^{13}C NMR of Cyanylated Flavodoxin from *Megasphaera elsdenii* and of Thiocyanate Model Compounds†

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ABSTRACT: Both of the thiol groups of *Megasphaera elsdenii* flavodoxin have been cyanylated using ^{13}C-enriched cyanide. This chemical modification increases the dissociation constant of the apoflavodoxin–flavin mononucleotide (FMN) complex from 0.4 nM to 2 μM. The thiocyanate carbons of the cyanylated cysteine residues in apoflavodoxin had ^{13}C chemical shifts of 109.4 ppm and 112.2 ppm, which were replaced by signals at 115.5 ppm and 109.6 ppm when FMN was bound. The signals at 109.4 ppm and 112.2 ppm due to the cyanylated apoflavodoxin were unstable at 28 °C, and they were slowly replaced signals at 114.5 ppm and 115.3 ppm which are attributed to an inactive form of the apoprotein, which does not bind FMN. At alkaline pH values or after prolonged incubation at neutral pH, the signals at 114.5 ppm and 115.3 ppm were replaced by signals at approximately 171 ppm. On the basis of results obtained with model compounds, the signals at 171 ppm are assigned to the 2-imino carbon of the 2-iminothiazolidine ring formed by the cyclization of the appropriate thiocyanate group. After determining the chemical shift of the thiocyanate carbon of model compounds in a range of solvents, we conclude that the thiocyanate carbons will have a minimal chemical shift of approximately 109 ppm in apolar solvents which do not contain hydrogen bond donors. In water, a more polar hydrogen-bonding solvent, the chemical shift increases to approximately 115 ppm. We also conclude that the chemical shift of a thiocyanate carbon can be used as a probe of its molecular environment. Finally, we discuss how the chemical shifts of the thiocyanate carbon of cyanylated apoflavodoxin, flavodoxin, and other proteins depend on the microenvironments of the thiocyanate group.

^{13}C NMR spectroscopy of proteins chemically modified by ^{13}C-enriched reporter groups can provide a powerful probe of protein structure and function (Malthouse, 1986). The cyanylation of a cysteine residue to produce a β-thiocyanatoalanine (S-cyanocysteine) residue provides a simple way of introducing a small ^{13}C-enriched group into a protein. Three methods for cyanylation have been used. The first involves the reaction of cyanide ion with the disulfide of a protein cystinyl residue (Catsimpoolas & Wood, 1966). In the second method, a cyanide ion reacts with a mixed disulfide formed by prior reaction of the thiolate ion of a cysteinyl residue with a reagent such as 5,5′-dithiobis(2,2′-nitrobenzoic acid) (DTNB)[1] (Vanaman & Stark, 1970; Degani et al., 1970). In the third method, the thiolate ion of a cysteinyl residue reacts with a thiocyanate, such as 2-nitro-5-thiocyanatobenzene or thiocyanatopurine ribose triphosphate, to yield a β-thiocyanatoalanine residue in a single step (Degani et al., 1970; Yount, 1975).

β-Thiocyanatoalanine can undergo two reactions, a cyclization reaction (Schöberl et al., 1951) or an elimination reaction (Degani & Patchornik, 1974). The cyclization reaction can also occur following the cyanylation of cysteine residues in peptides and proteins. The rates increase with increasing pH, but they are often slower than with the free cysteine derivative, and when the amino group of the β-thiocyanatoalanine residue is involved in a peptide bond, the cyclization reaction is accompanied by cleavage of the peptide bond (Catsimpoolas & Wood, 1966; Jacobson et al., 1973; Degani & Patchornik, 1974). The second reaction of β-thiocyanatoalanine, involving elimination of the thiocyanate group to give dehydroalanine and thiocyanate anion, has been demonstrated with small model compounds (Degani & Patchornik, 1974), but it does not appear to be a major reaction of cyanylated proteins.

Several investigations have used cyanylation to cleave a protein at the peptide nitrogen of a cysteine residue. For example, Jacobson et al. (1973) reported the cleavage of carboxypeptidase A and three other proteins when they were treated with 2-nitro-5-thiocyanatobenzoic acid under denaturing conditions. A notable conclusion from this work was that the cyclization reaction generally occurred only after disruption of the protein's native structure (Stark, 1977). Therefore, when conditions are such that cyclization does not occur, a ^{13}C-enriched thiocyanate carbon can be used as a probe of the environment of cysteine residues in proteins. The cyano group is particularly useful for this purpose, because, being small, it is less likely than more bulky substituents of thiols to interact sterically with neighboring functional groups.

Boettcher and Martinez-Carrion (1975) were the first to use $[^{13}C]CN^-$ in an attempt to study the active site of an enzyme by NMR when they cyanylated a single cysteine residue of glutamate aspartate transaminase thought to be at or near the active site. The NMR signal at ~109 ppm (Boettcher & Martinez-Carrion, 1975; Malthouse, 1986) due to the $[^{13}C]$thiocyanate group was not affected significantly by substrates or substrate analogs or by a change of pH in the range 5.6–8.9. Therefore, it was suggested that the thiocyanate carbon might be of only limited use as an active site probe (Boettcher & Martinez-Carrion, 1975).

In contrast, the NMR signal of a ^{13}C-enriched thiocyanate carbon introduced into lactate dehydrogenase was affected

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2,2′-nitrobenzoic acid); GSCN, γ-glutamyl-β-thiocyanatoalanyl-glycine; NACCN, *N*-acetyl-β-thiocyanatoalanine; 2PDS, 2,2′-dipyridyl disulfide; TNB, thionitrobenzoate; Tris, tris(hydroxymethyl)aminomethane.

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by binding of the substrate analogs oxalate and oxamate, shifting from 114.2 ppm in their absence to 112.9 ppm on formation of a ternary complex of enzyme, NAD^+ and analog (Waldman et al., 1986; the chemical shifts have been re-referenced relative to tetramethylsilane at 0 ppm, using internal dioxane at 67.4 ppm as a secondary reference). X-ray crystallography had shown that the cysteine residue was unlikely to be involved directly in substrate binding, and these authors therefore concluded that the 1.3 ppm change in chemical shift on binding substrate analogs was due to a conformational change that occurred on formation of the ternary complex and that affected the environment of the [^{13}C]thiocyanate group.

We have applied ^{13}C-cyanylation to the study of the small flavoprotein flavodoxin. This electron-transfer protein contains a molecule of noncovalently bound FMN, and the protein from *Megasphaera elsdenii* contains two cysteine residues, at positions 57 and 127, in a polypeptide chain of molecular mass 14.6 kDa (Mayhew & Ludwig, 1975; Mayhew & Tollin, 1992; Tanaka et al., 1973, 1974). Recent 2D-NMR studies have shown that neither of the two cysteines is at the FMN-binding site (van Mierlo et al., 1990). This can also be concluded by analogy with flavodoxin from *Clostridium beijerinckii* MP, for which the crystal structure has been determined (Burnett et al., 1974). However, experiments with thiol-modifying reagents indicate that at least one of the thiols is necessary for the binding of flavin; treatment of the holoprotein with phenylmercuric acetate or DTNB causes dissociation of FMN, while treatment of apoflavodoxin with 2 mol of *p*-(chloromercuri)benzoate or *N*-ethyl maleimide/mol of protein completely abolishes FMN binding (Mayhew, 1971a,b).

In the present study, *M. elsdenii* apoflavodoxin has been cyanylated by sequential treatment of the apoprotein with DTNB and potassium cyanide; the modified protein has been found to bind FMN, albeit less strongly than the native protein. The ^{13}C-NMR spectrum of the [^{13}C]thiocyanate derivative was found to change on FMN binding. The changes have been interpreted by comparing the spectra with those of model compounds in different solvent systems. Preliminary accounts of this work have been given (Malthouse et al., 1989; Doherty et al., 1991).

