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# Site-Specific Conversion of Cysteine Thiols into Thiocyanate Creates an IR Probe for Electric Fields in Proteins

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In this paper, we develop a straightforward and general method to introduce the thiocyanate nitrile stretch as a site-specific electric field probe for proteins. Electrostatics affect nearly all aspects of protein function, but general, site-specific probes for these fields are not yet available. The nitrile stretch is a particularly attractive probe because the frequency is in a relatively uncluttered region of the IR spectrum ($\bar{\nu}_{C\equiv N} \sim 2100-2240$ cm$^{-1}$) and is typically quite intense ($\epsilon \sim 50-1000$ M$^{-1}$ cm$^{-1}$), and $\bar{\nu}_{C\equiv N}$ is sensitive to electric fields, that is, it has a relatively large Stark tuning rate [$\sim$0.4−1.1 cm$^{-1}$/(MV/cm)].[1] In some cases, it is possible to deliver the nitrile probe on a substrate or inhibitor,[2] and it may prove possible to introduce nitrile-containing amino acids, such as 4-CN−Phe, site-specifically into proteins by semi-synthesis or nonsense suppression,[3] but both methods often yield smaller quantities of modified protein than typically used for biophysical studies and are not readily compatible with diverse expression systems or multi-subunit protein assemblies. Since the introduction of cysteine residues is used widely as a site-specific labeling strategy (e.g., for spin[4] or fluorescent labels), we exploit a well-known chemical modification of cysteine residues to form thiocyanates as a general method for introducing a very small IR-based probe of protein electric fields.

The strategy outlined in Scheme 1 for converting cysteine thiols into thiocyanates[5,6] is routinely employed as the first step in the selective cleavage of peptide bonds at cysteine residues.[7] Briefly, the protein in buffer at pH 7 is reacted with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent[8]) to form the mixed protein−thionitrobenzoic acid disulfide (PS−TNB), followed by displacement by cyanide ($CN^-$), to form the protein−thiocyanate (PS−CN).[9] The electronic absorption of 2-nitro-5-thiobenzoate (TNB) anion byproduct is conveniently monitored at 412 nm ($\epsilon_{412}$ = 13 600 M$^{-1}$ cm$^{-1}$)[8] to follow the course of reaction. We observed that the PS−CN products in the examples that follow were stable when stored at 4 °C over 4 days at pH 7,[10] consistent with previous reports.[11]

We have chosen three very different systems to demonstrate the versatility and scope of this method: modification of S-peptide bound to the ribonuclease S-protein (RNase S),[12] human aldose reductase (*h*ALR2), which has multiple cysteine residues and for which a number of CN-containing inhibitors related to diabetes control are available,[2,13] and the bacterial photosynthetic reaction center (RC), which is a multi-subunit, integral membrane protein containing many prosthetic groups.

The sensitivity of a vibrational frequency to an electric field is calibrated by vibrational Stark effect (VSE) spectroscopy.[14−16] Thiocyanate was introduced into RNase S and the VSE spectrum recorded (Figure 1). RNase S is a noncovalent complex between residues 1−20 (S-peptide) and 21−124 (S-protein) of bovine ribonuclease A[12] and is an extensively studied vehicle for the introduction of non-natural biophysical probes into proteins.[17,18] The S-peptide was prepared by solid-phase peptide synthesis with

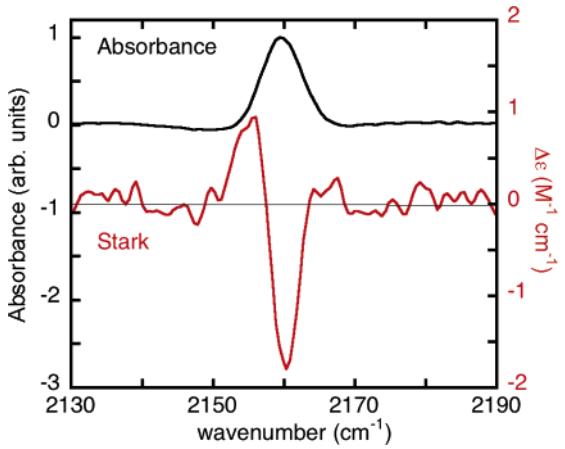
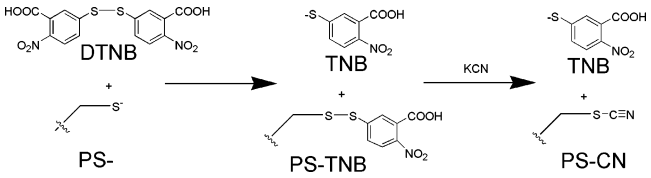



***Figure 1.*** IR absorption (top, left axis) and VSE (red, bottom, right axis) spectra for 11 mM RNase S with homocysteine introduced at position 13 and labeled with CN. The VSE spectrum is the field-on minus field-off difference spectrum obtained at 77 K in a 50/50 (v/v) glycerol/water glass. The spectrum is scaled to a path length of 1 cm, a concentration of 1 M and a field of 1 MV/cm.

***Scheme 1.*** Cysteine Cyanylation




homocysteine substituted at methionine 13, and the unique thiol of the peptide was labeled according to Scheme 1 (see Supporting Information for details) and combined with S-protein to form the labeled RNase S complex. Formation of the complex was observed by the shift of $\bar{\nu}_{C\equiv N}$ from 2161.2 cm$^{-1}$ (fwhm = 11.4 cm$^{-1}$, $\epsilon$ = 120 M$^{-1}$ cm$^{-1}$) for free, labeled S-peptide in buffer solution to 2155.4 cm$^{-1}$ (fwhm = 8.0 cm$^{-1}$, $\epsilon$ = 130 M$^{-1}$ cm$^{-1}$) for the complex. By analyzing the VSE spectrum, the Stark tuning rate[14,15,19] was determined to be 0.7 cm$^{-1}$/(MV/cm), comparable to the value observed in simpler model compounds in organic glasses.[1] Both $\epsilon$ and the Stark tuning rate are quite large, demonstrating the utility of thiocyanate as a probe.

