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Carbon-13 Nuclear Magnetic Resonance Spectroscopy of [2-^{13}C]Carboxymethylcytochrome *c*†

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ABSTRACT: Horse heart cytochrome *c* has been carboxymethylated under various reaction conditions using [2-^{13}C]bromoacetate. Direct analysis of reaction products using ^{13}C nuclear magnetic resonance spectroscopy shows that the protein can be much more extensively modified than has previously been assumed. The proximity of one carboxymethylmethionine residue to the paramagnetic center of the ferric protein allows it to be distinguished from a more constant carboxymethylmethionine residue on the basis of the chemical shift of its labeled methylene group. Refolding of cytochrome *c* after alkylation at low pH apparently gives a different configuration of modified methionine residues within the protein compared to that produced by alkylation at neutral pH in the presence of cyanide.

Cytochrome *c* is a small protein with a covalently linked heme prosthetic group which plays a major role in the electron transport mechanism associated with mitochondrial oxidation of organic acids. Since its biological function has been well defined and its structure at the amino acid sequence level determined for many widely varied animal and plant species, cytochrome *c* has become a prominent model protein system for the study of structure–function relationships. When this protein is perturbed from its native configuration the associated changes in biological activity, spectroscopic properties, and hydrodynamic properties give valuable clues in the still unsolved puzzle of how the molecular structure accommodates a functional requirement for electron transfer.

One of the first experimental techniques used for this purpose was selective chemical modification of native cytochrome *c*. Schejter and George (1965) found that carboxymethylation of cytochrome *c* in the presence of cyanide yielded a product with pH dependent spectral properties different from that of the native protein. Subsequently, carboxymethylation has become a prominent experimental tool in the study of cytochrome *c* and has greatly enhanced understanding the importance of the methionine-80 residue in the electron transfer function. Dramatic changes in electronic, ligand binding, and biological properties accompanying carboxymethylation at the sulfur of methionine-80 has made this particular residue the center of attention although other residues are also susceptible to alkylation.

The reaction product obtained by carboxymethylation under various conditions has been analyzed for carboxymethyl derivatives, either directly, or indirectly by loss of the parent amino acid. These methods require chemical degradation of the reaction product under conditions which could destroy some carboxymethyl derivatives, and has led to a variety of descriptions of reaction product from essentially equivalent preparations. Positive detection of some carboxymethyl derivatives of amino acids from carboxymethylcytochrome *c* in addition to *S*-carboxymethylmethionine has been reported by Babul and Stellwagen (1972): N^{δ}-carboxymethylhistidine, N^{ϵ}-carboxymethylhistidine, N^{δ},N^{ϵ}-dicarboxymethylhistidine, and N^{ϵ},N-dicarboxymethyllysine.

In order to determine the extent of carboxymethylation of cytochrome *c* directly without chemical degradation of the modified protein we have used [2-^{13}C]bromoacetate as the alkylating agent and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy for product analysis. As will be seen in the results presented here, the alkylated protein contains N^{ϵ}-monocarboxymethyllysine and glycolate esters in addition to those amino acid modifications previously detected.

Experimental Section

Sources of Materials. Bromoacetic acid enriched to 90+ atom % ^{13}C in the methylene carbon was a gift from Vernon Kerr and Donald Ott of The Los Alamos Scientific Laboratory. Amino acid and peptide derivatives were obtained from Foxx Chemical Company and Type VI horse heart cytochrome *c* was obtained from Sigma Chemical Company.

Carboxymethylation of Amino Acid and Peptide Derivatives. Amino acid and peptide derivatives were carboxymethylated by dissolving or suspending 100 μmol of each in separate aliquots containing 1.45 ml of 0.7 *M* sodium [2-

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‡ Additional support from the University Research Institute, University of Texas, Austin, Texas.

Table I: Methylene Carbon Resonances of [2-^{13}C]Carboxymethylated Model Compounds.

