pulses with their frequencies (ω_3) measured following a waiting time (T_w), the time between the second and third pulses (Fig. 4). Thus, the 2D spectra report on the connections among the frequencies of the vibrations in a system, and thereby provide a direct measure of the coupling of modes, vibrational energy transfer among modes, or for a single mode, fluctuations during T_w among probe environments or between different chemical or conformational states of the molecule. For example, the T_w -dependent changes in the 2D lineshapes and/or cross bands of an IR probe can be analyzed to follow the dynamics of a protein environment as it samples its distribution of conformational states.^{4–6,14,22,31,50} However, in a 2D IR experiment the information about the initial frequencies is stored during T_w *via* depleted ground state and excess excited state populations, and the ablation of the population states through vibrational relaxation leads to loss of the information, and consequently the decay of 2D IR bands. As a result, the longest T_w s for which 2D IR spectra

can be acquired, and so the timescale experimentally accessible for 2D IR spectroscopy to measure dynamics, are limited by the vibrational lifetime of the IR probe.

To evaluate CNSePhe as an extended timescale 2D IR probe, 2D IR spectroscopy was performed for 50 mM CNSePhe and CNPhe in H₂O and DMSO. Example 2D spectra taken with different T_w s for CNPhe and CNSePhe in H₂O, as well as for CNSePhe in DMSO, are shown in Fig. 4. At short T_w s, the spectra show elongation along the diagonal that reflects the inhomogeneity in frequencies due to interaction of the CN with the solvent molecules. With increasing T_w , the spectra become less elongated due to the fluctuation of the solvent-probe interactions. By a T_w of 2 ps, the 2D spectra for the samples in water are nearly symmetrical due to the rapid dynamics of H-bond interactions of water.⁵¹ In addition, the water solvent itself strongly absorbs the mid-IR pulses, leading to off-diagonal spectral features associated with water heating.⁴¹ For CNPhe, the 2D spectra at T_w of 10 ps (Fig. 4A) already show line width distortion due to reduction in signal strength as a result of the fast lifetime of CNPhe. In contrast to CNPhe, for CNSePhe the 2D IR spectra taken under equivalent experimental conditions at the same probe concentration can be obtained at longer T_w s (Fig. 4B), despite the lower transition dipole strength of CNSePhe. Acquisition of 2D IR spectra was possible for CNSePhe in DMSO with the very long T_w of 140 ps (Fig. 4C), due to a combination of the reduced interfering background signals and the longer lifetime of the CNSePhe in the aprotic solvent. The latter suggests that when H-bonds are excluded, CNSePhe could provide an even longer timescale probe of protein dynamics. The T_w -dependent changes in 2D lineshapes are much slower for the DMSO than the H₂O solvent, indicating that the dynamics of the DMSO solvent structure around the CN probe fluctuates much more slowly than the dynamics of H-bonding with water.

Conclusions

We have characterized CNSePhe in protic and aprotic polar solvents by linear, pump probe, and 2D IR spectroscopy to demonstrate its potential as an extended lifetime probe for 2D IR studies of proteins. The incorporation of the heavy Se atom to decouple the CN vibration does reduce the transition dipole strength, decreasing the intensity of spectroscopic signals, but also dramatically increases the vibrational lifetime, enabling collection of 2D IR spectra for analysis of molecular dynamics on longer timescales. In addition, the lifetime for CNSePhe shows remarkable sensitivity to the presence of H-bonding, suggesting that it could be a useful probe of solvation. Despite the attractive features of CNSePhe, a potential practical issue with its application as a probe for proteins is its oxygen sensitivity. Similar problems with chemical stability have been encountered previously for other selenocyanates.³³ Our attempts to incorporate CNSePhe into a protein using the amber codon approach with tRNA/tRNA synthetase pairs evolved for CNPhe under aerobic conditions resulted in an expressed protein, but the mass was approximately 12 Da lower than expected for CNSePhe introduction (ESI⁺), indicating that chemical modification of CNSePhe occurs in the cellular environment. Further efforts toward the incorporation of CNSePhe into proteins via this or alternate approaches, for example, under anaerobic conditions, are underway. Once incorporated, sequestration in a protein environment could protect the probe from oxygen or other reactive species, thus improving the stability of the probe. Another potential probe anticipated to have a long CN lifetime, albeit lower transition dipole strength, is cyano-

selenocysteine. Conveniently, protected selenocysteine is commercially available for use in peptide synthesis. In either case, the dramatic extension in 2D IR experimental timescale afforded by the large increase in lifetime of CNSe makes it a promising probe that merits further development for its potential to advance our understanding of protein dynamics.

