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Protein Probes

Cyano Groups as Probes of Protein Microenvironments and Dynamics**

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Protein dynamics and microenvironment heterogeneity are important for biological function; however, their exact role has remained unclear and controversial, mostly as a result of the difficulties associated with their direct experimental characterization. Although the temporal and potential structural resolution inherent to vibrational spectroscopy make it ideal for the study of proteins, spectral congestion precludes its straightforward application. In attempts to circumvent this problem, many recent efforts have focused on the use of environmentally sensitive probes that absorb IR light between 1800 and 2600 cm^{-1}, a region free of obscuring absorptions.[1–4] One such probe is the carbon–deuterium (C–D) bond, which may replace any nonexchangeable C–H bond, and another is the exogenous cyano (CN) chromophore, which may be appended to different amino acids.[1,5–14]

The CN probe has received a great deal of attention both experimentally[2,15–27] and computationally.[28–31] For example, its ability to characterize the microenvironments of small-molecule ligands,[15–18] free amino acids or peptides,[19–24] and a four-helix-bundle model of a membrane protein[25] has been reported. However, the probe has only been incorporated into two intact proteins, myoglobin[32] and ketosteroid isomerase.[26] Although these landmark studies demonstrate that the CN absorptions are observable and sensitive to their protein environment, only single positions were examined, and the effect on stability was not characterized; thus, the utility of the probe remains to be fully explored. For example, whereas the polarizability of the probe makes it a good IR chromophore, it may also result in the introduction of non-native interactions,[5,19] including metal-binding sites or hydrogen-bond acceptors. This possibility appears to have been examined in only one study, in which structural investigations of the large proteolytic fragment of ribonuclease A noncovalently bound to the small, CN-modified proteolytic fragment revealed that the probe introduced little structural perturbation.[2] In contrast, when His64 in myoglobin was replaced with *para*-cyano-L-phenylalanine, the Soret absorptions observed under some conditions suggested that the CN group introduced artificial interactions with the heme center.[32] Moreover, the use of CN probes under time-resolved conditions has not been examined. Herein, we explore the ability of site-specifically incorporated CN bonds to characterize cytochrome *c* (cyt *c*) under both equilibrium and time-resolved conditions.

Cyt *c* was synthesized with the CN group site-specifically appended to Tyr67 ((CN)Tyr67) or Phe82 ((CN)Phe82)), in both cases by the incorporation of *para*-cyano-L-phenylalanine at the corresponding position (Figure 1; see also the Supporting Information). Tyr67 and Phe82 are located in regions that are critical for biological function[33–35] and also proximal to the critical Met80 heme ligand.[36,37] To characterize the effects of the CN probe on the global structure of the protein, we first examined circular dichroism and absorption spectra of (CN)Tyr67 and (CN)Phe82 in 100 mM sodium phosphate (pH 8.0), that is, under conditions that should strongly favor folding of the protein. The circular dichroism spectrum and Soret absorption band of (CN)Phe82 were indistinguishable from those of the wild-type protein, whereas the rotational strength of (CN)Tyr67 was slightly diminished (see Figures S1 and S5 in the Supporting Information). Moreover, the Soret band of (CN)Tyr67 was red-shifted by approximately 2 nm to 416 nm, which is consistent with Met80 displacement. To further characterize the heme environments, we employed resonance Raman spectroscopy.

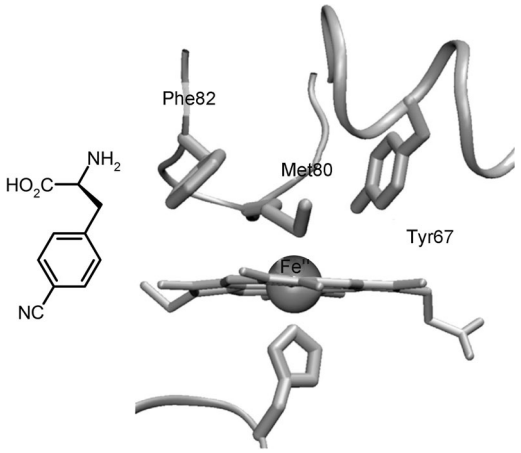

Figure 1. Left: Structure of *para*-cyano-L-phenylalanine. Right: Structure of the heme pocket of cyt *c* (PDB 1GIW) showing the heme iron atom and the side chains of Met80, Tyr67, and Phe82. The side chain of His18, the other protein-based heme ligand, is also shown.

