

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

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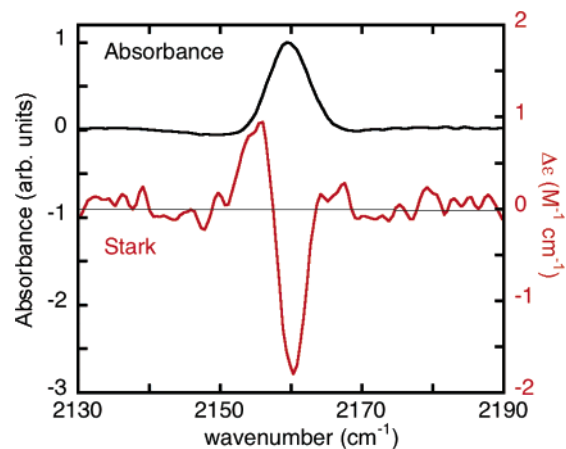
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In this paper, we develop a straightforward and general method to introduce the thiocyanate nitrile stretch as a site-specific electric field probe for proteins. Electrostatics affect nearly all aspects of protein function, but general, site-specific 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 ( $\bar{\nu}_{\text{C}\equiv\text{N}} \sim 2100\text{--}2240\text{ cm}^{-1}$ ) and is typically quite intense ( $\epsilon \sim 50\text{--}1000\text{ M}^{-1}\text{ cm}^{-1}$ ), and  $\bar{\nu}_{\text{C}\equiv\text{N}}$  is sensitive to electric fields, that is, it has a relatively large Stark tuning rate [ $\sim 0.4\text{--}1.1\text{ cm}^{-1}/(\text{MV}/\text{cm})$ ].<sup>1</sup> In some cases, it is possible to deliver the nitrile probe on a substrate or inhibitor,<sup>2</sup> 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,<sup>3</sup> 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<sup>4</sup> 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<sup>5,6</sup> is routinely employed as the first step in the selective cleavage of peptide bonds at cysteine residues.<sup>7</sup> Briefly, the protein in buffer at pH 7 is reacted with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent<sup>8</sup>) to form the mixed protein-thionitrobenzoic acid disulfide (PS-TNB), followed by displacement by cyanide ( $\text{CN}^-$ ), to form the protein-thiocyanate (PS-CN).<sup>9</sup> The electronic absorption of 2-nitro-5-thiobenzoate (TNB) anion byproduct is conveniently monitored at 412 nm ( $\epsilon_{412} = 13\,600\text{ M}^{-1}\text{ cm}^{-1}$ )<sup>8</sup> 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,<sup>10</sup> consistent with previous reports.<sup>11</sup>

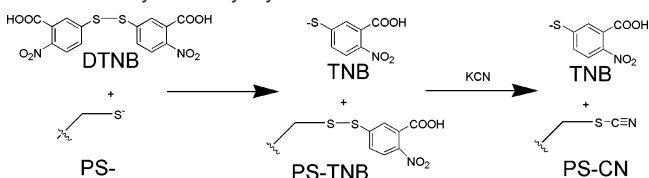
We have chosen three very different systems to demonstrate the versatility and scope of this method: modification of S-peptide bound to the ribonuclease S-protein (RNase S),<sup>12</sup> human aldose reductase (*h*ALR2), which has multiple cysteine residues and for which a number of CN-containing inhibitors related to diabetes control are available,<sup>2,13</sup> 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.<sup>14–16</sup> 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<sup>12</sup> and is an extensively studied vehicle for the introduction of non-natural biophysical probes into proteins.<sup>17,18</sup> 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  $\bar{\nu}_{\text{C}\equiv\text{N}}$  from  $2161.2\text{ cm}^{-1}$  (fwhm =  $11.4\text{ cm}^{-1}$ ,  $\epsilon = 120\text{ M}^{-1}\text{ cm}^{-1}$ ) for free, labeled S-peptide in buffer solution to  $2155.4\text{ cm}^{-1}$  (fwhm =  $8.0\text{ cm}^{-1}$ ,  $\epsilon = 130\text{ M}^{-1}\text{ cm}^{-1}$ ) for the complex. By analyzing the VSE spectrum, the Stark tuning rate<sup>14,15,19</sup> was determined to be  $0.7\text{ cm}^{-1}/(\text{MV}/\text{cm})$ , comparable to the value observed in simpler model compounds in organic glasses.<sup>1</sup> 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.<sup>20</sup> Cysteine 298, which lies in the active site of the enzyme,<sup>20,21</sup> has been shown to be particularly reactive toward cysteine-thiol-modifying reagents.<sup>21,22</sup> 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  $2159.4\text{ cm}^{-1}$  with

