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Rapid Report

Selective Incorporation of Nitrile-Based Infrared Probes into Proteins via Cysteine Alkylation†

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ABSTRACT: The nitrile stretching vibration is increasingly used as a sensitive infrared probe of local protein environments. However, site-specific incorporation of a nitrile moiety into proteins is difficult. Here we show that various aromatic nitriles can be easily incorporated into peptides and proteins via either thiol alkylation or arylation reaction.

The C≡N (nitrile) stretching vibration has recently emerged as a valuable infrared (IR) probe of the conformation and local environment of biological molecules (*1*−*26*) because of its sensitivity to various factors (*13*, *14*, *17*), such as local electric field and hydrogen bonding interactions (*27*). For example, it has been used to probe insertion of a peptide into membranes (*6*), protein−ligand interactions (*25*), and the dehydration status of an antimicrobial peptide encapsulated in reverse micelles (*8*). For chemically synthesizable peptides, site-specific incorporation of a nitrile group can be readily achieved via the use of nitrile-derivatized non-natural amino acids, such as cyanoalanine (Ala_{CN}) and *p*-cyanophenylalanine (Phe_{CN}). For proteins that cannot be chemically synthesized, however, selective incorporation of a nitrile moiety is rather difficult. Currently, only the chemical method developed by Boxer and co-workers is available, which directly converts a cysteine thiol into a thiocyanate (*7*). Additionally, it has been shown that Phe_{CN} can be incorporated into proteins by using an orthogonal tRNA-synthetase pair (*28*, *29*), but the techniques involved are time-intensive and available to only a handful of laboratories worldwide. Thus, it would be quite helpful to develop an alternative and easier method for selective incorporation of different nitrile moieties into proteins. Here, we show that cysteine alkylation and arylation reactions under mild conditions can be used for such a purpose.

We tested the feasibility of the proposed method on four cyanobenzyl derivatives (Scheme 1), based on the consideration that the oscillator strength and stark tuning rate of aromatic nitriles are normally larger than those of alkyl nitriles (*3*, *4*). As shown (Scheme 1), these model probes can be quite easily attached to the cysteine side chain via either thiol alkylation or arylation (*30*−*32*). Similar to that of Phe_{CN} (*3*), the C≡N stretching frequency of these nitrile derivatives in water is found to be in the range of 2233−2241 cm^{-1} (Table 1 and Figure S1 of the Supporting Information), with the exact value depending on the molecular structure (e.g., the band of the benzylic derivatives shows an approximately 3 cm^{-1} shift to a higher energy as compared to that of the aryl derivatives). Because any interactions that decrease or increase the electron density of the C≡N bond will result in an increase or decrease, respectively, in the C≡N stretching vibrational frequency of nitriles (*33*), these results can be understood qualitatively in the context of the effect of an activating substituent on the cyanobenzyl ring (i.e., sulfur vs methylene and para vs ortho position with respect to the nitrile group). Furthermore, in comparison with those obtained in water, the C≡N stretching bands of these probes in tetrahydrofuran (THF) show a 7−8 cm^{-1} shift toward a lower frequency and also a concomitant decrease in the bandwidth by a factor of approximately 2 (Table 1), demonstrating the potential utility of these aromatic nitriles as local environmental probes.

Moreover, it is interesting to note that in aqueous solution the C≡N stretching bandwidths of **2** and **4** are noticeably larger than those of **1** and **3**. This finding is consistent with the study of Waegele et al. (*34*), which showed that the C≡N stretching vibration of an aromatic nitrile can be influenced by direct interactions between the nitrile group and solvent molecules and also indirectly by the solvation status of the aromatic ring. In other words, the larger bandwidth of **2** and **4** arises most likely from their asymmetric molecular shape (with respect to the nitrile group), which leads to a more heterogeneous solvation of the respective aromatic ring and hence a broader vibrational transition.