MATERIALS AND METHODS

Materials. 5,5′-Dithiobis(2,2′-nitrobenzoic acid) (DTNB), dithiothreitol, *N*-acetylcysteine, glutathione (reduced), and commercial FMN were obtained from Sigma Chemical Co., Dorset, England. 2,2′-Dipyridyl disulphide (2PDS), cysteine ethyl ester hydrochloride, and 99 atom % [^{13}C]KCN were from Aldrich Chemical Co., Dorset, England, as were all of the deuterated solvents (≥98 atom % ^{2}H) used for NMR spectroscopy. [^{13}C,^{15}N]NaCN (99 atom % ^{13}C, 99 atom % ^{15}N) was obtained from MSD Isotopes, Montreal, Canada. Sodium lactate was from E. Merck, Darmstadt, Germany. Yeast extract was supplied by Oxoid, Basingstoke, England. All other chemicals were obtained either from Riedel-de-Haën AG, Hanover, Germany, or from BDH Ltd., Dorset, England.

DTNB was dissolved in 60 mM potassium phosphate buffer, pH 7.5; the solubility of DTNB in this buffer was approximately 17.5 mM. 2PDS was prepared as a saturated solution in water (1–1.5 mM). The concentrations of these solutions were determined spectrophotometrically using absorbance coefficients of 18 000 M^{-1} cm^{-1} at 323 nm for DTNB (Brocklehurst & Little, 1973) and 10 200 M^{-1} cm^{-1} at 281 nm for 2PDS (Malthouse & Brocklehurst, 1976).

The concentrations of cysteine-containing compounds were determined routinely by reacting the thiol with at least a 10-fold molar excess of DTNB at pH 9.5 and measuring the amount of thionitrobenzoate anion (TNB) released spectrophotometrically. The absorbance coefficient of TNB in alkaline solution is 13 600 M^{-1} cm^{-1} at 412 nm (Ellman, 1959). The absorbance coefficient of 2-thiopyridone is 8080 M^{-1} cm^{-1} at 343 nm (Stuchbury et al., 1975).

Preparation of Model Compounds. [*cyanato*-^{13}C]-*N*-acetyl-β-thiocyanatoalanine ([*cyanato*-^{13}C]NACCN) was prepared by sequential treatment of *N*-acetylcysteine with 2PDS and [^{13}C]KCN at room temperature. The progress of both reactions was followed by monitoring the release of 2-thiopyridone; aliquots of the reaction mixture were diluted into 100 mM potassium phosphate buffer, pH 7.5, and the concentration of 2-thiopyridone was determined spectrophotometrically. In a total volume of 1.67 L, the reaction vessel for the first step contained 35.9 mM ammonium carbonate, pH 9.5; 0.90 mM *N*-acetylcysteine; and 1.01 mM 2PDS. The reaction was complete within 2 min of adding 2PDS. [^{13}C]-KCN in water was then added to a final concentration of 2.0 mM; the cyanolysis reaction was complete after about 50 min. The reaction mixture was concentrated to a volume of approximately 200 mL by rotary evaporation and then freeze-dried overnight. 2-Thiopyridone and excess 2PDS were removed by four extractions with chloroform. The remaining white solid was dried by rotary evaporation and then dissolved in 4 mL of ^2H_2O. The pH was adjusted from 8.4 to 1.9 by addition of HCl, and the sample was freeze-dried overnight. The [*cyanato*-^{13}C]NACCN was separated from remaining ammonium chloride by extracting it with [$^2H_6$]acetone.

Cyanylation of cysteine, cysteine ethyl ester, and glutathione was performed by first reacting the cysteine-containing compound with DTNB and then adding [^{13}C]KCN to the resulting mixed disulfide. The two reactions were monitored by following the release of TNB spectrophotometrically. A slight excess (9–11 molar %) of DTNB was added to the thiol (21–24 mM) in 50 mM ammonium carbonate, pH 9.5; the reaction with DTNB was complete within 2 min. The resulting reaction mixture was freeze-dried to dryness and then dissolved in a minimal volume of 25% (v/v) ^2H_2O (the thionitrobenzoate derivative of cysteine is much less soluble than the other mixed disulfides, so that the most concentrated solution of the compound obtained was 9.75 mM). A 1.6–3.0-fold molar excess of [^{13}C]KCN was added to this solution; the cyanolysis reaction reached more than 95% of completion within about 5 min. Ten minutes after the addition of [^{13}C]KCN, the pH was lowered (from 8.7–9.0) to approximately 6.5.

Growth of Bacteria and Preparation of Cell-Free Extracts. *M. elsdenii*, strain LCl of Elsden et al. (1956), was grown in an iron-poor lactate/yeast extract medium (Mayhew & Massey, 1969) according to the procedures of Walker (1958). After 48 h of growth in cultures of 20 and 40 L, cells were concentrated using a Millipore Pellicon microfiltration system and then harvested by continuous flow centrifugation at 38000*g*. Cell-paste was freeze-dried and then ground into a fine powder with a pestle and mortar; the powder was stored at −20 °C.

Cell-free extracts were prepared by suspending the powdered cells (1 g/10 mL) in 20 mM potassium phosphate buffer, pH 7.0, which had previously been deaerated and equilibrated with N_2, and stirring the suspension for 1 h under N_2 at 37 °C. The mixture was centrifuged at 20000*g* at 4 °C; the resulting precipitate was re-extracted twice. The supernatants

from all three extractions were combined to give the cell-free extract.

Preparation of Flavodoxin, Apoflavodoxin, and FMN. Flavodoxin was purified by the method of Mayhew and Massey (1969); a slight change in the published procedure was that the first fractionation with DEAE-cellulose was performed batchwise rather than on a column. A total of approximately 1.4 g of flavodoxin, with a ratio of absorbance at 272 nm to absorbance at 445 nm (A_{272}/A_{445}) of 5.01, was purified from 200 g of dry cells. The concentration of flavodoxin was determined using an absorbance coefficient of 10 200 M^{-1} cm^{-1} at 445 nm (Mayhew & Massey, 1969).

Apoflavodoxin was prepared by treating flavodoxin with trichloroacetic acid (TCA) (Wassink & Mayhew, 1975). The white precipitate was dissolved in 0.1 M potassium phosphate buffer plus 0.3 mM EDTA, pH 7.0, and dialyzed overnight against several changes of 10 mM potassium phosphate buffer plus 0.3 mM EDTA, pH 7.0. The concentration of apoflavodoxin was determined using an absorbance coefficient of 26 700 M^{-1} cm^{-1} at 278 nm (Mayhew & Massey, 1969). In addition, it was standardized by fluorescence titration into FMN (Wassink & Mayhew, 1975), to check for inactivation during storage at 4 °C.

The FMN removed from flavodoxin was freed of TCA and neutralized as described by Mayhew and Wassink (1980) and was retained for use in FMN binding experiments.

The dissociation constant (K_d) of the FMN–apoflavodoxin complex was determined by fluorescence titration of apoprotein in FMN at pH 6.0 (Wassink & Mayhew, 1975; Mayhew, 1971c). For the cyanylated apoprotein, the K_d was determined from the x-axis intercept of a plot of the reciprocal of the change in fluorescence versus the reciprocal of the concentration of added apoprotein (Benesi & Hildebrand, 1949).

Preparation of Cyanylated Apoflavodoxin. Apoflavodoxin was modified with DTNB by mixing the protein with a small molar excess of the reagent (approximately 2.5 mol/mol of apoprotein). The reaction was carried out in 50 mM Tris-HCl, pH 8.0, at room temperature and was complete (> 95%) within 20 min. The stoichiometry of the reaction was determined by monitoring the release of TNB. The modified apoprotein was separated from TNB and excess DTNB by gel filtration on a column of Sephadex G-25 (Pharmacia) which was equilibrated with 0.1 M sodium acetate buffer, pH 5.5.

