beyond 20 000. They may be useful in cases where homonuclear ^1H–^1H methods fail because of overlap or spin diffusion problems. Selective ^{13}C or ^{15}N labeling coupled with isotope filtering can alleviate spectral overlap problems associated with homonuclear ^1H–^1H approaches;[24] however, these methods still rely on the nuclear Overhauser effect for sequential assignments, and it will be useful to have alternative methods such as this that are immune to spin diffusion effects.

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Detailed Analysis of Carbon-13 NMR Spin Systems in a Uniformly Carbon-13 Enriched Protein: Flavodoxin from *Anabaena 7120*

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The two-dimensional (2D) resonance-offset-compensated ^{13}C-$\{^{13}C\}$ double quantum correlation (DQC) NMR experiment discussed in the previous paper[1] has been used to obtain one-bond carbon–carbon correlations in oxidized flavodoxin, a protein of molecular weight ~21 000 (Figure 1). The flavodoxin was purified from a cyanobacterium, *Anabaena 7120*, that had been grown on [26% ^{13}C]CO_2 as the sole carbon source. Carbon spin systems of an alanine and tyrosine residue have been outlined in Figure 1, beginning at the carbonyl carbon and following through to the end of the side chain.[2] Although not obvious from the scale of Figure 1, many additional spin systems can be resolved in spectral expansions. The resolution and sensitivity are exemplified by one row of the spectrum (shown at the bottom of Figure 1) containing the $^{13}C_\zeta$–$^{13}C_\epsilon$ correlation of the outlined tyrosine residue. This type of information, when combined with proton-carbon correlations,[3-5] will be useful in the assignment of the proton spin systems of amino acid residues.

The ^{13}C–^{13}C correlation results have been used to assign several of the carbon resonances of the noncovalently bound flavin mononucleotide cofactor of the flavodoxin. Table I shows assignments resulting from observed correlations between $^{13}C_{7_a}$–$^{13}C_7$, $^{13}C_7$–$^{13}C_6$, $^{13}C_6$–$^{13}C_{5_a}$, and $^{13}C_{8_a}$–$^{13}C_8$ of the isoalloxazine ring as well as from