Parent Compound	δ (ppm) (±0.05) from Me_4Si	Assignment
Carbobenzoxymethionine methyl ester	48.3	*S*-Carboxymethyl
Carbobenzoxymethionylglycine methyl ester	48.4	*S*-Carboxymethyl
Carbobenzoxyglycylmethionine methyl ester	48.3	*S*-Carboxymethyl
Carbobenzoxyhistidylglycine methyl ester	50.1	N^{δ}-Monocarboxymethyl
Carbobenzoxyhistidylglycine methyl ester	50.8	N^{δ},N^{ϵ}-Dicarboxymethyl
Carbobenzoxyhistidylglycine methyl ester	51.5	N^{ϵ}-Monocarboxymethyl
Carbobenzoxyhistidylglycine methyl ester	53.1	N^{δ},N^{ϵ}-Dicarboxymethyl

^{13}C]bromoacetate at pH 3.3. Reactions were allowed to proceed for 4 days at 22–25° before spectra were obtained.

Preparation of Cytochrome c Derivatives. Ferricytochrome *c* was prepared by dialysis of an aqueous cytochrome solution against 10^{-4} *M* $K_3Fe(CN)_6$, followed by dialysis in distilled water and lyophilization. Ferrocytochrome *c* was prepared by adding solid $Na_2S_2O_4$ (ca. 0.5 mg/ml) to an aqueous cytochrome *c* solution. Ferricytochrome *c* (8–10 mg/ml) was carboxymethylated at room temperature in 0.18 *M* bromoacetate buffered to pH 7.0 with either 0.1 *M* Tris or 0.1 *M* NH_4^+ ammonium phosphate buffer (except where noted). The reaction was terminated by dialysis in 0.05 *M* NH_4^+ ammonium phosphate buffer (pH 7.0). Carboxymethylcytochrome *c* was recovered by lyophilization. Carboxymethylation of cyanocytochrome *c* was performed in 0.1 *M* NaCN, as above. Carboxymethylferrocytochrome *c* was prepared by treating an aqueous solution of carboxymethylferricytochrome *c* with solid $Na_2S_2O_4$ (ca. 0.5 mg/ml). The pH of solutions of carboxymethylcytochrome *c* was adjusted with phosphoric acid or concentrated potassium hydroxide solution during titrations. Carbonyl carboxymethylferrocytochrome *c* was prepared by bubbling CO slowly through an aqueous solution of carboxymethylferrocytochrome *c* and maintaining the resulting samples in an atmosphere of CO.

NMR spectroscopy. Proton decoupled ^{13}C NMR Fourier transform spectra were obtained at 25° and 25.2 MHz with a Varian XL-100-15 spectrometer interfaced to a Data General Supernova computer, using the deuterium resonance (15.4 MHz) from a D_2O capillary as a lock signal. Chemical shift values were calculated relative to the methyl carbon resonance of neat tetramethylsilane (Me_4Si) measured under the same conditions. Free induction decays of 40-μsec rf pulses were accumulated as 4096 data points in the time domain when observing a 5000-Hz region spanning the methylene carbon region. Data acquisition time was 409 msec and preliminary T_1 measurements indicated very short relaxation times for all carboxymethylmethylene resonances (ca. 40–160 msec) in the oxidized cytochromes. Relaxation times in reduced samples were found to be longer and spectra used in determination of relative abundances were obtained over a 1250-Hz range with an added delay of 1.000 sec between pulses. Total recovery time per pulse was 1.819 sec, more than five times longer than the longest observed T_1 value.

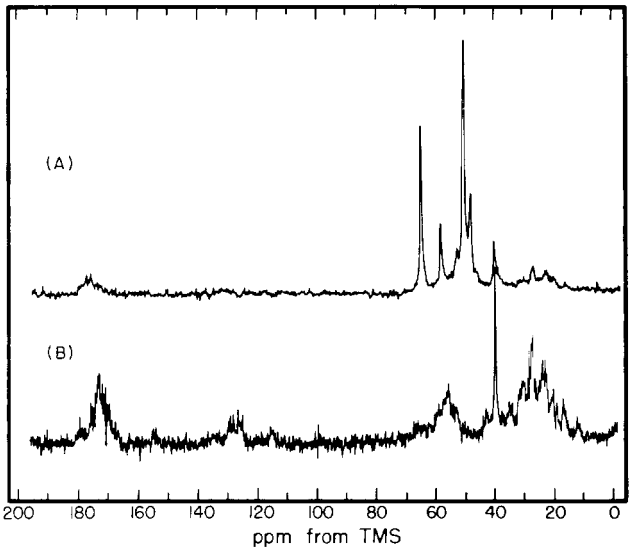

FIGURE 1: The 5000-Hz ^{13}C NMR spectra of [2-^{13}C]carboxymethylferricytochrome *c* and natural abundance carbon ferricytochrome *c*. (A) [2-^{13}C]Carboxymethylferricytochrome *c*, 10000 pulses. Carboxymethylation reaction time was 6 days at pH 7 in 0.1 *M* cyanide. (B) Ferricytochrome *c*, 49681 pulses.

