# Carbon-13 Magnetic Resonance of Carboxymethylated Human Carbonic Anhydrase B. Chemical Shift and Spin–Lattice Relaxation Studies†

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ABSTRACT: We have previously prepared $N^{\tau}$-carboxymethylhistidine-200 human carbonic anhydrase B using 90% [1-$^{13}$C]bromoacetate and have observed the $^{13}$C NMR resonance of the enriched carboxylate now covalently attached in the active site. We report here chemical shift studies of the zinc-free carboxymethylated enzyme and its $Co^{2+}$-substituted form, as well as relaxation studies of the resonance in the zinc enzyme at three frequencies (15.04, 25.15, and 90.5 MHz). The chemical shift and relaxation data are both consistent with the immobilization of the carboxylate at pH 8 and its approach or coordination to the zinc. The relaxation data indicate that lowering the pH to 5.5 leads to internal motion of the carboxymethyl moiety, consistent with the chemical shift evidence for the disruption of the proposed zinc–carboxylate coordination. Inhibitor binding at either pH 5.5 or 8.0 eliminates whatever internal motion might be present. The relaxation data have been interpreted using theoretical calculations on dipolar and chemical shift anisotropy contributions. The combined results indicate that the catalytic consequences of the carboxymethylation may be due to the proposed zinc–carboxylate coordination and need not result from the disruption of any role that histidine-200 might play in the catalytic mechanism.

The chemical modification of active sites of enzymes has played a key role in our understanding of their structure and function (cf. Hirs & Timasheff, 1972). Modifications of individual residues leading to catalytic activity changes frequently provide a starting point for assessing their role, if any, in the catalysis. Such studies have been frequently carried out on human erythrocyte carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1), especially its low specific activity isozyme HCAB.[1] This zinc metalloenzyme has an unusually nucleophilic histidine-200 that is readily modified by active-site directed alkylating reagents such as bromoacetic acid (for a general review, see Lindskog et al., 1971).

We have recently used $^{13}$C nuclear magnetic resonance ($^{13}$C NMR) to study $N^{\tau}$-carboxymethylhistidine-200 HCAB, or CmHCAB, prepared by reaction of HCAB with 90% [1-$^{13}$C]bromoacetate (Strader & Khalifah, 1976). The modified enzyme is known to retain intrinsic but diminished residual catalytic activity toward the hydration of $CO_2$ (Khalifah & Edsall, 1972) and the hydrolysis of various esters (Whitney, 1970; Whitney et al., 1967). The basis for the carboxymethylation effects on the enzyme activity has not yet been elucidated. It has sometimes been assumed that the alteration in activity reflects a crucial role for histidine-200 in the catalysis (Wang, 1969; Pocker & Dickerson, 1968), although this conclusion has been frequently disputed (cf. Whitney, 1970; Khalifah & Edsall, 1972). Our $^{13}$C NMR studies of the enriched carboxylate of CmHCAB have interestingly suggested that the carboxylate may actually interact with the essential zinc atom or its aquo ligand (Khalifah, 1977; Khalifah et al., 1977). Such a hypothesis has important mechanistic implications toward understanding the effects of carboxymethylation, as was first proposed and recognized by Coleman (1975). We report here $^{13}$C NMR studies on CmHCAB, its metal-free derivative (apoCmHCAB), and its $Co^{2+}$-substituted form, including spin–lattice relaxation measurements, that may have bearing on the above hypothesis. The relaxation studies take explicit note of the possible contributions of the chemical shift anisotropy mechanism to the relaxation of immobilized and rotating carboxylate carbons.

## Materials and Methods

*Enzyme Preparations.* HCAB was prepared from hemolysate in gram quantities by the affinity chromatography method previously described (Khalifah et al., 1977). Enriched CmHCAB was prepared by reacting HCAB with 90% [1-$^{13}$C]bromoacetate under conditions that lead to the exclusive $N^{\tau}$-carboxymethylation of histidine-200 (Strader & Khalifah, 1976; Khalifah et al., 1977). ApoCmHCAB was initially prepared by the *o*-phenanthroline dialysis method at pH 5 (Lindskog & Nyman, 1964) and more recently by the much more rapid pyridine-2,6-dicarboxylic acid method at the milder pH 7 that has been previously used with HCAB (Hunt et al., 1977; Kidani et al., 1976). Our kinetic studies on zinc removal show that this latter chelator is equally efficient with CmHCAB. Enriched $Co^{2+}$–CmHCAB was prepared either by carboxymethylation of $Co^{2+}$–HCAB (Whitney & Brandt, 1976) or by the stoichiometric addition of "Specpure" $CoSO_4$ to enriched apoCmHCAB.