The mixed disulfide of apoflavodoxin and TNB was treated with [^{13}C]KCN to cyanolyze the disulfide. The reaction was performed at room temperature in 0.2 M Tris-HCl buffer, pH 8.0. Low molecular weight components were removed by gel filtration on Sephadex G-25 at 4 °C, with 50 mM potassium phosphate buffer, pH 6.0 as eluant. Protein-containing fractions were identified from absorbance measurements at 278 nm, and concentrated by ultrafiltration in an Amicon Diaflo cell fitted with a PM10 membrane. The concentration of cyanylated apoflavodoxin was determined at 278 nm by assuming that the absorbance coefficient in the UV region is the same as that of the native protein.

NMR Spectra. NMR spectra were obtained using a Bruker WP-80 wide-gap spectrometer operating at 20.115 Hz for ^{13}C nuclei. Protein solutions, of total volume 0.7–1.2 mL, contained 25% (v/v) ^2H_2O in 10-mm-diameter sample tubes. The spectral conditions for protein samples were the following: 0.926 s acquisition time; 1 W broadband ^{1}H decoupling power; 6 μs pulse width (90° pulse = 12 μs); 220 ppm spectral width; 1 Hz exponential weighting factor; 28 °C. For low molecular weight compounds, the sample size was 1–5 mL in 10-mm-diameter sample tubes. Unless indicated otherwise, the spectral conditions for these compounds were the same as for the protein samples, except that a pulse width of 0.7 μs was used (0.7 μs = 10° pulse; the spin lattice relaxation time of the thiocyanate carbon of [*cyanato*-^{13}C]NACCN in 25% (v/v) ^2H_2O was estimated to be approximately 70 s by the progressive saturation technique). For samples in nondeuterated solvents, a concentric tube (diameter = 4 mm) containing 99.8 atom % ^2H_2O was used to obtain a deuterium lock signal.

Table I: ^{13}C Chemical Shifts of the Cyano Carbons of Low Molecular Weight Thiocyanates[a]

compound	solvent	solvent dielectric constant	chemical shift (ppm)
ethyl thiocyanate			
	ethyl thiocyanate	nk[b]	112.15 ± 0.06
	[^{2}H]chloroform	4.8	111.97 ± 0.06[c]
	[$^2H_{12}$]cyclohexane	2.0	109.31 ± 0.06[d]
	[$^2H_6$]benzene	2.3	111.57 ± 0.06[e]
[*cyanato*-^{13}C]NACCN			
	H_2O (pH 5.8–10.0)	78.5	115.65 ± 0.01
	^2H_2O (pD 5.9)	78.3	115.63 ± 0.01
	[$^2H_6$]dimethyl sulfoxide	46.7	112.82 ± 0.01
	[$^2H_4$]methanol	32.6	113.20 ± 0.03
	[*hydroxy*-^{2}H]ethanol	24.3	112.38 ± 0.01
	[$^2H_6$]acetone	20.7	112.59 ± 0.03
	[*hydroxy*-^{2}H]-*tert*-butanol	10.9	112.11 ± 0.05
	[*formyl*-^{2}H]methyl formate	8.5	112.81 ± 0.01
	[^{2}H]chloroform	4.8	111.66 ± 0.05
[*cyanato*-^{13}C]GSCN			
	25% (v/v) ^2H_2O-(pD 6.5)	78.5	115.14 ± 0.05

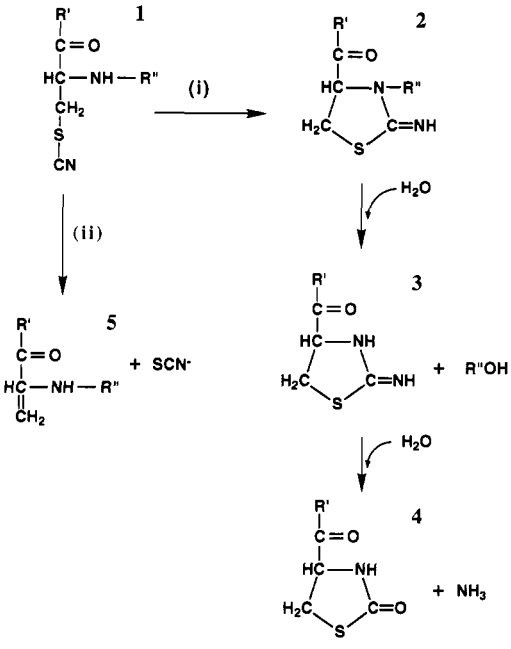
[a] The preparation of the ^{13}C-labeled compounds and the spectral acquisition conditions were as described in Materials and Methods. NMR spectra were recorded at 28 °C, except when the solvent was [*hydroxy*-^{2}H]-*tert*-butanol, when the temperature was 30 °C. The dielectric constants given are those cited for the nondeuterated solvents by Weast (1981). [b] nk = not known. [c] Mean value for 10%, 4.88%, and 2.2% (v/v) solutions of ethyl thiocyanate in [^{2}H]chloroform; the mean was 111.97 ● 0.06 (SD), resolution ± 0.05 ppm. [d] The NMR spectrum was recorded at three concentrations of ethyl thiocyanate (11.3%, 5.0%, and 1.9% (v/v)); extrapolation to 0% (v/v) gave the value indicated with an error within the resolution of ±0.05 ppm. [e] Determined using a 20% (v/v) solution of ethyl thiocyanate in [$^2H_6$]benzene.

Referencing of NMR Signals. All ^{13}C chemical shifts are quoted relative to tetramethylsilane (TMS) at 0 ppm. The methyl groups of [*hydroxy*-^{2}H]ethanol, [*hydroxy*-^{2}H]-*tert*-butanol, and [*formyl*-^{2}H]methyl formate had chemical shifts of 57.59, 31.39, and 50.88 ppm, respectively, when 5–10% TMS was used as an internal standard. Cyclohexane-d_{12} had a chemical shift of 26.26 ppm when 10% TMS was used as an internal standard. The chemical shifts of compounds dissolved in these solvents were determined in the absence of TMS using the appropriate solvent signal as a secondary standard. For all the other organic solvents used, the appropriate ^{13}C solvent signal was used as a secondary reference using the values of Wehrli et al. (1988). In aqueous solutions, 1,4-dioxane (2.5% v/v) at 67.40 ppm was used as a secondary standard.

RESULTS AND DISCUSSION

^{13}C-NMR Spectra of Thiocyanate Compounds in Various Solvents. The chemical shifts of the thiocyanate carbons of ethyl thiocyanate, [*cyanato*-^{13}C]NACCN, and [*cyanato*-^{13}C]-γ-glutamyl-β-thiocyanatoalanyl-glycine ([*cyanato*-^{13}C]GSCN) were determined in a range of solvents (Table I). In

Scheme I



	R'	R"	Compound 1
a	OH	H	β-thiocyanoalanine
b	OH	acetyl	N-acetyl-β-thiocyanoalanine
c	OC_2H_5	H	β-thiocyanoalanine ethyl ester
d	glycine	γ-glutamyl	γ-glutamyl-β-thiocyanoalanyl-glycine

water, the thiocyanate carbons of [*cyanato*-^{13}C]NACCN (structure **1b**, Scheme I) and [*cyanato*-^{13}C]GSCN (structure **1d**, Scheme I) had chemical shifts at 115.7 and 115.1 ppm, respectively (Table I). As [*cyanato*-^{13}C]NACCN was insoluble in benzene and cyclohexane, the chemical shift of the thiocyanate carbon in these solvents was obtained using ethyl thiocyanate. The chemical shifts of the thiocyanate carbons of ethyl thiocyanate and [*cyanato*-^{13}C]NACCN are similar in [^{2}H]chloroform (Table I), and so we expect the chemical shift of the thiocyanate carbon of ethyl thiocyanate in benzene and cyclohexane to be similar to that of the thiocyanate carbon of [*cyanato*-^{13}C]NACCN in these solvents. However, as the ethyl thiocyanate used was not ^{13}C-enriched, a high concentration had to be used (2–10% v/v) in order to determine the chemical shift of the thiocyanate carbon. The chemical shift of the thiocyanate carbon of ethyl thiocyanate (Table I) was independent of solute concentration (2–10% v/v ethyl thiocyanate) in chloroform and was similar to the value of 112.1 ppm reported by Simons (1983). However, it varied with concentration when the solvent was cyclohexane, and therefore the value in Table I was obtained by extrapolation to zero solute concentration.