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The resonance Raman spectrum of (CN)Phe82 was identical to that of the wild-type protein, whereas the spectrum of (CN)Tyr67 was slightly perturbed (see Figure S6). Our results suggest that under these conditions, (CN)Phe82 adopts an overall fold similar to that of the wild-type protein, with an intact Met80–Fe bond, whereas (CN)Tyr67 adopts a slightly perturbed fold, possibly with Met80 displaced from the heme center by the CN group.

We next monitored the changes in the Soret absorption during the guanidine hydrochloride (GdnHCl) induced unfolding of cyt *c* and the two CN-modified variants to determine whether the CN probes impact stability. Both the line width and the absorption maximum of the Soret band of all three proteins shifted as a function of GdnHCl concentration and showed a single transition between the limiting values observed at 0 and 6 M GdnHCl (Figure 2). However, introduction of the CN group at either position 67 or position 82 resulted in significant destabilization of the protein, whereby the denaturation midpoint was decreased by 0.7 and 0.9 M, respectively.

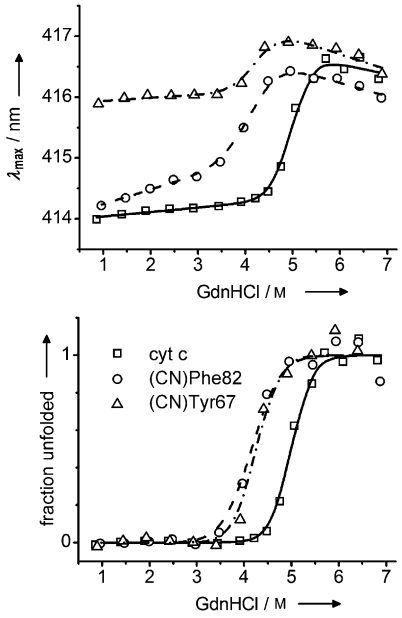

Figure 2. GdnHCl-dependent Soret band maximum (top) and fractional concentration of unfolded protein (bottom) for cyt *c* (squares), (CN)Tyr67 (triangles), and (CN)Phe82 (circles). The apparent midpoints are 5.0, 4.3, and 4.1 M GdnHCl for cyt *c*, (CN)Tyr67, and (CN)Phe82, respectively. Lines are sigmoidal fits to the data.

To explore the ability of the CN probe to report on its local microenvironment, we characterized the steady-state IR absorptions of (CN)Tyr67 and (CN)Phe82 under conditions that favor folding (Figure 3, Table 1). Both proteins showed a single, strong CN absorption. However, the CN absorption of (CN)Phe82 was centered at 2235 cm^{-1} and was thus similar to that of the free CN-modified amino acid under the same conditions, whereas that of (CN)Tyr67 was centered at 2229 cm^{-1}; this shift is consistent with ligation to iron.[38,39]

We next explored the ability of the CN probe to characterize protein dynamics under time-resolved conditions

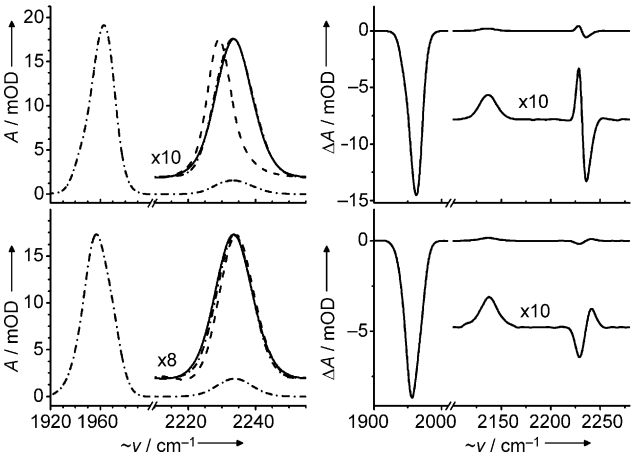

Figure 3. Left: IR spectra of reduced (CN)Tyr67 (top) and (CN)Phe82 (bottom) at 3.2 M GdnHCl in the presence of CO (dashed–dotted line). For comparison, the CN stretch absorptions of the folded (dashed line) and unfolded (solid line) reduced proteins are also shown. Right: LIDS of (CN)Tyr67–CO (top) and (CN)Phe82–CO (bottom) at 3.2 M GdnHCl.