Results

Methylene Carbon Chemical Shifts in Carboxymethylated Model Compounds. The carboxymethylation of blocked methionine, methionine dipeptides, and a histidine dipeptide produced methylene carbon resonances similar to those found by Nigen et al. (1973). Table I lists the chemical shifts of these resonances relative to Me_4Si.

Background Spectra from Natural Abundance ^{13}C in Cytochrome c. A typical protein ^{13}C NMR spectrum is generated from the natural abundance ^{13}C in cytochrome *c* (1.1 atom %). The spectra of oxidized ferricytochrome *c* and reduced ferrocytochrome *c* are similar but not identical. A strong resonance which appears at 40.5 ppm corresponding to the 19 lysine ε carbons is the most prominent feature in the methylene carbon region. A 5000-Hz natural abundance ^{13}C NMR spectrum of ferricytochrome *c* is shown in Figure 1 along with a corresponding spectrum of [2-^{13}C]carboxymethylferricytochrome *c*. This natural abundance ^{13}C NMR spectrum of cytochrome *c* is essentially the same as that reported by Oldfield and Allerhand (1973).

Methylene Carbon Resonances in Carboxymethylated Cytochrome c. The methylene carbon region of cyanocytochrome *c* extensively carboxymethylated with [2-^{13}C]bromoacetate is illustrated in Figure 2. Prominent resonances in this region include: 64.5 ppm, corresponding to glycolate esters of aspartic acid and glutamic acid residues (Nigen et al., 1973); 58.2 ppm, the methylene carbon of dicarboxymethyllysine; 53.1 ppm, N^{δ},N^{ϵ}-dicarboxymethylhistidine; and 40.5 ppm, the natural abundance ^{13}C of the ε-methylene carbon of lysine. Between 49 and 52 ppm there is a complex resonance signal dominated by the methylene carbon of monocarboxymethyllysine (50.4 ppm) but derived also from the methylene carbon of N^{δ},N^{ϵ}-dicarboxymethylhistidine (50.8 ppm), which is partially resolved in spectra at higher resolution (Figure 3), and the methylene carbon of N^{ϵ}-monocarboxymethylhistidine (51.5 ppm) or N^{δ}-monocarboxymethylhistidine (50.1 ppm). The observed carboxymethylmethylene resonances correspond to those described by Nigen et al. (1973) in carboxymethylated myo-

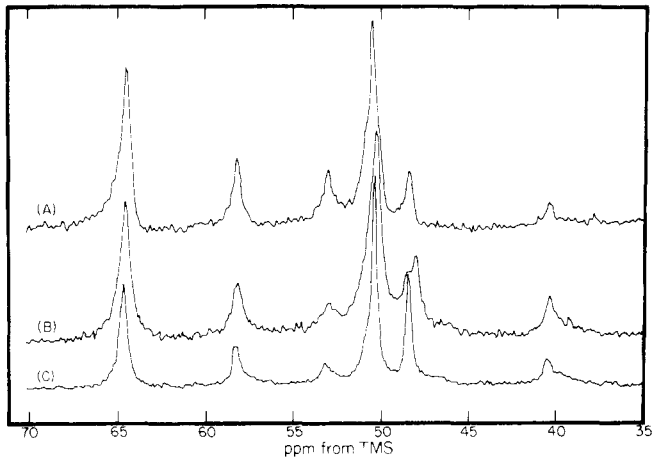

FIGURE 2: ^{13}C NMR spectra of [2-^{13}C]carboxymethylcytochrome *c*. (A) Cytochrome *c* carboxymethylated 6 days in the absence of cyanide; 200 mg/ml of ferric form in H_2O at pH 5.82; 5000 Hz, 10000 pulses, expanded. (B) Cytochrome *c* carboxymethylated 6 days in the presence of 0.1 *M* cyanide; 200 mg/ml of ferric form in H_2O at pH 6.25; 5000 Hz, 10000 pulses, expanded. (C) Cytochrome *c* carboxymethylated 6 days in the presence of 0.1 *M* cyanide; 200 mg/ml of ferrous CO complex in H_2O at pH 6.25; 5000 Hz, 10000 pulses, expanded.