Experimental

Preparation of CNSePhe

CNSePhe was synthesized according to published procedures from *p*-amino-phenylalanine and KSeCN and purified by reverse-phase HPLC (ESI[†]).³⁵ Solutions of B50 mM CNSePhe or CNPhe were prepared by dissolving the solid in Ar-purged H₂O (pH < 1) or DMSO. To determine the extinction coefficient for the IR bands of the CN, solutions of CNSePhe in 65/35% methanol/H₂O were characterized by both UV/vis and FT IR, and an extinction coefficient of 7.2 mM⁻¹ cm⁻¹ at 240 nm was used to determine concentration.³⁵ For CNPhe and CNAla, solutions at known concentration were directly prepared. The solutions were loaded between two 1 mm thick CaF₂ windows separated by a 38.1 μm or 127 μm Teflon spacer for FT IR spectroscopy, or a 76.1 μm spacer for pump-probe and 2D IR spectroscopy. The CNSePhe was briefly exposed to air when weighing the solid and loading the IR cell, but otherwise prepared anaerobically and stored in a sealed vial under Ar.

FT IR spectroscopy

FT IR spectra were acquired using an Agilent Cary 670 FT IR spectrometer with a liquid N₂-cooled MCT detector. Absorption spectra were generated from transmission spectra of CNSePhe, CNPhe, or cyano-alanine in H₂O, DMSO and/or 65 : 35 methanol : H₂O and reference transmission spectra of the pure solvents. A slowly varying baseline was removed by subtraction of a polynomial fit of the background from the spectra.

Pump probe spectroscopy

Pump and probe beams of ~180 fs pulses centered at 2160 cm⁻¹ generated from an optical parametric amplifier pumped by a Ti : Sa oscillator/regenerative amplifier (Spectra Physics) were focused at the sample, the probe beam was spectrally dispersed with a spectrograph onto a 32-element MCT array detector, and the pump-induced change in the transmitted probe beam intensity was measured as a function of the pump-probe delay. A waveplate/polarizer pair was placed before and a polarizer after the sample, all set at magic angle, to exclusively measure population dynamics.⁵² Complete experimental details are provided in ESI.[†] For the aqueous samples, a broad combination bend-libration band of H₂O occurs at ~2130 cm⁻¹ and is excited by our laser pulses. Intra and intermolecular vibrational relaxation of water modes are very fast relative that of CNSePhe,^{53,54} so do not complicate analysis of the long CN pump-probe signal decay. However, a long-lasting heating-induced signal from water occurs, and only decays on long ns timescales. For the aqueous solution data, the contribution from the persistent water heat signal was removed by subtraction of the spectrum taken at the longest delay time (200 ps) from those for all of the shorter delay times. The DMSO solvent has no absorbance in the spectral region of the CN probe.

2D IR spectroscopy

2D IR experiments were performed as described previously.^{50,55} Briefly, the mid-IR beam was split into three separate beams, which were separately delayed and focused into the sample in a BOXCARS geometry. To generate a 2D IR spectrum for a given T_w , the time between the first two pulses, τ , was scanned, with the time interval between the second and third pulses set at T_w . The third order signal emitted by the sample in the phased-matched direction was overlapped with a fourth mid-IR beam, the local oscillator, for heterodyned detection. The combined beams were frequency-dispersed by a spectrograph onto a 32-element MCT array detector. Frequency-resolved detection directly generates the ω_3 (probe) axis of the 2D spectrum, whereas Fourier transformation of the interferograms measured along τ generate the ω_1 (pump) axis of the 2D spectrum. Additional detail about experimental set up and data processing are available in ESI.†