The chemical shift of the thiocyanate carbon was minimal in cyclohexane and maximal in water (Table I). Cyclohexane is apolar and does not form hydrogen bonds, whereas water is a polar solvent which readily acts as a hydrogen bond donor, and therefore it appears that the chemical shift increases with an increase in the polarity of the solvent and/or in its ability to act as a hydrogen bond donor. Similar solvent effects have been observed for carbonyl carbons in the same solvents (Maciel & Ruben, 1963; Maciel & Natterstad, 1965; Stothers, 1972), and it was concluded that while there is a general trend for chemical shifts to increase with an increase in solvent polarity and/or with an increase in the solvent's ability to act as a hydrogen bond donor, no strict linear relationships can be demonstrated. From the results in Table I, it is clear that similar conclusions apply to the solvent-dependent changes in the chemical shifts of thiocyanate carbons.

The results described above show that the thiocyanate carbons of cyanylated cysteine residues in proteins have the potential to act as probes of their environment. A chemical shift of approximately 109 ppm indicates that the thiocyanate group is not hydrogen-bonded and that it is in an apolar environment equivalent to a cyclohexane solvent. But protein hydrogen bond donors may form hydrogen bonds with thiocyanate groups buried within a protein, and these hydrogen bonds may be as strong as, or stronger than, those formed between the thiocyanate group and water. Therefore, while chemical shifts of 115–116 ppm are expected for thiocyanate groups in a fully aqueous environment, buried thiocyanate carbons may have similar or larger chemical shifts, depending on the polarity of their environment and on the strengths of their hydrogen bond interactions.

The chemical shift of the thiocyanate carbon of [*cyanato*-^{13}C]NACCN was only slightly perturbed, from 115.1 ± 0.05 ppm to 115.6 ± 0.05 ppm, on ionization of its carboxy group ($pK_a' = 2.73 \pm 0.12$). As the mean distance of the carboxy oxygens from the thiocyanate carbon is ~4.4 Å, it is apparent that negatively charged groups at this distance have little effect on the chemical shift of a thiocyanate carbon in fully aqueous media. However, it is likely that a much larger titration shift would be observed if a group formed a good hydrogen bond to the thiocyanate carbon.

Stability of β-Thiocyanatoalanine-Containing Compounds. When an excess of [^{13}C]KCN was added to cysteine–TNB in water at pH 6.5, no signal around 115 ppm was observed, showing that β-thiocyanatoalanine (structure **1a**, Scheme I) was absent. However, a signal at 174.5 ppm was detected, and it was assigned to 2-iminothiazolidine-4-carboxylic acid formed by the cyclization of β-thiocyanatoalanine (structure **2a**, Scheme I) (Schöberl et al., 1951). When cysteine was replaced by its ethyl ester in this experiment, the only signal detected was at 174.5 ppm due to the ethyl ester of 2-iminothiazolidine-4-carboxylic acid (structure **2c** Scheme I). Therefore, both β-thiocyanatoalanine and its ethyl ester cyclize rapidly ($t_{1/2} < 1$ h) at pH 6.5 (Scheme I).

The cyclization process is much slower in β-thiocyanatoalanine derivatives which do not contain a free amino group (Degani & Patchornik, 1974). Therefore, the thiocyanate carbons of both [*cyanato*-^{13}C]NACCN and [*cyanato*-^{13}C]GSCN had stable signals ($t_{1/2} > 100$ h), at 115.6 ppm and 115.1 ppm, respectively, at neutral pH values (Table I). With a raise in pH from 6.6 (Figure 1a) to 9.8 (Figure 1b–i), the signal at 115.1 ppm due to the thiocyanate carbon of [*cyanato*-^{13}C]-GSCN decreased in intensity, while there was a concomitant increase in the intensity ($t_{1/2} \sim 1$ h) of a new signal at 168.6 ppm (Figure 1) due to the formation of a cyclic 2-iminothiazolidine derivative (structure **2d** or **3d**, Scheme I). Degani and Patchornik (1974) have shown that at pH 10 [*cyanato*-^{13}C]GSCN is converted to 2-iminothiazolidine-4-formylglycine (structure **3d**, Scheme I) and glutamic acid, with the latter cyclizing to 2-pyrrolidone-5-carboxylic acid. Therefore, the signal at 168.6 ppm, is assigned to 2-iminothiazolidine-4-formylglycine (structure **3d**, Scheme I). The signal at 157.4 ppm (Figure 1) was due to a small amount of free cyanide (Table II).

[*cyanato*-^{13}C]NACCN was the most stable of the cyanylated cysteine derivatives studied. When the pH was raised to 10.0, the signal at 115.6 ppm (Table I) disappeared slowly ($t_{1/2} \sim 100$ h) and was replaced by a signal at 167.6 ppm.

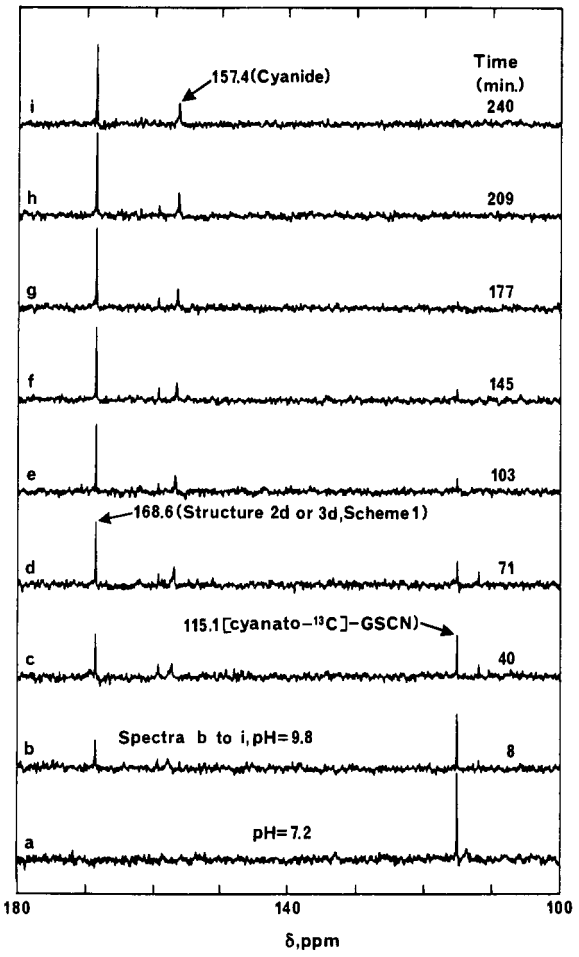


FIGURE 1: ^{13}C-NMR time course of the cyclization of [*cyanato*-^{13}C]-γ-glutamyl-β-thiocyanatoalanyl-glycine at pH 9.8. Glutathione was cyanylated by sequential treatment with DTNB and [^{13}C]KCN at pH 8.8. The pH was adjusted to 6.6, and the sample was divided into two equal aliquots. The pH of one aliquot was adjusted to 7.2, and the spectrum (a) was recorded after 48 h. The pH of the other aliquot was adjusted to 9.8 and the spectra (b–i) were recorded sequentially, commencing at 8, 40, 71, 103, 145, 177, 209, and 240 min, respectively, after elevation of the pH. Each spectrum resulted from 2048 accumulations (total accumulation time/spectrum = 31.6 min). The concentration of cyanylated glutathione was calculated from the amount of TNB released in the cyanolysis step: 25.8 mM [*cyanato*-^{13}C]-γ-glutamyl-β-thiocyanatoalanyl-glycine in 25% (v/v) D_2O, 100 mM potassium phosphate.