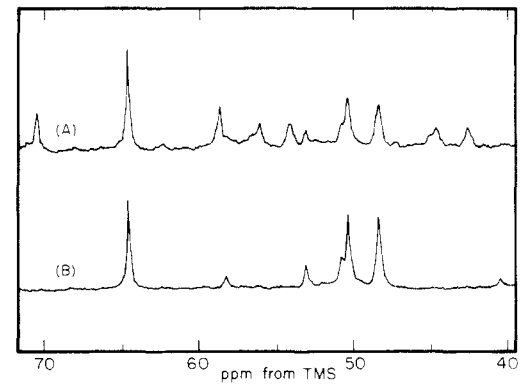

FIGURE 3: ^{13}C NMR spectra of [2-^{13}C]carboxymethylferrocytochrome *c* alkylated for 6 days in the presence of 0.1 *M* cyanide. (A) Proton coupled spectrum; 1250 Hz, 14000 pulses, 1.819 sec. recovery time between pulses, 25°. (B) Proton noise decoupled spectrum; 1250 Hz, 3000 pulses, 1.819 sec recovery time between pulses, 25°.

globin and carboxymethylated peptides. The particular assignments listed here are based on their interpretation, which we find acceptable and reasonable. There are also prominent resonances appearing in the region between 48 and 49 ppm which can be ascribed to the methylene carbon of carboxymethylmethionine based on resonance signals observed for carboxymethylated methionine peptides.

Analysis of the spectra in Figure 2 shows that carboxymethylation in the absence of cyanide, where position 65 is the only reactive methionine (Stellwagen, 1968), gives a single carboxymethylmethionine resonance at 48.4 ppm. However, carboxymethylation in the presence of cyanide, where methionines at position 65 and position 80 are both reactive (Schejter and Aviram, 1970), gives two resonances for carboxymethylmethionine. One of these corresponds to the 48.4 ppm species generated by carboxymethylation of methionine-65, and a new upfield resonance appears at 48.2 ppm, presumably corresponding to carboxymethylmethionine at position 80. Upon reduction and carbonylation the position 80 carboxymethylmethionine resonance moves back to 48.4 ppm.

No other shifts in resonance positions were observed in the ferric protein although line broadening was apparent. Most resonance lines appeared to be broadened by factors ranging from 1.5 to 2 in the oxidized form of the protein. The effect of pH on the position of the methylene resonances was determined over a wide range on the reduced and carbonylated protein and over a smaller range on the oxidized protein. It was found that the major component of the complex peak centered at 50.4 ppm was shifted downfield as the pH was raised above 8.5. The peak position during titration closely followed that of N^{ϵ}-carboxymethyllysine. The positions of the other methylene peaks in the carboxymethylated protein and in carboxymethylated model peptides were not significantly affected by titration over the high pH range. However, the methylene peak from glycolate esters rapidly disappeared and was replaced by a glycolate methylene resonance due to ester hydrolysis.

The only significant shift in methylene resonance positions during titration over the low pH range occurred below 4.5 where the glycolate ester peak was shifted upfield at lower pH due to protonation of the carboxyl groups.

A carboxymethylcytochrome *c* preparation obtained by reaction at pH 3 (Ando et al., 1965; Tsai and Williams, 1965; Babul and Stellwagen, 1972) in 0.2 *M* citrate buffer showed significant methylene resonances only at the positions of the methylene carbons of lysine (from natural abundance ^{13}C) and the methionine *S*-carboxymethylmethylene position. There apparently was a small amount of glycolate ester formed, but no detectable lysine or histidine derivatives. The carboxymethylmethionine resonance was similar to that shown in Figure 2, with a double peak system (48.5 and 48.3 ppm) in the ferric protein which collapsed to a single peak system (48.5 ppm) upon reduction and carbonylation. However, the upfield peak was the broader component of the *S*-carboxymethylmethionine resonance in the ferric protein in contrast to the broader downfield component shown in Figure 2.