Considering the fact that the synthesis of **1** and **2** involves conditions much milder [e.g., the reaction solution contains only a small amount of organic solvent, and similar conditions have been used in chemical modification of proteins (*35*)] than those used in the synthesis of **3** and **4** and that the extinction coefficient of the C≡N stretching vibration of **1** and **3** is about one order of magnitude larger than that of **2** and **4** (Figure S1 of the Supporting Information), only probe **1** is used in the subsequent proof-of-principle tests involving peptides and proteins.

First, the method of cysteine alkylation is applied to two cysteine mutants of mastoparan-X (MpX), W3C and A8C. These mutants are chosen because upon association with calmodulin (CaM) the side chains of Trp3 and Ala8 of MpX are known to situate inside the peptide−protein binding groove and, as a result, become less solvent-exposed (*36*). It is found that both peptides are efficiently labeled by *p*-cyanobenzyl bromide with >80% yield (determined by LC−MS) under the conditions specified in Scheme 1 (the corresponding nitrile-containing peptides are hereafter termed W3C-CN and A8C-CN). The FTIR spectra of W3C-CN (Figure 1) and A8C-CN (Figure S2 of the Supporting Information) also support the site-specific incorporation of probe **1** into these peptides, as their C≡N stretching bands in

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Scheme 1

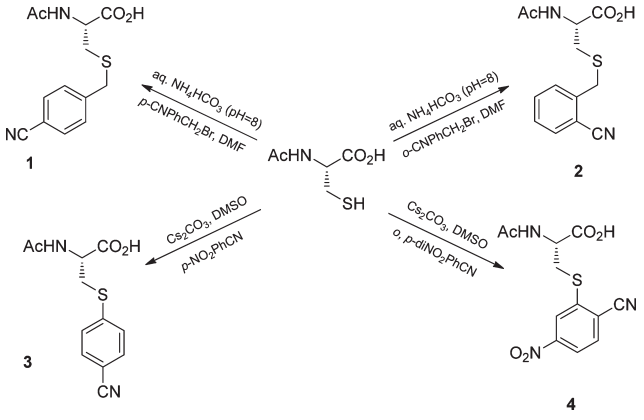

Table 1: Band Positions (ν), Full Widths at Half-Maximum ($\Delta\nu$), and Estimated Molar Extinction Coefficients (ε) of the C≡N Stretching Vibration of Various Probes in H_2O and THF

	1	2	3	4
ν (cm^{-1}, H_2O)	2236.6	2240.3	2233.7	2237.9
$\Delta\nu$ (cm^{-1}, H_2O)	11.8	14.4	11.2	16.4
ν (cm^{-1}, THF)	2228.5	2232.3	2226.8	2229.6
$\Delta\nu$ (cm^{-1}, THF)	7.4	7.8	6.1	7.1
ε (M^{-1} cm^{-1})	210 ± 60	22 ± 10	240 ± 60	24 ± 10

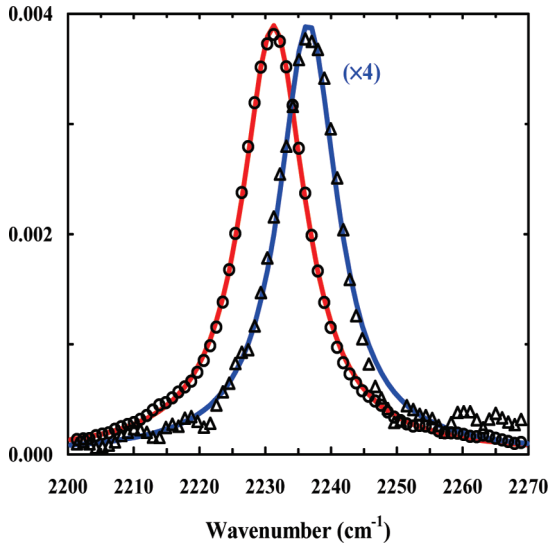

FIGURE 1: C≡N stretching bands of W3C-CN obtained in the presence (○) and absence (△) of CaM [50 mM HEPES buffer (pH 7.4) and 30 mM $CaCl_2$]. In the former case, the concentrations of W3C-CN and CaM were estimated to be 1−2 mM. Lines are respective fits of these data to a Lorentzian function with the following parameters: for W3C-CN, ν = 2236.5 cm^{-1} and $\Delta\nu$ = 10.7 cm^{-1}, and for the W3C-CN−CaM complex, ν = 2231.2 cm^{-1} and $\Delta\nu$ = 11.7 cm^{-1}.