by using a procedure previously reported for photoinitiating the unfolding of cyt *c*.[14] Briefly, at subdenaturing concentrations of GdnHCl and saturating concentrations of CO, cyt *c* adopts an unfolded state in which CO replaces Met80 at the heme center. Whereas folding after CO photodissociation with a single pulse is limited by fast CO rebinding,[40,41] persistent photoirradiation leads to the accumulation of a long-lived photostationary folded state, and when the irradiation is halted, CO rebinding initiates unfolding.[14] For native cyt *c*, the photostationary state is most efficiently formed at a GdnHCl concentration of about 4 M; however, because the transition midpoint for the CN-modified variants is decreased by about 0.8 M GdnHCl (see above), we used 3.2 M GdnHCl in the present study. With this lower concentration of denaturant, the IR spectrum of (CN)Phe82 showed a strong absorption at 1957 cm^{-1}, which we assigned to heme-bound CO in the unfolded protein, and an absorption at 2234 cm^{-1}, which was identical to the CN absorption of the unfolded reduced protein (Figure 3, Table 1). In contrast, the spectrum of (CN)Tyr67 showed a strong absorption within the range expected for heme-bound CO that was well fit by two Gaussian functions of approximately equal amplitude (centered at 1957 and 1965 cm^{-1}); we assigned these peaks to the CO-bound unfolded and folded proteins, respectively. The CN stretch absorption was well fit by a single Gaussian function, which, as in (CN)Phe82, was identical to the corresponding absorption in the unfolded, reduced protein. Thus, under these conditions, (CN)Phe82 appears to adopt a CO-bound unfolded state, whereas (CN)Tyr67 appears to exist as a mixture of the CO-bound folded and CO-bound unfolded states.

Next, we examined whether persistent CO photodissociation induced the population of a photostationary state in (CN)Phe67 and (CN)Phe82 by measuring the light-induced difference spectra (LIDS) of (CN)Tyr67–CO and (CN)Phe82–CO in 3.2 M GdnHCl after preirradiation for

Table 1: Spectral fit parameters for steady-state spectra and LIDS (standard deviation for the last significant digit is given in parentheses).

	(CN)Tyr67			(CN)Phe82			Cyt *c*[a]		
	relative amplitude	ν [cm^{-1}]	fwhm [cm^{-1}]	relative amplitude	ν [cm^{-1}]	fwhm [cm^{-1}]	relative amplitude	ν [cm^{-1}]	fwhm [cm^{-1}]
reduced protein									
CN stretch, folded	1	2228.9(2)	9.0(2)	1	2234.5(2)	11.6(2)			
CN stretch, unfolded	1	2233.7(2)	12.7(1)	1	2233.5(2)	12.8(2)			
CO-bound protein[b]									
CO stretch	1	1957.5(5)	27.0(3)	1	1957.5(1)	26.7(2)	1	1955.6(2)	25(1)
	0.98(4)	1964.6(1)	17.1(4)	0.06(1)	1973.7(2)	10.7(3)	0.10(3)	1974(1)	11.5(6)
CN stretch	0.16(1)	2233.6(1)	12.9(2)	0.110(2)	2233.7(1)	12.3(1)			
LIDS[b]									
CO bleach	−1	1957.5[c]	27.0[c]	−1	1957.5[c]	26.7[c]	−1	1955.6[c]	25[c]
	−0.94(7)	1964.6[c]	17.1[c]	−0.10(1)	1973.7[c]	10.7[c]	−0.12 (3)	1974[c]	11.5[c]
CO induced abs.	0.10(2)	1969.5(6)	17.6(4)				0.11(2)	1966(1)	17(1)
	0.025(6)	2135.9(5)	21(3)	0.018(6)	2136(1)	21(2)	0.026(7)	2136(1)	25(1)
CN bleach	−0.089 (4)	2233.6[b]	12.9[c]	−0.10(2)	2233.7[c]	12.3[b]			
CN induced abs.	0.113(5)	2228.8(1)	7.6(1)	0.09(2)	2235.2(3)	12.4(2)			

