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General Strategy for the Bioorthogonal Incorporation of Strongly Absorbing, Solvation-Sensitive Infrared Probes into Proteins

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Supporting Information

ABSTRACT: A high-sensitivity metal-carbonyl-based IR probe is described that can be incorporated into proteins or other biomolecules in very high yield via Click chemistry. A two-step strategy is demonstrated. First, a methionine auxotroph is used to incorporate the unnatural amino acid azidohomoalanine at high levels. Second, a tricarbonyl (η5-cyclopentadienyl) rhenium(I) probe modified with an alkynyl linkage is coupled via the Click reaction. We demonstrate these steps using the C-terminal domain of the ribosomal protein L9 as a model system. An overall incorporation level of 92% was obtained at residue 109, which is a surface-exposed residue. Incorporation of the probe into a surface site is shown not to perturb the stability or structure of the target protein. Metal carbonyls are known to be sensitive to solvation and protein electrostatics through vibrational lifetimes and frequency shifts. We report that the frequencies and lifetimes of this probe also depend on the isotopic composition of the solvent. Comparison of the lifetimes measured in H2O versus D2O provides a probe of solvent accessibility. The metal carbonyl probe reported here provides an easy and robust method to label very large proteins with an amino-acid-specific tag that is both environmentally sensitive and a very strong absorber.

INTRODUCTION

Infrared (IR) spectroscopy is an attractive method for monitoring the environment of biomacromolecules because IR modes are affected by hydrogen bonding, solvation, and the electrostatic environment.1−6 In the past few years, because of the efforts of Skinner and coworkers as well as other researchers, the correlation between environmental statics and vibrational frequencies has been developed to such an extent that it is now possible to quantitatively calculate IR spectra from molecular dynamics simulations using rigorous protocols, thereby allowing for atomic-level interpretations of experimental spectra.7−10 As a result, there is considerable interest in determining structure and probing the environment at specific locations in proteins using IR, which can be accomplished with isotopic substitution or non-natural labels at specific residues. Isotopic labeling with 13C18O backbone labels or C−D groups in side chains can provide site-specific resolution, and these probes are structurally nonperturbative, but it can be difficult to incorporate a single label into a protein.11−17 Small, unnatural amino acids are also commonly used as IR probes, such as azidohomoalanine, p-cyanophenylalanine, p-azidophenylalanine, or thiocyanates, with the latter being introduced via modification of cysteine side chains.18−26 While these probes are small enough to be minimally perturbative and absorb in an otherwise transparent region of the IR spectrum, they have modest extinction coefficients, so that millimolar protein concentrations are usually required. Many proteins are not soluble at such high concentrations, and aggregation can be a major problem. Thus, their use is limited to stable, highly soluble proteins that express well.

We and others have taken an alternative approach and traded for a strong extinction coefficient by using metal carbonyls as the vibrational tag.27,28 Metal carbonyls offer a very attractive compromise between sensitivity and size; they are larger than standard probes based on unnatural amino acids but are still relatively small and have significantly higher extinction coefficients than standard unnatural amino-acid-based probes or isotopic labels. The frequencies of the metal carbonyl stretches fall in a transparent region of the protein IR spectrum and are well-resolved from water and protein modes. The carbonyl stretch frequencies are sensitive to the electrostatic environment, and their lifetimes are sensitive to solvation. They have been used to study the electrostatic environment and dynamics of a range of biological and nonbiological systems including the dynamics of lipid bilayers, the active site of cytochrome c oxidase, the hydration of ubiquitin and alpha-synuclein, and the dynamics of organic polymers and glasses.27,29−32 A desirable property is their strong extinction coefficient, on the order of 4100 M−1 cm−1, which is considerably larger than that of commonly used probes such as the nitrile (cyano) group, which has an extinction coefficient | 7953

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on the order of 20–240 M\(^{-1}\) cm\(^{-1}\).\(^{33,34}\) The weak oscillator strength of many other probes leads to an even poorer relative signal for higher order spectroscopies, such as two-dimensional infrared (2D IR) spectroscopy, where the signal scales with the fourth power of the transition dipole.\(^35\) Thus, metal carbonyl tags enable solvation and environment studies at much lower protein concentrations than are possible with many other vibrational tags.