*Chemicals.* Ninety percent [1-$^{13}$C]bromoacetic acid was purchased from Koch Isotopes. Specpure grade $CoSO_4$ was a product of Johnson Matthey Chemicals. Deuterium oxide (99.8%) was from Aldrich Chemicals and was dithizone-ex-

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[1] Abbreviations used: HCAB, human erythrocyte carbonic anhydrase isozyme B; apoHCAB, zinc-free HCAB; CmHCAB, $N^{\tau}$-carboxymethylhistidine-200 HCAB (see Figure 5 for imidazole ring notation according to the IUPAC-IUB CBN (1975)); apoCmHCAB, zinc-free CmHCAB; $Co^{2+}$–CmHCAB, $Co^{2+}$-substituted CmHCAB; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Tris, tris(hydroxymethyl)aminomethane; Bistris, *N,N*-bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; CSA, chemical shift anisotropy.

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tracted and redistilled as necessary. Pyridine-2,6-dicarboxylic acid, diphenylthiocarbazone (dithizone), and Dowex chelating resin were from Sigma.

*NMR Samples and Measurements.* $^{13}C$ NMR spectra were mostly recorded on a JEOL PFT/EC 100 Fourier transform spectrometer at a $^{13}C$ frequency of 25.15 MHz. Comparative measurements were also carried out at 15.04 MHz using a JEOL FX60Q instrument and at 90.5 MHz using a Bruker HXS-360 instrument. All measurements were at 25 °C. Chemical shifts are reported relative to $(Me)_4Si$ using internal referencing with dioxane whose shift was assumed to be 67.40 ppm. Spin-lattice relaxation rates were measured by the inversion-recovery method of Vold et al. (1968) under full $^1H$ noise decoupling. Nuclear Overhauser effect (NOE) measurements were carried out using a gated-decoupling technique (Opella et al., 1976) with waiting times exceeding four times the $T_1$ values. Calculations of the spin-lattice relaxation rates ($R_1 \equiv 1/T_1$) were carried out using an iterative nonlinear least-squares method (Gerhards & Dietrich, 1976) in order to minimize the critical dependence of the calculated $T_1$ values on the "infinite" wait spectrum. Enzyme samples were usually 3–8 mM in 0.2 ionic strength buffers and were predialyzed extensively against EDTA. Stringent precautions were also taken to remove paramagnetic ion contamination from glassware. Control experiments were carried out using dithizone-extracted $D_2O$ and Chelex treated buffers as described below. All pH readings are apparent meter readings uncorrected for isotope effects. $D_2O$ samples were approximately 95% in the isotope, while $H_2O$ samples contained about 10% $D_2O$ for locking purposes.

## Theoretical Considerations

*Dipolar Contributions to Relaxation.* The relaxation of a $^{13}C$-labeled nucleus commonly occurs by dipolar interactions with adjacent intramolecular proton neighbors (Lyerla & Grant, 1972). This dipolar mechanism has been theoretically described for the cases where (a) the dipolar vector motion is purely isotropic, or (b) an additional superimposed axial rotation occurs along with the overall isotropic rotation (Doddrell et al., 1972; Led et al., 1975). Under full $^1H$ noise decoupling conditions, the dipolar spin-lattice and spin-spin relaxation rates ($R \equiv 1/T$) are given by (Led et al., 1975)

$$R_1^D = (1/20)\hbar^2\gamma_C^2\gamma_H^2[J_0(\omega_H - \omega_C) + 3J_1(\omega_C) + 6J_2(\omega_H + \omega_C)] \quad (1)$$

$$R_2^D = (1/40)\hbar^2\gamma_C^2\gamma_H^2[4J_0(0) + J_0(\omega_H - \omega_C) + 3J_1(\omega_C) + 6J_1(\omega_H) + 6J_2(\omega_H + \omega_C)] \quad (2)$$

The NOE is then given by

$$\text{NOE} = 1 + \frac{\gamma_H}{\gamma_C}\left[\frac{6J_2(\omega_H + \omega_C) - J_0(\omega_H - \omega_C)}{J_0(\omega_H - \omega_C) + 3J_1(\omega_C) + 6J_2(\omega_H + \omega_C)}\right] \quad (3)$$