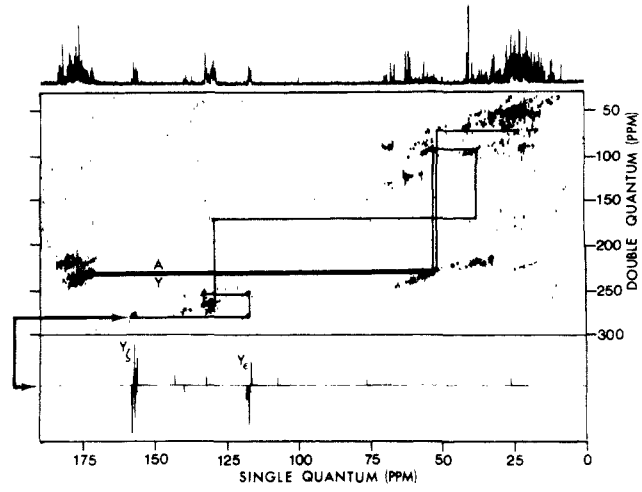

Figure 1. ^{13}C–^{13}C correlations in oxidized *Anabaena 7120* flavodoxin obtained by two-dimensional $^{13}C\{^{13}C\}$DQC NMR. The sample was 0.4 mL of 3.5 mM [26% Ul-^{13}C]flavodoxin in ^2H_2O containing 100 mM phosphate buffer at pH 7.5. A 5 mm (OD) NMR tube was used. Data were collected on a Bruker AM-500 spectrometer, with a ^{13}C frequency of 125.76 MHz. The total acquisition (time, 84 h) consisted of 256 blocks, each containing the average of 1792 free induction decays (8192 data points each). The 90° carbon pulse was 7 μs. WALTZ-16[13] decoupling (500 MHz proton) was used during acquisition to collapse ^{13}C–^1H splittings. The experiment was optimized for $^{13}C_\alpha$–$^{13}C_o$ couplings (~55 Hz) by setting the total delay in the double-quantum propagator to 9.26 ms. The raw data were multiplied by a Lorentzian-to-Gaussian function and zero-filled to 32 768 data points in ω_2 and multiplied by a cosine function and zero-filled to 1024 data points in ω_1 before Fourier transformation in each dimension. Noise was reduced in the spectrum by using the program "MAKEUP".[14] ^{13}C chemical shifts are referenced to tetramethylsilane. Single- and double-quantum chemical shifts are plotted along the horizontal and vertical axes, respectively. Each correlation is represented at the double-quantum frequency by two antiphase doublets, one at the single-quantum frequency of each component. The one-dimensional projection in the ω_2 dimension is plotted at the top. Connectivities are outlined beginning at the carbonyl carbons for an alanine (A) and tyrosine (Y) residue (see Table I for chemical shifts). The row of the spectrum with a double-quantum chemical shift of 272.3 ppm is shown at the bottom of the figure. The antiphase doublets in this row representing the $^{13}C_\zeta$–$^{13}C_\epsilon$ correlation of the outlined tyrosine residue are indicated.

correlations between the ribityl chain carbons. Flavin carbon resonances have been assigned previously in other flavoproteins by incorporating ^{13}C-labeled flavin into unlabeled apo-protein.[6-8] Because of the presence of several closely spaced resonances, it was necessary to incorporate two or more selectively enriched flavins into each protein, either independently or as mixtures with varying levels of enrichment. The present results show that such assignments can be made directly with a single uniformly labeled sample without the necessity of organic syntheses and cofactor reconstitution. Three of the ribityl carbon assignments have been extended to the ribityl protons via a proton-carbon correlation experiment[5] (see Table I).

The outlined tyrosine carbon spin system demonstrates the potential of this experiment for assigning aromatic spin systems. Ring carbon assignments can be extended via proton-carbon correlations to ring protons.[3,5] This through-bond technique circumvents ambiguities that may arise by using through-space (NOESY) connectivities between C_β–H's and aromatic ring side chain protons to assign aromatic ring protons.[9] Additional quaternary carbon resonances (such as tyrosine $^{13}C_\zeta$) also can be assigned directly in this manner (Table I).

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Table I. Representative ^{13}C and ^{1}H Spin Systems Identified on the Basis of Two-Dimensional ^{13}C-^{13}C and ^{1}H-^{13}C Connectivities

group[a]	carbon atom	chemical shifts[b] (ppm) ^{13}C (±0.1)	attached ^{1}H (±0.02)
ribose	1′	52.2	
	2′	71.2	2.79
	3′	74.6	3.60
	4′	71.0	4.21
	5′	63.8	
isoalloxazine ring	5a	139.4	
	6	129.8	
	7	141.9	
	7a	20.3	
	8	152.8	
	8a	23.0	
alanine-A	o	172.8	
	α	51.2	
	β	21.4	
tyrosine-A	o	171.8	
	α	52.3	
	β	34.9	
	γ	127.7	
	δ	131.4[c]	
	ε	116.0[c]	
	ζ	156.3	
threonine-A	o	170.8	
	α	59.9	
	β	66.3	
	γ	16.4	

[a] Sequence-specific assignments have not been made yet for the amino acid spin systems. [b] ^{13}C chemical shifts are relative to TMS. ^{1}H chemical shifts are relative to TSP. [c] The two tyrosine $^{13}C_\delta$ and $^{13}C_\epsilon$ carbons appear to have degenerate chemical shifts.