Metal carbonyls have previously been incorporated into proteins in a number of different ways. They have been attached to lysine, histidine, and cysteine residues with a variety of linkages, including our previous work with a methanethiosulfonate derivative to cysteine.\(^27,28,34,36\)–\(^43\) Linkage via cysteine is the most common way to attach non-native chromophores, but cysteine cannot always be incorporated into proteins because the addition of a cysteine residue can interfere with proper oxidative refolding, and introduction of a cysteine can sometimes lead to undesired disulfide-linked aggregates. A bioorthogonal strategy will be required if multiple probes need to be incorporated specifically. Here we present a simple and general bioorthogonal approach for the selective incorporation of metal carbonyl IR probes in a quantitative yield. The method involves the recombinant incorporation of azidohomoalanine using a methionine auxotrophic \(E.\ coli\) strain together with a newly designed metal carbonyl compound that is attached to the protein via the copper(1)-catalyzed azide–alkyne cycloaddition reaction, or Click chemistry.

Our strategy is outlined in Figure 1. The protein of interest is modified by the selective incorporation of azidohomoalanine (Aha), the azido analogue of Met. Aha can be recombinantly incorporated into proteins using Met auxotrophs.\(^44,45\) The advantage of this approach is that proteins generally express well in Met auxotrophs, although expression yields are usually lower than with nonauxotrophs, and very high levels of Aha incorporation are obtainable. The Click reaction is usually quantitative, resulting in a high level of incorporation of the probe. The disadvantage of the strategy is that the protein cannot contain other Met residues if single specific labeling is desired. However, Met is a relatively rare amino acid and can often be substituted by Leu. Aha can also be incorporated into polypeptides by Fmoc solid-phase peptide synthesis without the need for a protecting group on the azido side chain.\(^46\) We illustrate the approach using the C-terminal domain of the ribosomal protein L9 (CTL9) as a model system (Figure 1). This 92 residue protein forms a mixed \(\alpha−\beta\) structure and folds cooperatively.\(^47,48\) We targeted a surface site, residue 109, located in the loop between the second \(\alpha\) helix and the second \(\beta\) strand.

**EXPERIMENTAL SECTION**

**Protein Expression and Purification.** A copy of the wild-type CTL9 gene in the pET3a plasmid was used for mutagenesis. The codon for lysine at position 109 was mutated to that coding for methionine (ATG) using the QuickChange site-directed mutagenesis protocol from Stratagene. \(E.\ coli\) B834 cells were transformed with pET3a-CTL9-K109M. Cells were grown in M9 medium to an optical density (OD\(_{600}\)) of 0.8 to 1.0 at 37 °C, then harvested and resuspended twice in M9 salt solution. Cells were then resuspended in 1 L of M9 medium supplemented with 17 amino acids (no tyrosine, cysteine, or methionine) and 50 mg of azidohomoalanine (Aha). Cells were lysed, and protein was purified from the supernatant by cation-exchange chromatography, followed by reverse-phase HPLC on a C8 preparative column. For HPLC purification, an A-B
gradient system was used in which buffer A consisted of 0.045% (v/v) solution of hydrochloric acid (HCl) in water, and buffer B consisted of 90% (v/v) acetonitrile, 10% (v/v) water, and 0.045% (v/v) HCl. HCl was used as the ion pairing agent instead of TFA as residual TFA can interfere with IR measurements.