[a] From Ref. [14]. [b] For (CN)Tyr67 and (CN)Phe82, 3.2 M GdnHCl; for cyt *c*, 4.0 M GdnHCl. [c] Fixed during fit (see the Supporting Information). fwhm = full width at half maximum.

90 s with the second harmonic of a Nd:YAG laser (532 nm, pulse width: ca. 10 ns, repetition rate: 20 Hz). In both cases, the LIDS showed a strong bleach signal at approximately 1960 cm^{-1} with the same line shape as the corresponding steady-state absorption of the heme-bound CO, and an induced absorption at 2136 cm^{-1}, which was assigned to the stretch vibration of unbound CO (Figure 3, Table 1).[42] (CN)Phe82–CO also showed a minor induced absorption (10% amplitude relative to the bleach signal) at 1970 cm^{-1}, which must correspond to a photostationary state with CO bound to the heme center, as previously observed for cyt *c*–CO.[14] A similar minor induced absorption of (CN)Tyr67 may be masked by the strong bleach signal of CO bound to the folded protein. Overall, the LIDS spectral features associated with CO reveal that during irradiation, CO is dissociated, and the majority of the protein (ca. 90%) forms a long-lived photostationary state.

Interestingly, the LIDS of the modified proteins exhibited a derivative-like absorption at the spectral position of their respective CN stretch vibration (Figure 3, Table 1). For both proteins, this feature was well fit by two Gaussian functions. One function has a negative amplitude and was assigned to the bleach signal of the CN absorption in the CO-bound state; the other has a positive amplitude and was assigned to an induced absorption in the photostationary state. The frequency and line width of the bleach signals were identical to those observed for the unfolded proteins, whereas those of the induced absorptions were identical to those of the respective folded states. Thus, the data suggest that the photostationary state of each modified protein is at least partially folded.

To further confirm that the changes observed in the LIDS reflect differences in folding, we examined the LIDS for (CN)Tyr67 in the presence of saturating CO as a function of GdnHCl concentration (see the Supporting Information). The LIDS at 1.2 M GdnHCl, under which conditions the protein is fully folded, showed a strong bleach signal at 1965 cm^{-1} due to depopulation of the CO-bound folded state, and a derivative-shaped feature at approximately 2230 cm^{-1}, which again was well fit by two Gaussian functions, one assigned to the bleach of the CN absorption in the CO-bound folded state, and the other to an induced absorption in the photostationary folded state. In contrast, at 4.0 M GdnHCl, under which conditions the protein is fully unfolded (in the presence or absence of CO), the bleach signal at 1955 cm^{-1} was greatly diminished, and no change was observed in the CN stretching region. Thus, it is likely that the observed changes in the LIDS do indeed reflect changes in the folded state of the protein. In all, the LIDS data suggest that during the irradiation of both (CN)Tyr67 and (CN)Phe82, a single major species accumulates that is at least partially folded.

We next employed rapid-scan FTIR spectroscopy to characterize the accumulation and decay of the CO bleach at about 1960 cm^{-1} for both (CN)Tyr67 and (CN)Phe82 (Figure 4). In each case, the buildup of the CO bleach signal was well fit by two exponential functions, and its decay was well fit by three exponential functions (Table 2). Interpreta-

Table 2: Fit parameters for postirradiation decay kinetics.

		a_1 [%]	t_1 [s]	a_2 [%]	t_2 [s]	a_3 [%]	t_3 [s]
(CN)Tyr67							
	CO bleach	47 ± 3	2.7 ± 0.6	43 ± 2	15 ± 2	7 ± 5	700 ± 200
	CN bleach	43 ± 9	3.1 ± 0.3	47 ± 9	16 ± 2	7 ± 4	600 ± 200
(CN)Phe82							
	CO bleach	32 ± 3	4.3 ± 0.8	62 ± 3	32 ± 8	5 ± 4	1100 ± 500
	CN bleach	35 ± 8	9 ± 4	56 ± 2	41 ± 9	6 ± 5	700 ± 500
cyt *c*[a]							
	CO bleach	5 ± 2	2.1 ± 0.9	95 ± 2	20 ± 4		

[a] From Ref. [14].