Here $\gamma_H$ and $\gamma_C$ are the gyromagnetic ratios, $\omega_H$ and $\omega_C$ are the Larmor frequencies, and $\tau_C$ is the overall isotropic rotational correlation time. The so-called spectral densities $J_k(\omega)$ depend on $\tau_C$ and $r_i^{-6}$ (the C–$H_i$ distances) when no internal motion is present. However, if internal motion, characterized by a correlation time $\tau_G$, is also present, then the $J_k(\omega)$ will additionally depend on $\tau_G$ and the angles $\theta_i$ between each C–$H_i$ vector and the axis of internal rotation (cf. equations given by Doddrell et al., 1972; Led et al., 1975). The above treatment ignores cross-correlation effects (Werbelow & Grant, 1975).

*Chemical Shift Anisotropy Contributions.* It has been recently emphasized that nonprotonated carbonyl and aromatic carbons in proteins could have significant contributions from the chemical shift anisotropy (CSA) mechanisms that become especially large at high magnetic fields (Norton et al., 1977). The carboxylate carbon $^{13}C$ shielding tensor has appreciable anisotropy that has been thoroughly characterized by solid-state NMR techniques (Pines et al., 1972, 1974; Griffin et al., 1975). Theoretical calculations of the CSA contributions to $^{13}C$ relaxation have been described for the case of pure isotropic motion only (Norton et al., 1977), but the formalism encompassing internal axial rotation has been developed by Hull & Sykes (1975) in their $^{19}F$ studies and is adapted to the carboxylate rotation case here. Following Hull & Sykes, we denote the diagonalized chemical shift tensor components by $\sigma_{ii}$ ($i$ = 1 to 3) and the components of the traceless part of the shift tensor by $\delta_x$, $\delta_y$, and $\delta_z$, where $\delta_x = \sigma_{11} - (1/3)\Sigma\sigma_{ii}$, and so on. An asymmetry parameter $\eta$ is defined as equal to $(\delta_x - \delta_y)/\delta_z$. The contributions of the CSA mechanism to $R_1$ and $R_2$ will then be given by (Hull & Sykes, 1975):

$$R_1^{CSA} = (3/20)\omega_C^2\delta_z^2\sum C_iJ_i(\omega_C) \quad (4)$$

$$R_2^{CSA} = (1/40)\omega_C^2\delta_z^2\sum C_i[3J_i(\omega_C) + 4J_i(0)] \quad (5)$$

where the sum runs from 0 to 2. The spectral densities are here defined by

$$J_i(\omega) = 2\tau_i(1 + \omega^2\tau_i^2)^{-1} \quad (6)$$

For the case where internal motion, characterized by a rotational correlation time $\tau_G'$, is present in addition to the overall $\tau_C$, the $\tau_i$ will be given by[2]

$$(\tau_0)^{-1} = (\tau_C)^{-1} \quad (7)$$

$$(\tau_1)^{-1} = (\tau_C)^{-1} + (\tau_G')^{-1} \quad (8)$$

$$(\tau_2)^{-1} = (\tau_C)^{-1} + 4(\tau_G')^{-1} \quad (9)$$

The $C_i$ coefficients are complicated functions (see eq 15–17 of Hull & Sykes, 1975, for full details) of the asymmetry $\eta$ and the Euler angles $\beta$ and $\gamma$ that relate the rotation of the principal axes of the diffusion tensor *into* those of the chemical shift tensor. For the simple case of pure isotropic rotation, i.e., when $(\tau_G')^{-1} = 0$, all the $J_i(\omega_C)$ are equal and we note that $\Sigma C_i = 1 + \eta^2/3$. This is the case utilized by Norton et al. (1977), although their notation differs somewhat.[3]

We have carried out $R_1^{CSA}$ and $R_2^{CSA}$ calculations for immobilized and rotating carboxylate carbons, discussed further below (see Discussion), whose chemical shift and diffusion tensors have the orientations shown in Figure 1. Numerical values of the $\sigma_{ii}$ components of a typical carboxylate were obtained by averaging the values for the carboxylates of glycine, alanine, tartarate, and acetic acid (Pines et al., 1972, 1974; Griffin et al., 1975). Table I lists these averaged values as well as the $\delta$s and $\eta$. The Euler angles $\beta$ and $\gamma$ for rotation of the carboxylate around the $C_M$–$C_O$ axis are also listed in the Table. *For these angles,* the $C_i$ assume the rather simple

[2] These equations imply a different convention for defining $\tau_G'$ in terms of the diffusion tensor components (Hull & Sykes, 1975) than is commonly used in the treatment of dipolar interactions (Doddrell et al., 1972). This inconsistency of definition is of no consequence in our work, since $\tau_G$ and $\tau_G'$ refer to rotations around different bonds.