The thiocyanate electric field probe was introduced into reactive cysteine residues of the globular protein *h*ALR2. *h*ALR2 has seven cysteines, none of which participates in disulfide bonds.[20] Cysteine 298, which lies in the active site of the enzyme,[20,21] has been shown to be particularly reactive toward cysteine−thiol-modifying reagents.[21,22] When reacted with 1.1 equiv of DTNB for 10 min, 1 equiv of TNB was released, and after displacement by cyanide, the LC-MS determined that the mass was 26 Da higher than that of the unmodified protein. The FTIR spectrum of the labeled protein (Figure 2, red) exhibited a narrow peak at 2159.4 cm$^{-1}$ with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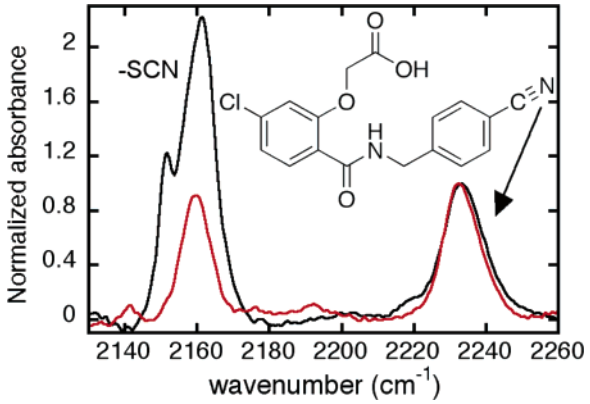


***Figure 2.*** Room temperature IR absorption spectra for *h*ALR2 in a 1:1 complex with the CN-containing inhibitor shown (2232 $cm^{-1}$) and bearing one (red) or multiple (about 2−3 cysteines labeled, see text, black) thiocyanate labels (2160 $cm^{-1}$ region). Both spectra are scaled to the absorption of the nitrile group in the inhibitor.

approximately equal intensity, but very different peak position, to a nitrile on a bound inhibitor which makes a 1:1 complex with the protein (see Supporting Information and Figure 2). It is possible to modify additional labile cysteines by driving the reaction with the addition of 10 mM DTNB over 4 h, after which we measured between 2.2 and 2.8 total equivalents of TNB released.[23] The integrated area in the 2160 $cm^{-1}$ region of multiply labeled *h*ALR2 is between 2 and 3 times that of the singly labeled protein (normalized to the inhibitor as an internal standard). Multiply labeled *h*ALR2 shows peaks at 2151.3 and 2161.3 $cm^{-1}$, the latter presumably the sum of contributions from the first labeled peak centered at 2159.4 $cm^{-1}$ with another shifted to slightly higher energy. Importantly, this higher energy vibration contributing to the 2161.3 $cm^{-1}$ peak and the peak at 2151.3 $cm^{-1}$ both report environments in the enzyme that are distinct from that of the most reactive site. Detailed assignments based on removal of individual cysteines will be reported elsewhere.

In a final demonstration of the utility of this method, we employed Scheme 1 to label the RC of *Rb. capsulatus*. Native cysteines were removed, and a single cysteine replaced Ile(L150), a moderately buried residue located on a short helix on the RC periplasmic surface. Following successive reactions with DTNB and cyanide for 8−16 h each, the overall labeling efficiency was estimated at 60% (see Supporting Information). As shown in Figure 3, narrow peaks were observed at 2159 $cm^{-1}$ for $-S^{12}CN$ and 2110 $cm^{-1}$ for $-S^{13}CN$ with approximately equal intensities for identically treated RC samples, consistent with homogeneous, site-specific labeling. The isotope shift ($\bar{\nu}_{C\equiv N}$ = 49 $cm^{-1}$) is as expected; this simple isotope labeling strategy is especially useful for confirming that small peaks are real in the presence of large backgrounds or baseline offsets as often occurs with aqueous protein samples.

We have demonstrated the versatility of a simple reaction scheme to introduce very small, stable nitrile electric field probes into proteins with three very diverse systems. For each system, work is underway to measure electric fields and changes that occur during function, and these will be reported in full publications.

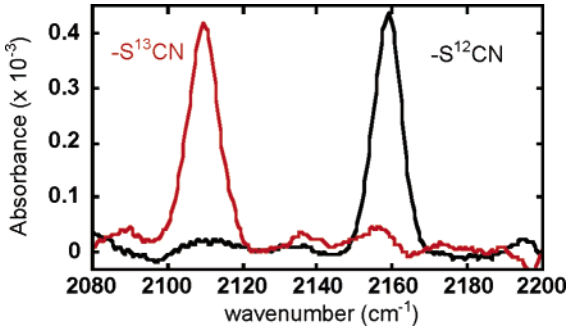


***Figure 3.*** Room temperature IR spectra for the a *Rb. capsulatus* photosynthetic reaction center mutant with a unique cysteine [I(L150)C/C(L92/98/108/246/247)A] labeled with $-S^{12}CN$ (black) or $-S^{13}CN$ (red). Spectra are for samples of approximately equal concentration.

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**Supporting Information Available:** Experimental procedures and product characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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