Extent of Carboxymethylation as a Function of Reaction Time. The extent of carboxymethylation of various amino acid residues was observed by ^{13}C NMR spectroscopy at 24 hr and at 6 days in reaction systems including and excluding cyanide. Reaction in the presence of cyanide was observed also after a 10-hr period.

Analytical spectra, such as those shown in Figure 3, were used to estimate relative abundances of the several derivatives in the 6-day product and to establish the relationships between proton-coupled and decoupled peak intensities for calculation of effective nuclear Overhauser enhancements (NOE) in the decoupled spectra. Relative abundances and ^{13}C-proton coupling constants reported in Table II were taken directly from the proton-coupled spectra. Data given in Table III for other products were calculated from peak intensities in broad-band proton-decoupled spectra using the NOE values observed for the various classes of $^{13}CH_2$ resonances. The peak at 58.3 ppm representing dicarboxymethyllysine was masked in the coupled spectra by one of the ester components and the NOE was assumed to be equal to that for monocarboxymethyllysine. Most of the abundances could be obtained directly from peak areas taking into account two equivalent methylene carbons for a single N^{ϵ},N^{ϵ}-dicarboxymethyllysine residue. However, the composite peak could be resolved only into that portion due to N^{δ},N^{ϵ}-dicarboxymethylhistidine (assuming it was equivalent in area to the N^{ϵ},N^{ϵ}-dicarboxymethylhistidine peak)

Table II: Relative Abundances of Carboxymethyl Derivatives in the 6-day Alkylation Product of Cyanocytochrome *c*.[a]

Derivative	Chemical Shift (ppm)	NT_1 (msec)	$^1J_{CH}$ (Hz)	NOE $(1+\eta)$	Rel Abundance
S-Carboxymethylmethionine	48.4 ± 0.1	0.48 ± 0.08	145 ± 1	2.35 ± 0.24	2.00[b] ± 0.14
N^{δ}-Monocarboxymethylhistidine	(50.1[c])				(0.43[d])
N^{ϵ}-Monocarboxymethyllysine	50.4 ± 0.1	0.43 ± 0.09	144 ± 1	2.26 ± 0.23	1.74 ± 0.12
N^{ϵ}-Monocarboxymethylhistidine	(51.5[c])				
N^{δ},N^{ϵ}-Dicarboxymethylhistidine(δ)	50.8 ± 0.1	0.32 ± 0.03			
(ϵ)	53.1 ± 0.1	0.26 ± 0.05	145 ± 1	2.18 ± 0.22	0.57 ± 0.04
$N^{\epsilon},N^{\epsilon}$-Dicarboxymethyllysine	58.3 ± 0.1	0.38 ± 0.03		(2.26[e])	(0.23[f])
Glycolate esters	64.5 ± 0.1	0.62 ± 0.15	149 ± 1	1.81 ± 0.18	2.63 ± 0.18

[a] Product reduced with sodium dithionite in an atmosphere of argon; spectra taken at pH 7; relative abundances obtained from proton-coupled spectra and nuclear Overhauser enhancements, by comparison of proton-coupled and -decoupled spectra, except as noted. [b] Basis for relative abundances. [c] Not observed as a resolved signal. [d] Appears as a shoulder on the upfield side of the large peak at 50.4 ppm; relative abundance calculated on the assumption that one histidine was carboxymethylated (Stellwagen, 1966). [e] Average of values for similar derivatives. [f] Calculated from the peak integral in decoupled spectra. In coupled spectra the peak is masked by upfield component of the ester triplet. The relative peak intensity is twice this value, as there are two carboxymethyl groups in the derivative.

Table III: Relative Abundances of Carboxymethyl Derivatives in Various Preparations of Carboxymethylcytochrome *c*.[a]

	Alkylated Cyanocytochrome *c*			Alkylated Cytochrome *c*	
Derivative	10 hr	24 hr	144 hr	24 hr	144 hr
S-Carboxymethylmethionine	1.2 ± 0.1	1.3 ± 0.1	2.0[b]	0.5 ± 0.1	1.0[b]
N^{δ}-Monocarboxymethylhistidine					
N^{ϵ}-Monocarboxymethyllysine	0.9[c] ± 0.1	1.4 ± 0.1	2.2 ± 0.2	1.3 ± 0.1	2.6 ± 0.2
N^{ϵ}-Monocarboxymethylhistidine					
N^{δ},N^{ϵ}-Dicarboxymethylhistidine	0.1 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	0.9 ± 0.1
$N^{\epsilon},N^{\epsilon}$-Dicarboxymethyllysine	Trace	0.1 ± 0.1	0.2 ± 0.1	Trace	0.7 ± 0.1
Glycolate esters	0.2 ± 0.1	0.5 ± 0.1	2.6 ± 0.3	0.6 ± 0.1	4.0 ± 0.4