This signal at 167.6 ppm at pH 10.0 lies on the titration curve for 2-iminothiazolidine-4-carboxylic acid (Table II), indicating that cyclization of [*cyanato*-^{13}C]NACCN was accompanied by cleavage of the amide bond (see structures **2b** and **3b**, Scheme I).

In order to determine whether or not 2-iminothiazolidines were hydrolyzed to 2-ketothiazolidines (structures **4a–d**, Scheme I), glutathione was cyanylated using [^{13}C,^{15}N]NaCN. At pH 6.8, the signal at 115.2 ppm was coupled to ^{15}N with J_{CN} = 13.0 Hz (Figure 2a). With a raise in the pH to 9.7 (Figure 2b–i), the signal from the thiocyanate carbon at 115.2 ppm was replaced by a signal at 168.6 ppm, which was also coupled to ^{15}N (J_{CN} = 19.4 Hz). This shows that the ^{13}C–^{15}N bond is intact and that the 2-iminothiazolidine is not hydrolyzed to the 2-ketothiazolidine.

The minor doublet at 113.1 ppm (J_{CN} = 18.4 Hz) in Figure 2a was due to excess [^{13}C,^{15}N]NaCN. Two minor doublets are also evident in Figure 2 (spectra c–i), one at 162.1 ppm and the other at 134.0 ppm, and these had J_{CN} values of 20.5 and 14.0 Hz, respectively. These signals were also just detectable in the spectra of the [2-^{13}C]ITC-glycine produced from [^{13}C]GSCN when large numbers of transients were accumulated (not shown). The carbon of unenriched potassium thiocyanate has a chemical shift of 134.0 ppm, which is independent of pH in the range pH 2.2–13.5. The signal at 134.0 ppm in Figure 2 is therefore assigned to thiocyanate formed by β-elimination from β-thiocyanatoalanine (pathway ii, Scheme I). We are unable to assign a structure to the species giving rise to the signal at 162.1 ppm; this signal is not due to cyanide ion, which has a chemical shift of 151.5 ppm at pH 9.7 (Table II).

The chemical shifts of the 2-iminothiazolidine compounds were pH-dependent. Table II shows that the differences in chemical shifts between the protonated and unprotonated species of the iminothiazolidine derivatives studied are all in the range 6.8–7.8 ppm. 2-Iminothiazolidine-4-carboxylic acid has chemical shifts which are somewhat lower than those of the other two iminothiazolidines, and its pK_a (8.91) is considerably higher. These differences may be due to the presence of the negatively charged carboxy group attached to the ring of 2-iminothiazolidine-4-carboxylic acid. A similar but slightly lower pK_a, at 8.48, for 2-iminothiazolidine-4-carboxylic acid was reported by Gawron et al. (1962).

Cyanylation of Apoflavodoxin. When DTNB was added to apoflavodoxin at pH 8.0 (2.5 mol of DTNB/mol of apoflavodoxin), 1.9 mol of TNB was released per mol of apoflavodoxin, showing that both cysteine residues of apoflavodoxin were chemically modified by DTNB. The UV–visible absorption spectrum of the modified protein had absorption maxima at 278 nm and 323 nm. The absorption band centered at 323 nm is identical to that of DTNB (Ellman, 1959) and it is attributed to the 5-thio-2-nitrobenzoate derivative of the modified protein. This mixed disulfide of apoprotein and TNB did not bind FMN. On addition of excess dithiothreitol, the absorption peak at 323 nm was replaced by an absorption maximum at 412 nm, and the apoprotein's affinity for FMN was restored to that of native apoflavodoxin (K_d = 0.4 nM). This shows that chemical modification of apoflavodoxin with DTNB does not denature the protein irreversibly.

When a 230-fold excess of KCN was added to the mixed disulfide of apoflavodoxin and TNB at pH 8.0, the increase in absorbance at 412 nm showed that all of the TNB was released from the protein. Low molecular mass molecules were removed by gel filtration and the absorbance spectrum of the cyanylated apoflavodoxin was identical to that of unmodified apoflavodoxin, confirming that no TNB remained bound to the protein.

In contrast to the mixed disulfide of apoflavodoxin and TNB, the cyanylated apoprotein bound FMN. The dissociation constant for the protein–FMN complex, as measured by fluorescence titration, was 2 μM. Although cyanylation results in 5000-fold weaker binding of FMN, this is the first chemical modification to the thiols of *M. elsdenii* flavodoxin that has not abolished flavin binding. The spectrophotometric changes that occurred when cyanylated apoflavodoxin was added to FMN were similar to those observed during the binding of flavin to the untreated apoprotein (Mayhew, 1971b). A spectrophotometric titration showed that the stoichiometry of binding was 0.72 mol of FMN/mol of cyanylated apoprotein. The fraction of cyanylated apoflavodoxin which did not bind FMN is henceforth described as "inactive" apoprotein; "active" apoprotein is used to denote protein which did bind FMN.

^{13}C-NMR Spectra of ^{13}C-Cyanylated Apoflavodoxin and Flavodoxin. When ^{13}C-cyanylated apoflavodoxin was incu-

Table II: Effect of pH on the ^{13}C Chemical Shifts of [2-^{13}C]-2-Iminothiazolidine-Containing Compounds and [^{13}C]Cyanide[a]

compound	chemical shifts (ppm)		pK_a	titration shift (ppm)
	low pH	high pH		
[2-^{13}C]ITC	174.61 ± 0.02	166.89 ± 0.03	8.91 ± 0.02	7.72 ± 0.04
[2-^{13}C]ITC-EE	175.30 ± 0.029	168.50 ± 0.03	7.44 ± 0.03	6.80 ± 0.04
[2-^{13}C]ITC-glycine	175.82 ± 0.02	168.61 ± 0.02	7.14 ± 0.02	7.21 ± 0.03
[^{13}C]cyanide	112.70 ± 0.06	166.41 ± 0.13	9.29 ± 0.01	53.71 ± 0.14

[a] Cysteine, cysteine ethyl ester, and glutathione were cyanylated and cyclized to produce [2-^{13}C]ITC (structure **2a**, Scheme I), [2-^{13}C]ITC-EE (structure **2c**, Scheme I), and [2-^{13}C]ITC-glycine (structure **3d**, Scheme I) respectively, as described in the text. Samples (0.01–0.1 M) were titrated in 25% (v/v) ^2H_2O using HCl or KOH. pH-dependent changes in chemical shift were analyzed as described by Malthouse et al. (1985).