Preparation of the Metal Carbonyl Probe. General Procedures. All procedures were carried out under a nitrogen atmosphere using Schlenk line techniques. Glassware and needles were oven-dried prior to use. DMF was vacuum-distilled with CaC2. THF was used as received from a solvent purification system. Re(CO)5 was a gift from Chuck Casey and oxidized to CpRe(CO)3 using a previously reported method.33 All other chemicals were obtained from Sigma-Aldrich.

\[(\eta^5-I\text{C}_5\text{H}_5)\text{Re}(\text{CO})_3, A \text{ solution of} \text{CpRe}(\text{CO})_3 (780.9 \text{ mg}) \text{ in} \text{THF} \text{ (10 mL)} \text{ was cooled in a dry ice/acetone bath.} \text{n-BuLi (1.6 mL) was added after 10 min, after which the solution was stirred for 20 min.} \text{I}_2(\text{CH}_2)_{2} (656 \text{ mg}) \text{ was quickly added. The solution was brought to room temperature and stirred for 30 min. The material was purified with a silica column with hexanes/ benzene to yield 810.9 mg of (\eta^5-I\text{C}_5\text{H}_5)\text{Re}(\text{CO})_3 \text{ in 76% yield.}}\]

\[(\eta^5-H\text{C}≡\text{CC}_5\text{H}_4)\text{Re}(\text{CO})_3, \text{ the synthesis of} (\eta^5-H\text{C}≡\text{CC}_5\text{H}_4)\text{Re}(\text{CO})_3 \text{ follows a method previously reported.}49 (\eta^5-I\text{C}_5\text{H}_5)\text{Re}(\text{CO})_3 (810.9 \text{ mg}) \text{ was added to a flask under an argon atmosphere. To that, tributylethynylstannane (TBES, 1.6 mL) was added over 10 min, after which the solution was stirred for 20 min. I}_2(\text{CH}_2)_{2} \text{ (656 mg) was quickly added. The solution was brought to room temperature and stirred for 30 min. The material was purified with a silica column with hexanes/ benzene to yield 810.9 mg of (\eta^5-I\text{C}_5\text{H}_5)\text{Re}(\text{CO})_3 \text{ in 76% yield.}}\]

Site-Specific Protein Labeling. CTL9-K109Aha was dissolved in labeling buffer, which consisted of 20 mM MOPS and 150 mM NaCl. Protein concentration was determined by absorbance at 280 nm. A stock solution of the labeling agent consisted of 20 mM MOPS and 150 mM NaCl solution was made at pH 7.0 and 25 °C. Thermal denaturation data were fit to the following expression:

\[
\Delta G^o(T) = \Delta H^o(T)\left(1 - \frac{T}{T_m}\right) - \Delta C_P^o \left(T_m - T\right) + T \ln\left(\frac{T}{T_m}\right)
\]

where \(T_m\) is the midpoint temperature, \(T\) is the temperature, \(\Delta H^o(T_m)\) is the change in enthalpy for the unfolding reaction at \(T_m\) and \(\Delta C_P^o\) is the change in heat capacity, which was set to 1.07 kcal-mol\(^{-1}\)-deg\(^{-1}\), as determined by previous experiments.47

CD monitored urea denaturation experiments were performed on an AVIV 202SF spectrophotometer. Samples were 20 μM protein in 10 mM MOPS and 150 mM NaCl. Experiments were performed at pH 7.0 and 25 °C. The concentration of urea was determined by measuring the refractive index. Denaturation curves were fit to the equation:

\[
f = a_i + b_i[\text{denaturant}] + (a_d + b_d[\text{denaturant}]) \exp\left(-\frac{\Delta G^o_{\text{denaturant}}}{RT}\right)
\]

\[
\Delta G^o_{\text{denaturant}} = \Delta G^o_{\text{H}_2\text{O}} - m[\text{denaturant}]
\]
the intercept and $b_d$ is the slope of the curve in the post-transition region.

**FTIR Spectroscopy.** FTIR spectra were recorded on a Bruker Tensor 27 FTIR spectrometer equipped with a liquid-nitrogen-cooled mercury cadmium telluride (MCT) detector. The spectra of the free metal carbonyl compound in various solvents were the result of 16 scans, and the concentration of compound was 1 mM. The protein spectra at pH 6.6 and pH 1.6 were the result of 256 scans, and the protein concentration was $\sim 300 \mu M$. Spectra were recorded at a resolution of 1 cm$^{-1}$ using a Bruker BioATR II cell. The effective path length of the cell was approximately 6–8 μm. All experiments were performed at 20 °C.