[3] Norton et al. (1977) have used the term $\Delta\sigma$ to describe the quantity whose square is equal to $(3/2)[(\Sigma\sigma_{ii}^2) - (\Sigma\sigma_{ii})^2]$. This is not to be confused with the more frequent use (cf. Hull & Sykes, 1975) of this symbol to denote the quantity $\sigma_{33} - (\sigma_{11} + \sigma_{22})/2$. The two expressions become equal only for axially symmetric tensors. We denote the Norton et al. quantity by $\Delta\sigma'$ and list its numerical value for the average carboxylate in Table I for ease of comparison.

TABLE I: Parameters For Calculating the Chemical Shift Anisotropy Relaxation of a Typical Carboxylate Carbon.[a]

| Parameter | Definition or applicable relation | Numerical value |
|---|---|---|
| $\sigma_{11}$, $\sigma_{22}$, $\sigma_{33}$ | Diagonalized shift tensor components | −122.2, −56.5, +20.4 (ppm) |
| $\delta_x$, $\delta_y$, $\delta_z$ | Traceless shift tensor components | −69.4, −3.73, +73.9 (ppm) |
| $\eta$ | Asymmetry $(\delta_x - \delta_y)/\delta_z$ | −0.90 |
| $\beta$ | Counterclockwise rotation of $D_z$ into $\delta_z$ viewed from $+D_y$ | 90° |
| $\gamma$ | Counterclockwise rotation of $D_y$ into $\delta_y$ viewed from $+\delta_z$ | 180° |
| $C_0$ | $(\eta - 1)^2/4$ | 0.90 |
| $C_1$ | 0 | 0 |
| $C_2$ | $(3/4)(1 + \eta/3)^2$ | 0.370 |
| $\Delta\sigma'$ | $\{(3/2)[(\Sigma\sigma_{ii}^2) - (\Sigma\sigma_{ii})^2]\}^{1/2}$ | 124 ppm |

[a] See text for details. The $C_i$ expressions are obtained from eq 15-17 of Hull & Sykes (1975) by using $\beta = 90°$ and $\gamma = 180°$. $\Delta\sigma'$ is defined in footnote 3 of text in relation to the commonly used $\Delta\sigma$.

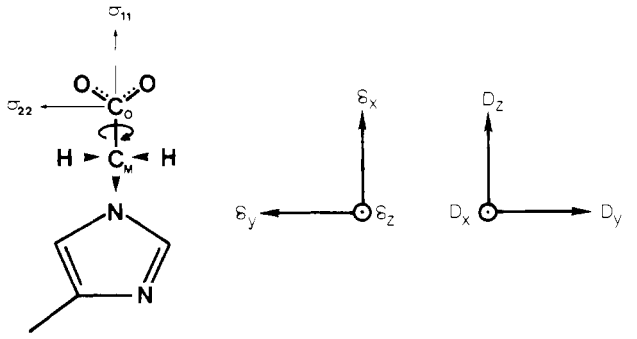


FIGURE 1: Chemical shift anisotropy of the carboxylate group and orientations of the chemical shift and diffusion tensors for the case of a carboxylate rotating around the $C_M$–$C_O$ axis with a correlation time $\tau_G'$. Note that $\sigma_{33}$, $\delta_z$, and $D_x$ are oriented perpendicular to the plane of the carboxylate, while all other components are in the plane. See Table I of text for further explanation of symbols and terms.