aqueous solution are centered at ~2236.5 cm^{-1} but shift to lower wavenumbers upon binding to CaM, as expected. In addition, the nitrile bandwidth of the free peptide is found to be slightly narrower than that of the peptide−CaM complex, likely because both bound and unbound peptides exist in the complex solution. A similar finding was also observed in a previous study (*3*).

Second, we tested the applicability of the cysteine alkylation reaction to proteins by applying it to human calmodulin-like protein CALM3, which contains a unique cysteine residue (Supporting Information). As shown (Figure 2), the IR spectrum of

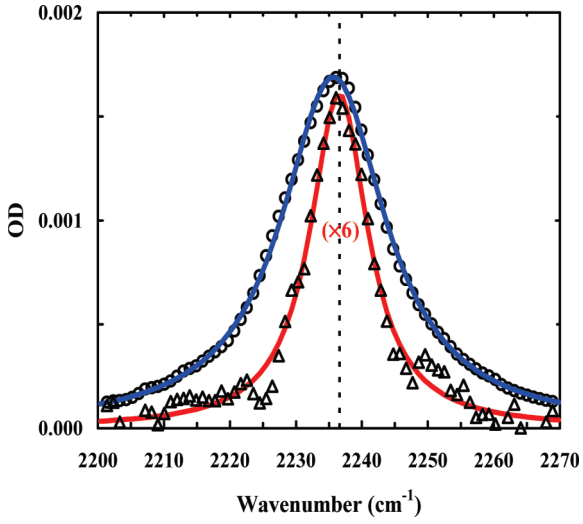

FIGURE 2: C≡N stretching band of the nitrile-labeled human calmodulin-like protein [in 50 mM HEPES buffer (pH 7.4)] in the absence (○) and presence (△) of Ca^{2+} (30 mM). The protein concentrations were approximately 1−2 mM (blue) and 200 μM (red), respectively. The solid lines are respective fits of these data to a Lorentzian function, and the thin dashed line indicates the peak position of the nitrile band of probe **1** in water.

the reaction product confirms the successful incorporation of probe **1** into the protein of interest, and the corresponding yield was estimated to be >50%. More importantly, the far-UV CD spectrum of the nitrile-labeled CALM3 is almost identical to that of the parent protein (Figure S3 of the Supporting Information), indicating that the labeling reaction does not change the structural integrity of the protein in question. The latter notion is further corroborated by the dependence of the bandwidth of the C≡N stretching vibration on Ca^{2+}. As indicated (Figure 2), upon addition of Ca^{2+}, the bandwidth of the IR transition is decreased from ~19 to ~11 cm^{-1}, a phenomenon expected to occur as Ca^{2+} is known to rigidify the calcium-binding domain (*37*, *38*) where the labeled cysteine is located. In addition, the peak position of this nitrile band (ν = 2236.0 cm^{-1}) does not change upon addition of Ca^{2+}, which is consistent with the fact that the labeled cysteine residue is exposed to solvent in CaM structures determined in the absence and presence of Ca^{2+} (*38*).

In conclusion, we have demonstrated a post-translational method allowing site-specific incorporation of nitrile-based IR probes into peptides and proteins via cysteine alkylation or arylation. Because this method involves relatively routine and mild reaction conditions, we expect that it will find wide application in biophysical studies of proteins.

SUPPORTING INFORMATION AVAILABLE

Materials, methods, and FTIR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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