**2D IR Spectroscopy.** All samples were prepared in 10 mM MOPS and 150 mM NaCl in H$_2$O at pH 7.0 or 10 mM dMOPS and 150 mM NaCl in D$_2$O and measured at an 800 μM protein concentration. Femtosecond mid-IR pulses ($\sim 1970$ cm$^{-1}$ center frequency, 200 cm$^{-1}$ bandwidth) were generated as follows. The 800 nm output of a 2 kHz Ti:sapphire KMLabs Wyvern regenerative amplifier (3.2 mJ, 100 fs pulses) is used to pump a home-built optical parametric amplifier (OPA) followed by difference frequency generation to generate 4 μJ pulses of mid-IR light centered around 5 μm. The mid-IR light is split into pump (97%) and probe (3%) beams. The pump beam is sent to a pulse shaper, which creates pulse pairs with controlled time delays and phases on a shot-to-shot basis.$^{51,52}$ The pulse delay was scanned from 0 to 4000 fs in 40 fs steps with a rotating frame frequency of 1850 cm$^{-1}$. The output from the pulse shaper and the probe was focused onto the sample using a pair of off-axis parabolic mirrors. The waiting time was scanned using a motorized translation stage that incremented in steps of 500 fs to 1 ps. The probe pulse was detected on a 64-channel MCT array after passing through a monochromator.

### RESULTS AND DISCUSSION

Incorporation of Aha is well-documented, and incorporation levels on the order of 90 to 95% are often reported. The highest level of incorporation is desirable, but a modest fraction of protein containing Met instead of Aha can be tolerated, at least for single labeling applications, because the Met-containing protein will be spectroscopically silent if no label is bound. We obtained a 92% level of incorporation using the B834 E. coli strain with CTL9 under control of the lac operon, as judged by liquid chromatography/mass spectroscopy (Figure 1), and 16 scans, and the concentration of compound was 1 mM. Spectra were recorded at a resolution of 1 cm$^{-1}$ using a Bruker BioATR II cell. The effective path length of the cell was approximately 6–8 μm. All experiments were performed at 20 °C.

2D IR Spectroscopy. All samples were prepared in 10 mM MOPS and 150 mM NaCl in H$_2$O at pH 7.0 or 10 mM dMOPS and 150 mM NaCl in D$_2$O and measured at an 800 μM protein concentration. Femtosecond mid-IR pulses ($\sim 1970$ cm$^{-1}$ center frequency, 200 cm$^{-1}$ bandwidth) were generated as follows. The 800 nm output of a 2 kHz Ti:sapphire KMLabs Wyvern regenerative amplifier (3.2 mJ, 100 fs pulses) is used to pump a home-built optical parametric amplifier (OPA) followed by difference frequency generation to generate 4 μJ pulses of mid-IR light centered around 5 μm. The mid-IR light is split into pump (97%) and probe (3%) beams. The pump beam is sent to a pulse shaper, which creates pulse pairs with controlled time delays and phases on a shot-to-shot basis.$^{51,52}$ The pulse delay was scanned from 0 to 4000 fs in 40 fs steps with a rotating frame frequency of 1850 cm$^{-1}$. The output from the pulse shaper and the probe was focused onto the sample using a pair of off-axis parabolic mirrors. The waiting time was scanned using a motorized translation stage that incremented in steps of 500 fs to 1 ps. The probe pulse was detected on a 64-channel MCT array after passing through a monochromator.