Supplementary Material

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Notes and references

1. Kim H and Cho M, Chem. Rev, 2013, 113, 5817–5847. [PubMed: 23679868]
2. Hamm P and Zanni M, Concepts and Methods of 2D Infrared Spectroscopy, Cambridge University Press, 2011.
3. Cho M, Two-Dimensional Optical Spectroscopy, CRC Press, Boca Raton, 2009.
4. Le Sueur AL, Horness RE and Thielges MC, Analyst, 2015, 140, 4336–4349. [PubMed: 26007625]
5. Fayer MD, Annu. Rev. Phys. Chem, 2009, 60, 21–38. [PubMed: 18851709]
6. Kim YS and Hochstrasser RM, J. Phys. Chem. B, 2009, 113, 8231–8251. [PubMed: 19351162]
7. Chin JK, Jimenez R and Romesberg FE, J. Am. Chem. Soc, 2001, 123, 2426–2427. [PubMed: 11456893]
8. Getahun Z, Huang CY, Wang T, Leon BD, DeGrado WF and Gai F, J. Am. Chem. Soc, 2003, 125, 405–411. [PubMed: 12517152]
9. Fafarman AT, Webb LJ, Chuang JI and Boxer SG, J. Am. Chem. Soc, 2006, 128, 13356–13357. [PubMed: 17031938]
10. Oh KI, Lee JH, Joo C, Han H and Cho M, J. Phys. Chem. B, 2008, 112, 10352–10357. [PubMed: 18671422]
11. Zimmermann J, Thielges MC, Yu W, Dawson PE and Romesberg FE, J. Phys. Chem. Lett, 2011, 2, 412–416.
12. King JT and Kubarych KJ, J. Am. Chem. Soc, 2012, 134, 18705–18712. [PubMed: 23101613]
13. Maj M, Ahn C, Kossowska D, Park K, Kwak K, Han H and Cho M, Phys. Chem. Chem. Phys, 2015, 17, 11770–11778. [PubMed: 25869854]
14. Thielges MC, Axup JY, Wong D, Lee HS, Chung JK, Schultz PG and Fayer MD, J. Phys. Chem. B, 2011, 115, 11294–11304. [PubMed: 21823631]
15. Bloem R, Koziol K, Waldauer SA, Buchli B, Walser R, Samatanga B, Jelesarov I and Hamm P, J. Phys. Chem. B, 2012, 116, 13705–13712. [PubMed: 23116486]