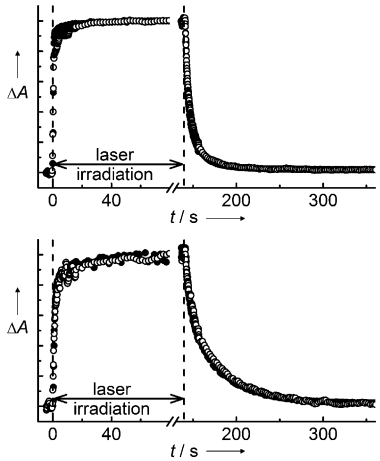

Figure 4. Time dependence of the normalized CO bleach (closed circles) and the CN bleach (open circles) for (CN)Tyr67–CO (top) and (CN)Phe82–CO (bottom).

tion of the processes underlying the bleach accumulation is complex and depends on relaxation rates and the delay between pulses. Interpretation of the decay kinetics is more straightforward and suggests that the unfolding of the photostationary state populates three distinct intermediates that are bound by CO. The small differences in amplitudes and time constants observed for the two proteins probably reflect their slightly different stabilities (Figure 2).

Finally, to explore the ability of the CN probe to directly characterize the protein dynamics associated with the unfolding of the photostationary state, we employed rapid-scan FTIR spectroscopy to characterize the accumulation and decay of the CN bleach and induced absorption signals at around 2230 cm^{-1} for (CN)Tyr67 and (CN)Phe82 (Figure 4). As for the CO probe, the spectral changes observed for the CN probe at both positions examined were well fit by two exponential functions for the buildup and three for the decay. In fact, the amplitudes and time constants observed with CN were identical to those observed with CO (Table 2). Because CO displaces two very different ligands from the heme center during rebinding (most likely Met80 in (CN)Phe82 and the nitrile ligand in (CN)Tyr67), the data suggest that the unfolding of the photostationary state is rate-limited by a step that follows ligand dissociation and probably involves large-scale protein rearrangements that enable CO access to an unsaturated iron center. Indeed, when adjusted for the different experimental conditions, the observed rates are similar to those measured for CO binding to folded states of cyt *c*.[43] A similar mechanism probably underlies unfolding of the native protein under physiological conditions. Indeed, previous experiments based on H/D exchange suggested that the native reduced protein unfolds through multiple steps,[44] and our results further suggest that three of the associated intermediates may be bound by CO.

In summary, our results provide additional evidence that CN probes should be of general utility for the site-specific characterization of proteins under both steady-state and time-resolved conditions. As expected on the basis of molar extinction coefficients,[45] comparison with the previously reported C–D studies reveals that the greatest advantage of the CN probe is its more intense absorptions. The relatively large polarizability of the C–N bond also makes it particularly useful for the characterization of local electric fields.[2,26,27,46–48] However, the results also suggest that the polarizability of the CN group predisposes it to microenvironment perturbation. Indeed, when incorporated at Tyr67, the CN probe appears to induce significant structural reorganization, which possibly involves heme misligation. This perturbation probably results, at least in part, from the proximity of Tyr67 to the iron center (Figure 1) and indicates that the CN probe is likely to be inappropriate for the characterization of some environments. Moreover, the destabilization observed upon incorporation of the probe at both sites examined suggests that some level of destabilization is likely to be general and highlights the care that must be employed when these probes are used for the characterization of folding. This propensity toward microenvironment perturbation stands in sharp contrast to the behavior of C–D bonds, which, although weaker IR chromophores, are strictly nonperturbative. Thus, it seems likely that a combination of both CN and C–D probes will prove most useful for the characterization of protein microenvironments and dynamics.

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