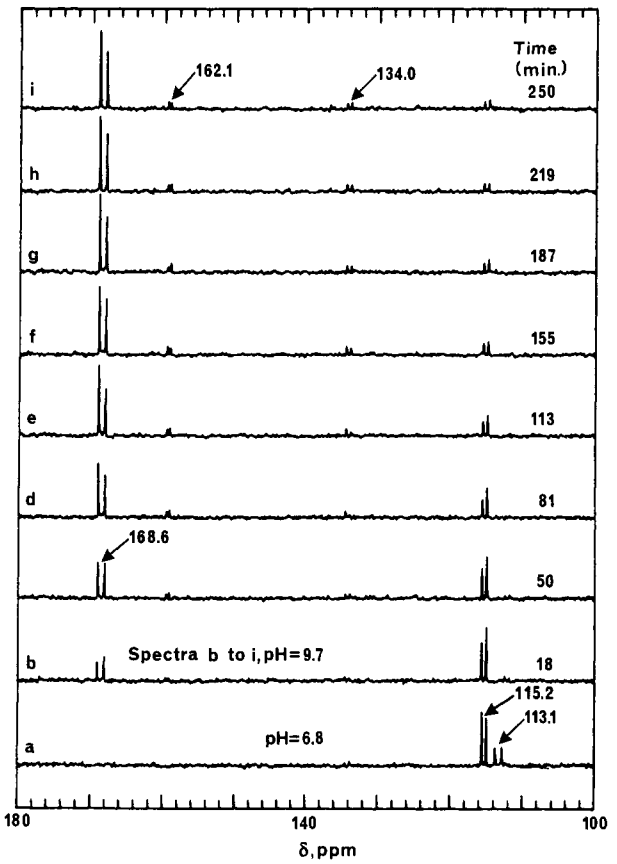


FIGURE 2: ^{13}C-NMR time course of the cyclization of [*cyanato*-^{13}C,^{15}N]-γ-glutamyl-β-thiocyanatoalanyl-glycine at pH 9.7. Glutathione was cyanylated by sequential treatment with DTNB and [^{13}C,^{15}N]NaCN at pH 8.6. The pH was adjusted to 6.6, and the sample was divided into two equal aliquots. The pH of one aliquot was adjusted to 6.8, and the spectrum (a) was recorded after 8 h. The pH of the other aliquot was adjusted to 9.7, and the spectra (b–i) were recorded sequentially, commencing at 18, 50, 81, 113, 155, 187, 219, and 250 min, respectively, after elevation of the pH. Each spectrum is the product of 1024 accumulations (total accumulation time/spectrum = 31.6 min). Concentrations were 48 mM [*cyanato*-^{13}C,^{15}N]-γ-glutamyl-β-thiocyanatoalanyl-glycine in 25% (v/v) D_2O, 196 mM potassium phosphate.

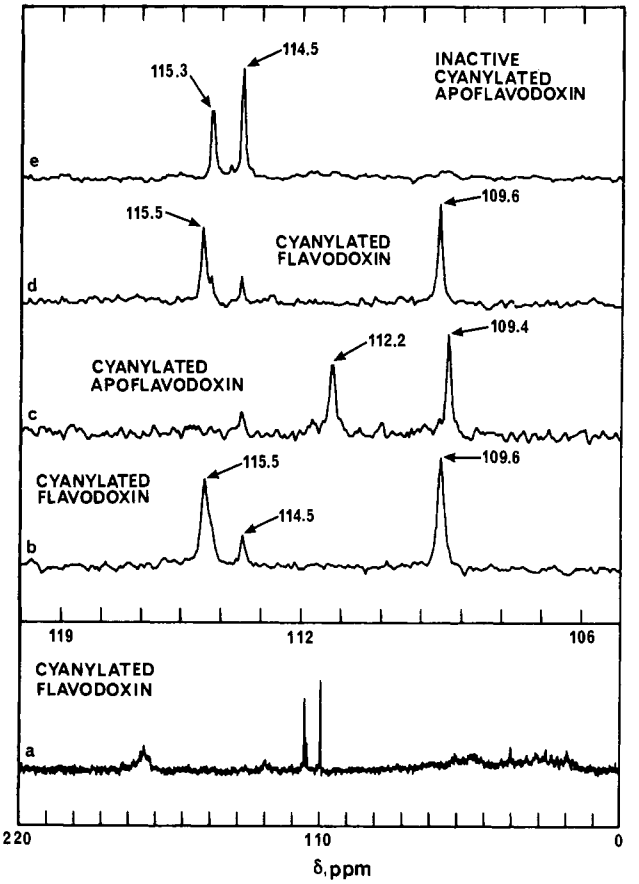


FIGURE 3: ^{13}C-NMR spectra of cyanylated flavodoxin and apoflavodoxin. Panels a and b show ^{13}C-cyanylated flavodoxin. ^{13}C-Cyanylated apoflavodoxin was incubated with a 1.5-fold molar excess of FMN immediately after separation of the modified protein from the other reaction products of cyanolysis. The cyanylated holoprotein was then concentrated by ultrafiltration to give 0.97 mM cyanylated flavodoxin in 25% (v/v) ^2H_2O and 37.5 mM potassium phosphate, pH 6.0. Number of accumulations = 78 259. (c) FMN was removed from the sample in (b) by TCA extraction to give 0.74 mM cyanylated apoflavodoxin in 25% (v/v) ^2H_2O and 37.5 mM potassium phosphate, pH 6.0. Number of accumulations = 61 840. (d) FMN was added to (c) to a final concentration of 1.45 mM to give 0.73 mM cyanylated flavodoxin in 25% (v/v) ^2H_2O and 37.5 mM potassium phosphate, pH 6.0. Number of accumulations = 80 000. (e) Inactive ^{13}C-cyanylated apoflavodoxin was prepared as described in the text. Excess [^{13}C]KCN was removed by gel filtration, and the protein was concentrated by ultrafiltration to give 1.98 mM cyanylated apoflavodoxin in 25% (v/v) ^2H_2O and 37.5 mM potassium phosphate, pH 6.3. Number of accumulations = 84 197.

bated with a 1.5-fold molar excess of FMN immediately after its preparation, the resulting proton-decoupled NMR spectrum at pH 6 showed two major signals, at 109.6 ppm and 115.5 ppm (Figure 3a,b). These signals were not evident in the NMR spectrum of native flavodoxin, nor could they be assigned to FMN (Malthouse et al., 1989). When the bound FMN was removed by TCA extraction, the signals at 109.6 ppm and 115.5 ppm disappeared, and new signals appeared at 109.4 ppm and 112.2 ppm (Figure 3c). These changes were reversible; the subsequent addition of FMN yielded cyanylated holoprotein (Figure 3d) with the major signals of Figure 3b.

Two minor signals, at 114.5 ppm and 115.3 ppm, are also evident in Figure 3, the former in all of the spectra shown and the latter in spectra d and e. At least one of these signals was observed in all preparations of ^{13}C-cyanylated (apo)flavodoxin studied. When excess [^{13}C]KCN was added to apoflavodoxin–$(TNB)_2$ which had been left at 28 °C for 30 h, only the signals at 114.5 ppm and 115.3 ppm were visible after gel