[a] Calculated from peak integrals in decoupled spectra, using NOE values given in Table II. [b] Bases for relative abundances. [c] Values in this row are for the composite peak minus the contribution from dicarboxymethylhistidine.

and that due to monocarboxymethyllysine plus both monocarboxymethylhistidine species.

From those data it is seen that even with a reaction time as short as 10 hr there is a significant amount of carboxymethylation of histidine and lysine and a significant amount of glycolate ester formed. Methionine appears to be the most readily alkylated amino acid, although in the absence of cyanide methionine-80 remains coordinated with the heme and is not reactive. Methionine-65, located on the surface of the protein, is the residue most susceptible to alkylation in the absence of cyanide.

Discussion

Extent of Carboxymethylation. From previous amino acid analyses of reaction products, the susceptibility of the methionine and histidine residues of cytochrome *c* toward carboxymethylation is well documented (Tsai and Williams, 1965; Stellwagen, 1966, 1968; Schejter and Aviram, 1970; Wüthrich et al., 1971; Babul and Stellwagen, 1972), but the extent to which other residues are modified by carboxymethylation of lysyl residues or formation of glycolate esters has been observed previously with native cytochrome *c* as a substrate. The significant amounts of these species observed in the undegraded product suggests that they are destroyed by the harsh hydrolysis conditions used to break the protein down into monomeric amino acids.

Thus it appears that a major problem in ascertaining the nature of carboxymethylation reaction products is that chemical analyses have required hydrolyses under conditions in which some carboxymethyl derivatives might be labile. The ^{13}C NMR method described here allows analysis of the carboxymethylation reaction product without any chemical degradation. It has shown that cytochrome *c* can be extensively modified by carboxymethylation and that the reaction with residues other than methionine proceeds over a long time period.

The progress of methionine carboxymethylation monitored by this method (see Table II) is comparable to the results of Stellwagen (1968) for alkylation both in the presence and in the absence of cyanide. The apparent biphasic reaction system, a fast reaction during the first 24 hr and a slower reaction which lasts over several days, is consistent with the data presented in Table II.

The absence of lysine and histidine reactivity toward alkylation at pH 3 is confirmed by the results obtained with the material prepared by the method of Babul and Stellwagen (1972). This was not unexpected since all nitrogens would probably be protonated at such a low pH. An unexpected result was obtained, however, in the *S*-carboxymethylmethionine resonance signal obtained with this preparation. The broad and narrow peak components were reversed from that observed in cytochrome carboxymethylated at neutral pH. This suggests that refolding of the much less extensively alkylated protein (reaction at pH 3) upon neutralization gives a significantly different conformation with respect to *S*-carboxymethymethionine residues than that generally obtained from carboxymethylation in the presence of cyanide at neutral pH.

Paramagnetic Effects. The two methionine residues in horse cytochrome *c* are of particular interest in the carboxymethylation reaction because of the differential effect on chemical properties of the protein caused by their modi-

fication. Methionine-65 is not normally in the region of the heme group and the chemical shift of the methylene group of its carboxymethyl derivative is not sensitive to the oxidation state of the iron. Thus the chemical shift is similar to that found in carboxymethylated methionine peptides. Methionine-80 is coordinated with the heme iron in the native protein. If methionine-80 is carboxymethylated, the methionine sulfur is displaced from the first coordination sphere and perhaps is replaced by lysine-79 in the ferric state to form a new low spin ($S = \frac{1}{2}$) complex (Keller et al. 1972). The position observed for the resonance of the methylene carbon of carboxymethylmethionine-80 indicates that even though this species is not coordinated to the heme iron, it still may be close enough to the paramagnetic center to experience a pseudocontact shift (LaMar, 1965). Such a phenomenon is further indicated by the shift to normal resonance position when the heme iron is converted to a diamagnetic species by reduction and carbonylation.