Figure 2. Urea-induced unfolding experiments demonstrate that the probe does not significantly modify the stability of the protein. Urea-induced unfolding experiments were conducted by monitoring the CD signal at 222 nm. (A) Wild-type CTL9 and (B) metal carbonyl-labeled CTL9. The solid lines are the best fit to eq 2. Protein samples were in 10 mM MOPS, 150 mM NaCl at pH 7.0 at 25 °C. Protein concentration was 20 μM.

values, although the slopes of the pretransition baselines are slightly different (Table 1, Figure 2). The $m$ value is believed to be related to the change in solvent-accessible surface area between the folded and unfolded states. The observation of similar $m$ values is additional evidence that the probe is nonperturbing. The midpoint of the thermal unfolding transition was determined by CD-monitored unfolding and is similar for the two variants, as is the value of $\Delta H^{\circ}$. The close agreement between the values of $\Delta H^{\circ}$ indicates that, as expected, the probe does not perturb the packing of the protein.

The fact that the probe does not perturb the stability of the protein or the cooperativity of its folding, as judged by the $m$ value for denaturant-induced unfolding and by $\Delta H^{\circ}$, is a significant observation. The metal carbonyl complex is very hydrophobic, and versions that are attached via long flexible
linkers may potentially interact with nearby hydrophobic patches on the protein surface and could thus perturb protein stability and modulate protein ligand interactions, which rely on binding to hydrophobic regions. The Click reaction leads to a rigid triazole linkage that will help to project the probe away from the protein and thereby minimize potentially deleterious interactions with the protein surface.

The frequency of the carbonyl vibrational mode of the metal complex is sensitive to the polarity of the environment, as illustrated in Figure 3A, which displays spectra of the rigid triazole linkage that will help to project the probe away from the protein and thereby minimize potentially deleterious interactions with the protein surface. The frequency of the carbonyl vibrational mode of the metal complex is sensitive to the polarity of the environment, as illustrated in Figure 3A, which displays spectra of the rigid triazole linkage that will help to project the probe away from the protein and thereby minimize potentially deleterious interactions with the protein surface.

Table 1. Thermodynamic Parameters for Wild-Type CTL9 and K109ReL1-CTL9

|          | ∆G° (kcal mol⁻¹) | m (kcal mol⁻¹ M⁻¹) | Tm (°C) | ∆H° (at 78 °C) (kcal mol⁻¹) |
|----------|-----------------|-------------------|--------|---------------------------|
| wild-type CTL9 | 6.00 ± 0.11     | 1.09 ± 0.02       | 78.1 ± 0.3 | 68.9 ± 2.0                |
| K109ReL1-CTL9 | 5.48 ± 0.27     | 1.03 ± 0.00       | 74.2 ± 0.2 | 65.4 ± 1.7                |

*Measurements were made in 10 mM MOPS, 150 mM NaCl at pH 7.0.

Vibrational frequencies and lifetimes are often sensitive to their environment. Frequencies are largely determined by the polarity of their surroundings. The frequency of most stretch modes, like those studied here, are higher in low dielectric constant liquids than in high dielectric liquids. Lifetimes depend on internal couplings, external couplings, and frequency fluctuations caused by solvent and solute structural dynamics. Thus, to a first approximation, they are sensitive to different solvent effects, although dielectric constants and structural fluctuations often mirror one another.

The FTIR spectra in Figure 3A are consistent with the expected trends, where the frequencies are higher in low dielectric constant liquids. We utilized 2D IR spectroscopy to examine the frequency and lifetime dependence of the metal carbonyl stretches in H₂O and D₂O. The 2D IR spectra can be interpreted in a similar manner to our previously published work that used a cysteine linkage to attach a metal carbonyl. Here we use 2D IR spectroscopy to measure the lifetimes. Results obtained from analysis of the diagonal peaks of the symmetric and asymmetric stretch modes are shown in Figure 4. Regarding the frequencies, a measurable 2 to 3 cm⁻¹ frequency shift was observed, with asymmetric stretch frequencies of 1930 and 1932 cm⁻¹ in H₂O versus D₂O, respectively, and symmetric stretch frequencies of 2021 and 2024 cm⁻¹. Because the dielectric constants of H₂O and D₂O are 80.4 and 80.1, one would not expect a large frequency shift due to appreciable electrostatic interactions. This frequency shift may be a consequence of accidental degeneracies. In our previous work using a Cys linker, we adjusted the linker length and found that the frequencies reflected the electrostatics of the solvent for long linker lengths and reflected the environment of the protein for short linker lengths. For the Click chemistry used here, the linker is about the same length as our longest cysteine linker (if measured starting from the backbone, not the end of the methionine) and so should reflect the solvation dynamics of the protein. From a comparison with the control experiments, for residue 109 on CTL9, it is clear that the label is dominated by solvent effects in both the native and denatured forms, as expected from its solvent-exposed position in the native state.