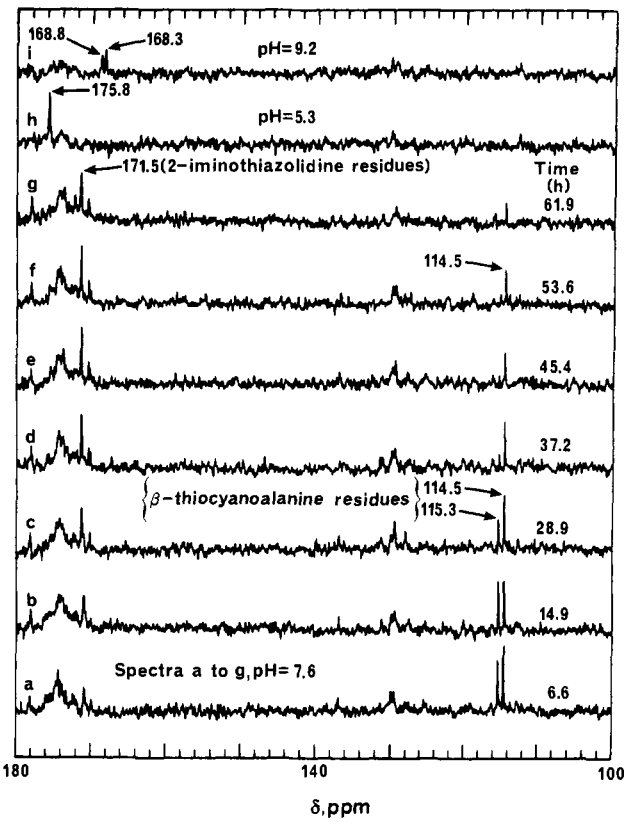


FIGURE 4: Iminothiazolidine formation in ^{13}C-cyanylated apoflavodoxin. (a–g) ^{13}C-NMR time course of the cyclization of the β-thiocyanatoalanine residues of ^{13}C-cyanylated apoflavodoxin at pH 7.6. Inactive ^{13}C-cyanylated apoflavodoxin was obtained by incubating the active protein at 25 °C for 3 days at pH 5.9. The pH was adjusted to 7.6 and the spectra (a–g) were recorded. Each of the spectra a–g represents 32 000 accumulations (total accumulation time/spectrum = 8.23 h). Spectra a–g were recorded 6.6, 14.9, 28.9, 37.2, 45.4, 53.6, and 61.9 h, respectively, after the pH was raised to 7.6. The sample contained approximately 2 mM ^{13}C-cyanylated apoflavodoxin in 25% (v/v) ^2H_2O and 56 mM potassium phosphate at pH 7.6. (h and i) ^{13}C-NMR pH titration of the product of the cyclization process: ^{13}C-NMR spectra after adjustment of the sample shown in (g) to pH 5.3 (spectrum h; 16 000 accumulations) and pH 9.2 (spectrum i; 48 000 accumulations).

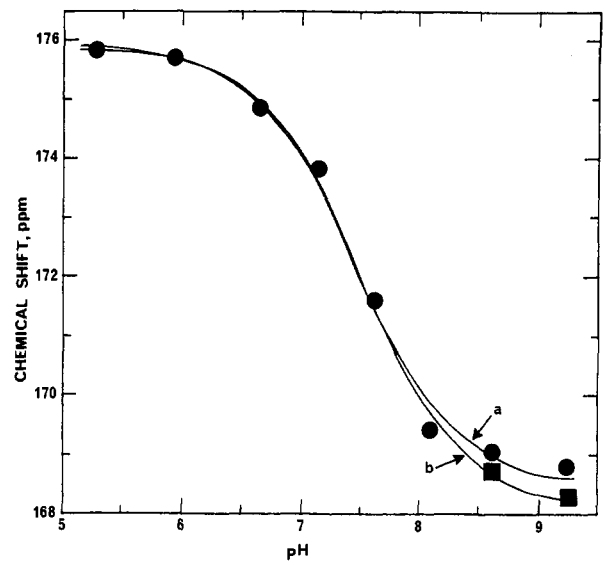


FIGURE 5: pH titration of the iminothiazolidine signals of cyanylated apoflavodoxin. The pH of the sample of Figure 4g was adjusted to the values indicated and the resulting ^{13}C-NMR spectra were recorded. The continuous lines were calculated using the equation $\delta_{obs} = (S_1 + S_2K_a/[H^+])/(1 + K_a/[H^+])$ and the following fitted parameters (Malthouse et al., 1985): for curve (a) $pK_a = 7.48 \pm 0.06$, $S_1 = 175.92 \pm 0.08$ ppm, and $S_2 = 168.48 \pm 0.09$ ppm; for curve (b) $pK_a = 7.52 \pm 0.04$, $S_1 = 175.88 \pm 0.05$ ppm, and $S_2 = 168.11 \pm 0.05$ ppm.

filtration to remove unreacted cyanide (Figure 3e). The NMR spectrum remained unchanged when a 9% molar excess of FMN was added to the sample, and no FMN was bound by the protein as judged by fluorescence measurements. Therefore, the signals at 114.5 ppm and 115.3 ppm were ascribed to an inactive form of cyanylated apoprotein, which could not bind FMN.

The results obtained with the model compounds (Table I) indicate that all of the resonances of the ^{13}C-cyanylated protein preparations in the range 109–116 ppm are due to the thiocyanate carbons of β-thiocyanatoalanine residues and that each signal represents a thiocyanate carbon in a different microenvironment.

The signals at 109.6 ppm and 115.5 ppm from the cyanylated holoprotein were stable for at least a week at 28 °C or 4 °C, and the signals at 109.4 ppm and 112.2 ppm due to the cyanylated apoprotein showed similar stability at 4 °C. However, at 28 °C and pH 6.5, the NMR signals of active cyanylated apoflavodoxin at 112.2 ppm and 109.4 ppm disappeared gradually ($t_{1/2} \sim 15$ h at pH 6.5) to be replaced by the signals at 115.3 ppm and 114.5 ppm of the inactive modified apoprotein. When the pH was raised to 7.6, the signals at 115.3 ppm and 114.5 ppm decreased slowly and a new signal appeared at approximately 171 ppm (Figure 4, spectra a–g). Figure 4 shows that the signal at 115.3 ppm disappeared more quickly than that at 114.5 ppm (the half-lives were 15 h and 38 h, respectively, at pH 7.6). Like the similar signals of the low molecular mass iminothiazolidine compounds described earlier (Figures 1 and 2), the signal at 171 ppm was pH-dependent (Figure 4, spectra h and i; Figure 5), indicating that the time course shown represents the cyclization of the two β-thiocyanatoalanine residues to the iminothiazolidine form. At the most alkaline pH value used (pH 9.2), the signal was resolved into two peaks at 168.8 ppm and 168.3 ppm (Figure 4, spectrum i), providing further evidence that both of the β-thiocyanatoalanine residues had cyclized. The pK_a values of the two iminothizaolidine species were determined to be 7.48 ± 0.06 and 7.52 ± 0.04 (Figure 5a,b). Cyclization also occurred at pH 6.5, with the signal at 114.5 ppm again disappearing more slowly than that at 115.3 ppm, but the process was approximately 5-fold slower at this lower pH value.

GENERAL DISCUSSION

From our studies on model compounds (Table I), we conclude that a thiocyanate carbon in water, a polar solvent with strong hydrogen-bonding capacity, will have a chemical shift of approximately 115 ppm, whereas a thiocyanate carbon in an apolar, non-hydrogen-bonding solvent such as cyclohexane will have a chemical shift at about 109 ppm. The signals at 114.5 ppm and 115.3 ppm in the inactive cyanylated apoflavodoxin (Figure 3e) could be due to thiocyanate carbons which are either fully exposed to water or are buried within the protein in an environment equivalent to that of water. In contrast, the signals at 109.4 ppm and 112.2 ppm in the active cyanylated apoprotein (Figure 3c) suggest that both thiocyanate groups are in environments in which the hydrogen-bonding capacity and/or polarity are less than those of water. The signal at 109.4 ppm indicates that one thiocyanate group is in an apolar environment devoid of hydrogen

bond donors, equivalent to a cyclohexane solvent (Table I).