If the 0.2-ppm upfield shift of the methylene group of [^{13}C]carboxymethylmethionine-80 in the ferric protein is to be attributed to dipolar interaction with a paramagnetic center, it is of interest to know if the corresponding space averaged position of the ^{13}C nucleus is reasonable. Carboxymethylferricytochrome *c* has been found to be low spin ($S = \frac{1}{2}$) by electron paramagnetic resonance (Wüthrich et al. 1971) but the *g* tensor components have not been reported. However, if one assumes axial symmetry with *g* tensor components equal to similar low spin ferric heme proteins, a surface of space average position for the ^{13}C nucleus corresponding to 0.2-ppm upfield shift can be constructed using the formulation of McConnell and Robertson (1958). This surface is within a 54° cone normal to the heme plane and is at a maximum distance of about 20 Å from the iron nucleus when the line joining the ^{13}C nucleus and the iron nucleus is perpendicular to the heme plane.

A surface of space average position of a ^{13}C nucleus corresponding to an 0.2-ppm upfield shift can be generated by assuming a rhombic *g* tensor equal to that of the low spin unmodified ferricytochrome *c*. Using the method of Horrocks and Greenberg (1973) based on magnetic susceptibilities determined from *g* tensor components, a complex surface is obtained. This surface is contained within a 73° cone, perpendicular to the plane of the heme, and is a maximum of about 20 Å from the iron nucleus when the line joining the ^{13}C nucleus and the iron nucleus is perpendicular to the heme plane.

Thus a dipolar interaction is a reasonable mechanism to account for the observed chemical shift of the methylene ^{13}C of carboxymethylmethionine-80. Although this resonance is the only one with an observable chemical shift, a similar shift of a minor component within the region of the complex peak 49–52 ppm could go undetected.

The small magnitude of the chemical shift observed for the methylene group of carboxymethylmethionine-80 would seem to eliminate the possibility suggested by Stellwagen (1968) that the carboxyl group of carboxymethylmethionine-80 may replace the sulfur methionine-80 as a first coordination sphere axial ligand when cytochrome *c* is carboxymethylated. A large contact shift would be expected for the ^{13}C methylene resonance if the adjacent carboxyl group were coordinated with the paramagnetic iron. Brunori et al. (1972) have suggested that lysine-79 replaces methionine-80 as the sixth ligand in carboxymethylcytochrome *c*. This or any other protein ligand occupying the sixth coordination position must be easily replaced by CO when the protein is in the reduced state since the carbonyl complex is readily formed.

Nuclear Relaxation. Cytochrome *c* has a molecular mass of about 13,000 and should exhibit a correlation time for isotropic reorientation, τ_R, of $1\text{–}2 \times 10^{-8}$ sec (Glushko et al., 1972; Hunkapiller et al., 1973). The spin-lattice relaxation time, T_1, of a ^{13}C atom rigidly locked into a system with that correlation time and interacting with two protons is approximately 25–35 msec, near the T_1 minimum, where the NOE approaches a limiting low value, near unity. However, internal motion will increase the NOE and, in the slow-motion regime for isotropic rotation, cause T_1 to decrease and then increase with decreasing correlation time for internal motion, τ_G. Calculations based on a simple model comprising a tetrahedral carbon rotating about a bond to a rigid framework (Doddrell et al., 1972) suggest that for isotropic rotation correlation times in the suggested range the NOE values will go through a maximum of 2–2.5, with internal correlation times of the order of 10^{-10} sec. The corresponding NT_1 values are expected to be between 40 and 900 msec. The peptide derivative most likely to fit the model is N^{ϵ},N^{ϵ}-dicarboxymethylhistidine, with internal rotation about the ^{13}C–N bond and with two interacting protons at the tetrahedral angle. The best calculated fit for NT_1 = 260 msec and NOE = 2.2 gives $\tau_R = 1\text{–}2 \times 10^{-8}$ sec and $\tau_G = 3\text{–}5 \times 10^{-11}$ sec to within the experimental error, perhaps fortuitously. Internal motion of the other derivatives is certainly more complex and, in fact, the observed combinations of NT_1 and NOE do not fit the expectations of either the simple model given above or of a single effective isotropic rotation with shorter correlation time, although the general concept of rapid internal motion superimposed upon slower isotropic rotation of the molecule as a whole appears to be correct in this instance. There is no apparent paramagnetic relaxation in reduced material and none is expected, as the visible spectra are characteristic of low-spin iron(II). Spin–rotation or scalar interaction with other nuclei, such as ^{14}N, with spin greater than $\frac{1}{2}$, are expected to make negligible contributions to relaxation for the observed ^{13}C correlation times.