16. King JT, Ross MR and Kubarych KJ, J. Phys. Chem. B, 2012, 116, 3754–3759. [PubMed: 22376278]
17. Woys AM, Mukherjee SS, Skoff DR, Moran SD and Zanni MT, J. Phys. Chem. B, 2013, 117, 5009–5018. [PubMed: 23537223]
18. Andrews SS and Boxer SG, J. Phys. Chem. A, 2000, 104, 11853–11863.
19. Waegle MM and Gai F, J. Phys. Chem. Lett, 2010, 1, 781–786. [PubMed: 20436926]
20. Zimmermann J, Thielges MC, Seo YJ, Dawson PE and Romesberg FE, Angew. Chem., Int. Ed, 2011, 50, 8333–8337.
21. Walker DM, Hayes EC and Webb LJ, Phys. Chem. Chem. Phys, 2013, 15, 12241–12252. [PubMed: 23771025]
22. Urbanek DC, Vorobyev DY, Serrano AL, Gai F and Hochstrasser RM, J. Phys. Chem. Lett, 2010, 1, 3311–3315. [PubMed: 21132120]
23. Fafarman AT and Boxer SG, J. Phys. Chem. B, 2010, 114, 13536–13544. [PubMed: 20883003]
24. Chung JK, Thielges MC and Fayer MD, J. Am. Chem. Soc, 2012, 134, 12118–12124. [PubMed: 22764745]
25. Bazewicz CG, Lipkin JS, Smith EE, Liskov MT and Brewer SH, J. Phys. Chem. B, 2012, 116, 10824–10831. [PubMed: 22909326]
26. Adhikary R, Zimmermann J, Dawson PE and Romesberg FE, Anal. Chem, 2015, 87, 11561–11567. [PubMed: 26523838]
27. Basom EJ, Maj M, Cho M and Thielges MC, Anal. Chem, 2016, 88, 6598–6606. [PubMed: 27185328]
28. Suydam IT and Boxer SG, Biochemistry, 2003, 42, 12050–12055. [PubMed: 14556636]
29. Maj M, Ahn C, Blasiak B, Kwak K, Han H and Cho M, J. Phys. Chem. B, 2016, 120, 10167–10180. [PubMed: 27532890]
30. Schultz KC, Supekova L, Ryu Y, Xie J, Perera R and Schultz PG, J. Am. Chem. Soc, 2006, 128, 13984–13985. [PubMed: 17061854]
31. Chung JK, Thielges MC and Fayer MD, Proc. Natl. Acad. Sci. U. S. A, 2011, 108, 3578–3583. [PubMed: 21321226]
32. Bian H, Li J, Wen X and Zheng J, J. Chem. Phys, 2010, 132, 184505.
33. Levin DE, Schmitz AJ, Hines SM, Hines KJ, Tucker MJ, Brewer SH and Fenlon EE, RSC Adv, 2016, 43, 36231–36237. [PubMed: 27114820]
34. Park KH, Jeon J, Park Y, Lee S, Kwon H-J, Joo C, Park S, Han H and Cho M, J. Phys. Chem. Lett, 2013, 4, 2105–2110.
35. Ganther HE, Bioorg. Med. Chem, 2001, 9, 1459–1466. [PubMed: 11408164]
36. Choi JH, Oh KI, Lee H, Lee C and Cho M, J. Chem. Phys, 2008, 128, 134506. [PubMed: 18397076]
37. Lee H, Choi JH and Cho M, Phys. Chem. Chem. Phys, 2010, 12, 12658–12669. [PubMed: 20830379]
38. Rogers P, Montague DC, Frank JP, Tyler SC and Rowland FS, Chem. Phys. Lett, 1982, 89, 9–12.
39. Lederman SM, Lopez V, Fairen V, Voth GA and Marcus RA, Chem. Phys, 1989, 139, 171–184.
40. Rodgers JM, Zhang W, Bazewicz CG, Chen J, Brewer SH and Gai F, J. Phys. Chem. Lett, 2016, 7, 1281–1287. [PubMed: 26990401]
41. Sueur ALL, Ramos S, Ellefsen J, Cook S and Thielges MC, Unpublished, 2017.
42. Lenchenkov V, She C and Lian T, J. Phys. Chem. B, 2006, 110, 19990–19997. [PubMed: 17020387]
43. Ohta K and Tominaga K, Chem. Phys. Lett, 2006, 429, 136–140.
44. Ohta K and Tominaga K, Chem. Phys, 2007, 341, 310–319.
45. Fafarman AT, Sigala PA, Herschlag D and Boxer SG, J. Am. Chem. Soc, 2010, 132, 12811–12813. [PubMed: 20806897]
46. Blasiak B, Ritchie AW, Webb LJ and Cho M, Phys. Chem. Chem. Phys, 2016, 18, 18094–18111. [PubMed: 27326899]
47. Choi JH, Raleigh D and Cho M, J. Phys. Chem. Lett, 2011, 2, 2158–2162. [PubMed: 22389750]

48. Cho M, J. Chem. Phys, 2009, 130, 094505. [PubMed: 19275407]
49. King JT, Baiz CR and Kubarych KJ, J. Phys. Chem. A, 2010, 114, 10590–10604. [PubMed: 20831231]
50. Basom EJ, Spearman JW and Thielges MC, J. Phys. Chem. B, 2015, 119, 6620–6627. [PubMed: 25955684]
51. Asbury JB, Steinel T, Kwak K, Corcelli SA, Lawrence CP, Skinner JL and Fayer MD, J. Chem. Phys, 2004, 121, 12431–12446. [PubMed: 15606264]
52. Tan HS, Piletic IR and Fayer MD, J. Opt. Soc. Am. B, 2005, 22, 2009–2017.
53. Steinel T, Asbury JB, Zheng J and Fayer MD, J. Phys. Chem. A, 2004, 108, 10957–10964. [PubMed: 19096727]
54. Kuo CH and Hochstrasser RM, Chem. Phys, 2007, 341, 21–28.
55. Park S, Kwak K and Fayer MD, Laser Phys. Lett, 2007, 4, 704–718.

Fig. 1.
(A) Chemical structure of CNSePhe. (B) FT IR absorbance spectra of CNSePhe in water (red) and DMSO (black).

Fig. 2.
Time-dependent pump–probe spectra of CNSePhe in H₂O (left) and DMSO (right).

Fig. 3. Time-dependent change in transmission at the fundamental CN frequencies (points) and exponential fits (lines) for CNPhe in H₂O (black), CNSePhe in H₂O (red), and CNSePhe in DMSO (blue).

Fig. 4. 2D IR spectra of (A) CNPhe in H₂O (B) CNSePhe in H₂O and (C) CNSePhe in DMSO.