When FMN is bound to cyanylated apoflavodoxin, signals at 115.5 ppm and 109.6 ppm are observed (Figure 3b,d). The signal at 109.6 ppm indicates that the thiocyanate group is not hydrogen bonded and is buried in an apolar environment. The signal at 115.5 ppm in the cyanylated holoprotein could result from the thiocyanate group being (1) fully exposed to water on the surface of the protein, (2) fully exposed to an aqueous environment but buried in a "pocket" of water below the surface of the protein, or (3) buried within the protein in an environment which is equivalent to water. The slow reaction of the native holoprotein with DTNB and other thiol reagents suggests that neither cysteine is readily accessible at the surface of the protein (Mayhew, 1971a). If cyanylation does not perturb the structure of the holoprotein, this would suggest that the thiocyanate group giving the signal at 115.5 ppm must be buried; i.e., either possibility (2) or (3) applies. However, since cyanylation results in a 5000-fold reduction in the affinity of the apoprotein for FMN, we cannot assume that it does not change the conformations of the apoprotein and holoprotein. Therefore, the signal at 115.5 ppm in the cyanylated holoprotein could arise from any of the possibilities (1), (2), or (3).

If the signal at 112.2 ppm moves to 115.5 ppm on FMN binding, then the binding of the flavin must only perturb the signal at 109.4 ppm to a value at 109.6 ppm. Alternatively, we cannot dismiss the possibility that the signal at 109.4 ppm moves to 115.5 ppm and the signal at 112.2 ppm moves to 109.6 ppm, on FMN binding. It is unlikely that the chemical shifts of the thiocyanate carbons will be perturbed by a direct interaction with FMN, because neither of the cysteine residues of *M. elsdenii* flavodoxin is at the flavin-binding site (Burnett et al., 1974; van Mierlo et al., 1990). This suggests that the changes in chemical shifts of the thiocyanate carbons of cyanylated apoflavodoxin on binding FMN must reflect changes in the microenvironments of the β-thiocyanatoalanine residues that result from conformational changes which accompany the binding of FMN by the cyanylated apoprotein.

Examination of the X-ray structure of the analogous flavodoxin of *C. beijerinckii* MP (Burnett et al., 1974) leads us to conclude that the sulfur atom of cysteine 53 (Cys 54 in *M. elsdenii* flavodoxin) is more closely surrounded by hydrophobic side chains than is the sulfur of cysteine 128 (Cys 127 in *M. elsdenii* flavodoxin). Also, the 2D-NMR results of van Mierlo et al. (1990) suggest that Cys 54 of *M. elsdenii* flavodoxin is buried more deeply within the protein than is Cys 127. This suggests that the signals at 109.4 ppm and 109.6 ppm may be due to the thiocyanate carbon of the cyanylated Cys 54 in the apo- and holoproteins, respectively, while the signals at 112.2 ppm and 115.5 ppm may be due to the thiocyanate carbon of the cyanylated Cys 127 residue in the apo- and holoproteins, respectively. However, such assignments are not unambiguous, since cyanylation must perturb the structure of the apoprotein to cause its decreased affinity for FMN.

The ^{13}C-NMR results clearly demonstrate that FMN binding changes the chemical shift of one of the thiocyanate carbons to a value of 115.5 ppm. However, this change of 3–6 ppm in chemical shift could result from a small structural change whereby the thiocyanate group is more effectively hydrogen bonded, but the overall conformation of the protein is not significantly perturbed. Two-dimensional NMR (van Mierlo, 1990) and temperature jump studies (Gast & Müller, 1978) have provided no evidence of conformational changes when unmodified apoflavodoxin binds FMN. However, the far-UV circular dichroism spectra of the apo- and holoproteins suggested a significant increase in random coil structure on removal of FMN from the holoprotein (D'Anna & Tollin, 1972), while the fact that thiol reagents react rapidly with the apoprotein but slowly with the holoprotein (Mayhew, 1971a,b) suggests that FMN binding causes a change in conformation, since the cysteines are not masked directly by the FMN. Therefore, although FMN binding to the unmodified apoflavodoxin must change the environment of the thiol groups, this change, and that which accompanies FMN binding by the cyanylated apoflavodoxin, may not result in any significant perturbation of the overall protein conformation.

We suggest that the NMR signals of the inactive cyanylated apoflavodoxin at 114.5 ppm and 115.3 ppm probably arise from β-thiocyanatoalanine residues which are exposed to water due to the denatured protein adopting a more extended conformation. The iminothiazolidine signal at 171 ppm is formed at the expense of the thiocyanate signals at 114.5 ppm and 115.3 ppm in the inactive cyanylated apoflavodoxin (Figure 4), suggesting that cyclization of the apoprotein's β-thiocyanatoalanine residues to give the iminothiazolidines occurs only after an initial disruption of the modified protein's tertiary structure has taken place. This is consistent with the observation of Stark (1977) that disruption of a protein's native structure generally precedes iminothiazolidine formation following cyanylation.

^{13}C-Cyanylation of a single cysteine residue of pig heart glutamate aspartate transaminase gave an NMR signal which varied between 108.8 ppm and 109.4 ppm, depending on the incubation conditions (Boettcher & Martinez-Carrion, 1975).[2] These chemical shift values are very similar to those observed for ethyl thiocyanate in cyclohexane (Table I), suggesting that the β-thiocyanatoalanine residue in the transaminase was in a non-hydrogen-bonding, hydrophobic environment, which changed little upon substrate binding. In contrast, cyanylation of pig heart lactate dehydrogenase gave rise to a β-thiocyanatoalanine residue (δ = 114.2 ppm) in what was apparently a relatively polar and/or hydrogen-bonding environment in the free enzyme (Waldman et al., 1986). Formation of a ternary complex of enzyme, NAD^+, and substrate analog caused the signal to move 1.3 ppm to 112.9 ppm (Waldman et al., 1986), suggesting that the residue's microenvironment became less polar and/or less hydrogen bonding. It is notable that no signals other that those which can be ascribed to β-thiocyanatoalanine residues were reported following cyanylation of either of the two enzymes from pig heart, indicating that no significant cyclization of the β-thiocyanatoalanines had occurred in these experiments.

We have shown that changes in the environment of the thiocyanate carbon of model compounds and cyanylated cysteine residues in proteins can alter the ^{13}C chemical shift of the thiocyanate carbon by at least 6 ppm. Our results with model compounds show that in an apolar environment devoid of hydrogen bond donors the thiocyanate carbon will have a chemical shift of $\sim$109 ppm. However, in water, a polar solvent which is a good hydrogen bond donor, its chemical shift is $\sim$115 ppm. Therefore, we conclude that the chemical shift of the thiocyanate carbon can be used as a probe of the environment of the thiocyanate group. Cyanylation of the thiol groups of cysteine residues in proteins is one of the smallest chemical modifications possible, and it is expected to produce

[2] These authors gave ^{13}C chemical shifts relative to the chemical shift of the guanidino carbon of arginine, which is assumed here to be 157.5 ppm relative to TMS, given that internal dioxane is at 67.4 ppm (Jardetzky & Roberts, 1981).

a minimal perturbation of the protein's structure. It is therefore not surprising that, since cyanylation of the thiol groups of *M. elsdenii* apoflavodoxin reduces its affinity for FMN 5000-fold, chemical modification of the thiols with larger substituents completely abolishes FMN binding (Mayhew, 1971b). The decrease in the affinity of apoflavodoxin caused by cyanylation shows that unmodified thiol groups are essential for optimal FMN binding. This suggests that oxidation of the thiol groups to SO, SO_2, or SO_3 should reduce the affinity of apoflavodoxin for FMN. Because cyanylation alters the conformation of flavodoxin, we cannot use the structure of the unmodified protein to determine unambiguously which cyanylated cysteine residue produces a given NMR signal. Such assignments may be facilitated by similar studies on other, analogous flavodoxins, by structural analysis of the cyanylated protein by 2D-NMR or X-ray crystallography, and by site-directed mutagenesis.

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