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Affinity Labeling of Catechol *O*-Methyltransferase by *N*-Haloacetyl Derivatives of 3,5-Dimethoxy-4-hydroxyphenylethylamine and 3,4-Dimethoxy-5-hydroxyphenylethylamine. Kinetics of Inactivation†

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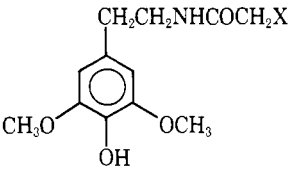
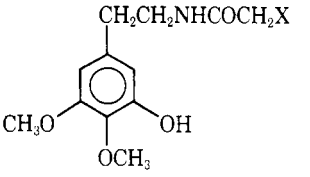
ABSTRACT: In an attempt to elucidate the relationship between the chemical structure and the catalytic function of catechol *O*-methyltransferase (COMT), several classes of affinity labeling reagents have been synthesized and their interaction with COMT has been studied. Earlier studies have shown that various *N*-haloacetyl derivatives of 3,5-dimethoxy-4-hydroxyphenylethylamine were effective affinity labeling reagents for this enzyme. In this report we have shown that *N*-haloacetyl derivatives of the isomeric 3,4-dimethoxy-5-hydroxyphenylethylamine also rapidly and irreversibly inactivate COMT and they satisfy many of the criteria established for affinity labeling reagents. This latter group of agents appear to modify a nucleophilic residue at the active site of COMT different from that modified by the 3,5-dimethoxy-4-hydroxyphenylethylamine series. Evidence to support this conclusion has been obtained by comparing the kinetics of COMT inactivation and the substrate protection profiles for these two classes of affinity labeling reagents.

The extraneuronal inactivation of catecholamines and the detoxification of many xenobiotic catechols is dependent upon the enzyme catechol *O*-methyltransferase (COMT)[1] (EC 2.1.1.6). COMT is a soluble, magnesium-requiring enzyme which transfers a methyl group from *S*-adenosylmethionine (AdoMet) to a catechol acceptor resulting in the formation of the meta and para O-methylated products (Axelrod and Tomchick, 1958; Molinoff and Axelrod, 1971; Flohe, 1974). In an effort to elucidate the relationship between the chemical structure and catalytic function of COMT we have synthesized various affinity labeling reagents for this enzyme. In earlier reports from this laboratory (Borchardt and Thakker, 1973, 1975) we have shown that *N*-iodoacetyl- and *N*-bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamines (**1a** and **b**) produce rapid inactivation of COMT and satisfy many of the criteria established for affinity labeling reagents. Since these derivatives of 3,5-dimethoxy-4-hydroxyphenylethylamine (**1a** and **b**) resemble meta-methoxylated products, it was of interest in our laboratory to synthesize and evaluate as affinity labeling reagents the corresponding derivatives of 3,4-dimethoxy-5-hydroxyphenylethylamine (**2a-c**), which resemble para methoxylated products. If both classes of inhibitors bind to the active site of COMT through the *o*-methoxy phenol functionality as proposed in Scheme I, then the re-

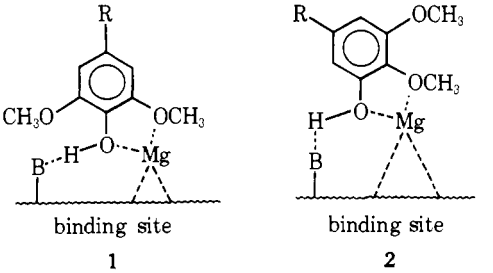


1a, X = I
b, X = Br



2a, X = H
b, X = I
c, X = Br

Scheme I



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[1] Abbreviations used are: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; COMT, catechol *O*-methyltransferase; DHA, 3,4-dihydroxyacetophenone; DCC, *N,N'*-dicyclohexylcarbodiimide.