The lifetimes of the symmetric and asymmetric stretches also change when exposed to H₂O versus D₂O, as measured by monoexponential fits. The symmetric stretch lifetimes are 5.17 ± 0.64 and 5.04 ± 0.66 ps in H₂O and D₂O, respectively, and the asymmetric stretch lifetimes are 6.10 ± 0.48 and 4.66 ± 0.66 ps, respectively, in H₂O and D₂O, respectively.
0.57 ps, respectively. This is a significant and easily measured difference. The solvent presumably changes the lifetime because of couplings and unexpected resonances. Kubarych et al. previously saw a similar 30% lifetime effect for a metal carbonyl complex; however, the lifetime was shorter in H₂O, not D₂O as observed here. This reversal is not unexpected because accidental degeneracies are very hard to predict. The two metal carbonyl complexes have very different structures and thus very different mode frequencies, making relaxation pathways for the two molecules difficult to anticipate. Nonetheless, it is clear that comparison of lifetimes in H₂O and D₂O provides a method to probe solvent accessibility.

**CONCLUSIONS**

In summary, we have presented a general method for the high-level incorporation of a metal carbonyl IR probe, which should be broadly applicable. The band position is sensitive to the environment, and solvent isotope effects can be exploited to interrogate accessibility. The ability to use the probe in conjunction with H₂O and D₂O isotope effects to probe the electrostatics and dynamics of the protein is very beneficial because the probe is easy to incorporate into proteins and is nonperturbative, maintaining the protein structure, at least when bound to the surface. The probe is likely to be more perturbing if introduced into the hydrophobic core of a protein, and this may limit some applications. The new methodology presented here complements strategies that target Cys side chains and is expected to facilitate IR-based investigations of protein ligand interactions and protein conformational changes as well as provide site-specific structural information.

An additional advantage of this class of probes is that the absorption bands are sharp, leading to the possibility of conducting multicolor experiments using different metal carbonyls. For example, one could attach a metal carbonyl of one sort to a cysteine and a different metal carbonyl via the Click reaction to a second site, thereby enabling structural and environmental probes at two sites simultaneously. Such a strategy could probe structure in a style similar to FRET or double electron–electron resonance (DEER) but with a shorter distance dependence. Of course, these labels are larger than native amino acids and some nongenetically encoded amino acids, and so they may alter protein stability if attached to sites in the interior of proteins. Nonetheless, they are similar in size or smaller than fluorescent or EPR probes, which have been widely used in the interior of proteins to measure solvent exposure and conformational changes. However, systematic studies have shown that small, seemingly conservative probes can perturb protein stability even when they do not perturb the overall fold. Of course, any potential effects on protein stability and structure can always be checked with standard assays such as the melting curves illustrated here. However, the utility of metal carbonyl vibrational labels is not in their relatively small size, but rather in their strong transition dipole strengths and environmental sensitivity, enabling studies at much lower protein concentrations and perhaps even 2D IR experiments in stained cells or tissues.

**ASSOCIATED CONTENT**

*Supporting Information*

HPLC data used to calculate the level of incorporation of the probe. CD and 1D NMR spectra of wild-type CTL9 and the metal carbonyl containing variant. Thermal unfolding curves for both proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

![Figure 4](dx.doi.org/10.1021/jp5008279j J. Phys. Chem. B 2014, 118, 7946–7953)
The authors declare no competing financial interest.

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