At least 154 of the expected ~210 $^{13}C_o$-$^{13}C_\alpha$ correlations were resolved by using the software package MADNMR.[2] This suggests that uniform ^{13}C labeling will support a heteronuclear approach to sequence-specific resonance assignments. The ^{13}C-^{13}C correlations, in combination with multiple-bond ^{13}C-^{1}H correlations or ^{13}C-^{15}N correlations from dual $^{13}C/^{15}N$-labeled proteins, or both, can be used to trace out the peptide backbone connectivities.[1,10,11]

Sensitivity considerations limit the application of the ^{13}C-$\{^{13}C\}$DQC experiment to proteins enriched with ^{13}C. Current methods for incorporating stable isotopes into biotechnology derived proteins have begun to alleviate this problem.[12] Carbon-13 enrichment levels of 20-30% represent a good compromise between improved sensitivity and decreased spectral simplicity. Higher enrichment levels might be useful for providing long-range carbon-carbon coupling constants for selectively enriched proteins[1] but would result in increased spectral overlap in a uniformly enriched protein.

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First Direct Observation of Pyridyne: Matrix Infrared Study of the Photolysis Products of 3,4-Pyridine Dicarboxylic Anhydride

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Heteroarynes have been proposed as likely intermediates in many organic reactions, principally those involving cycloaddition or cine-substitution.[1] However, only indirect evidence, based on trapping experiments to verify the presence of heteroaryne intermediacy, has been obtained. The reliability of such inferences is severely limited. Other mechanisms, e.g., addition-elimination, trans-halogenation, or addition ring opening-elimination ring closure(ANRORC), also can account for the formation of observed products.[1] Mass spectrometric analysis following the electron impact or the pyrolytic fragmentation of several heteroarene dicarboxylic anhydrides has been used to conjecture the structure of heteroarynes corresponding to certain m/z peaks.[2-5] Although diazabiphenylene, the dimer of 3,4-pyridyne, has been identified in the time of flight mass spectrometric and kinetic UV spectroscopic analysis of the products formed by flash photolysis of pyridine-3-diazonium-4-carboxylate,[6] no direct observation of any heteroaryne has yet been published.

In this report we present the first infrared spectrum of 3,4-pyridyne (3,4-didehydropyridine), generated via near UV photolysis ($\lambda > 340$ nm) of 3,4-pyridine dicarboxylic anhydride (3,4-PDA) in N_2 or Ar matrices. Similar experiments by Dunkin and McDonald were not successful;[7] apparently the photolytic conditions utilized in that study produced only decomposition products of the desired heteroaryne.

3,4-PDA (obtained from Aldrich and vacuum sublimed before use) was sublimed and codeposited for 2 h with Ar or N_2 (flow rate 2 mmol/min) on the CsI substrate of an Air Products CS202 Displex cryostat. Photolyses were conducted with a 200 W Hg-Xe arc lamp equipped with a water filter and various cutoff filters. Infrared spectra of the precursor and photolyzed products at 13 K were recorded with a BOMEM DA3.01 interferometric spectrometer.

As summarized in Scheme I, mild irradiation ($\lambda > 340$ nm and less than 100 min duration) of 3,4-PDA in N_2 or Ar matrices at 13 K readily fragmented the precursor to form CO, CO_2, and 3,4-pyridyne, which has a strong peak at 2085 cm^{-1} diagnostic of carbon-carbon triple bond formation. Subsequent irradiation with $\lambda > 210$-nm light immediately decomposed 3,4-pyridyne into HCN, diacetylene, acetylene, and cyanoacetylene as a result of alternative two-bond scissions. The infrared spectrum in the 2050-2300 cm^{-1} region prior to and following controlled photolysis (Figure 1) clearly demonstrates the formation of 3,4-pyridyne and its subsequent decomposition. The peak due to 3,4-pyridyne at 2085 cm^{-1} disappears upon shorter wavelength irradiation, and new peaks at 2101 cm^{-1} (HCN), 2181 cm^{-1} (diacetylene) and 2236 cm^{-1} (cyanoacetylene) begin to grow. Ten additional peaks below 2000 cm^{-1} show the same growth and decay pattern as the 2085-cm^{-1} band and are also attributable to 3,4-pyridyne (Table I).

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