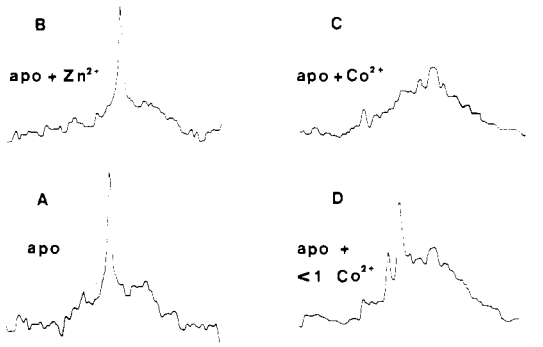


FIGURE 2: $^{13}$C NMR spectra (carbonyl region) at 25.15 MHz of derivatives of enriched CmHCAB. (A) ApoCmHCAB; (B) reconstituted CmHCAB; (C) $Co^{2+}$-CmHCAB; (D) EDTA-dialyzed $Co^{2+}$-CmHCAB. The tallest peak in A, B, and C is the resonance of the enriched carboxylate, while D shows the natural abundance background. Samples were 5-6 mM in enzyme at pH 8.0.

expressions shown in column 2 of Table I, column 3 giving the numerical values for the averaged carboxylate.

## Results

*Chemical Shift Studies.* The 90% enriched carboxylate covalently attached to $N^\tau$ of histidine-200 gives rise to a narrow and dominant resonance in the carbonyl carbon region of the $^{13}$C NMR spectrum of the enzyme (Khalifah et al., 1977). Figure 2A shows that a similar situation occurs in enriched apoCmHCAB, except for a shift in the resonance from 174.6 ppm to 175.6 ppm at pH 8.0. Reconstitution of CmHCAB by the addition of an equivalent of zinc to apoCmHCAB gives a spectrum (Figure 2B) indistinguishable from that of the original CmHCAB, i.e., the addition of $Zn^{2+}$ causes a 1.0-ppm *upfield* shift at this pH. The addition of $Co^{2+}$ to the enriched apoCmHCAB causes a progressive and, ultimately, a complete disappearance of the carboxylate resonance without intermediate broadening (Figures 2C and 2D), typical of a slow-exchange process. It is well established that $Co^{2+}$ goes to the $Zn^{2+}$ site in the enzyme, both metals being extremely tightly bound (Lindskog et al., 1971). The disappearance of the carboxyl resonance presumably indicates that it has shifted and/or broadened beyond ready detection due to the proximity of the carbon to the paramagnetic ion. This situation applies in the pH range of 5.7 to 9.8 and in the presence and absence of bound sulfonamide inhibitors like acetazolamide and *p*-carboxybenzenesulfonamide.

*$^{13}$C NMR Titration of Enriched ApoCmHCAB.* The chemical shift of the enriched carboxylate of apoCmHCAB was followed over the pH range of 5.0 to 9.6, as indicated by the experimental points in Figure 3. The resonance is seen to

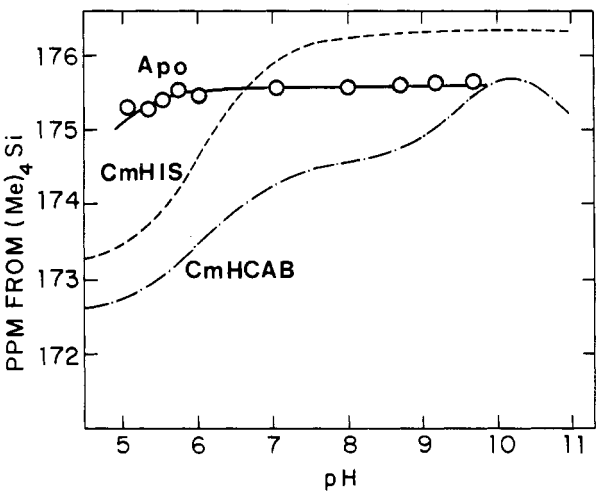


FIGURE 3: Enriched carboxylate titration shifts of apoCmHCAB (experimental points), CmHCAB (- · -), and the corresponding carboxylate of $N^\tau$-carboxymethylhistidine (- - -). Temperature was 25 °C and the ionic strength was 0.2.

be largely independent of pH except towards the low pH end. The pH titration behavior of the carboxylate of apoCmHCAB is quite different from that in CmHCAB or in the model compound $N^\tau$-carboxymethyl-L-histidine ($\tau$-CmHis). The behavior of the latter two is also shown in Figure 3 for comparative purposes and is taken from our previous work (Khalifah et al., 1977).

*$^{13}$C NMR Relaxation Studies.* The nuclear Overhauser

TABLE II: Spin-Lattice Relaxation Rates of the Enriched Carboxyl Carbon of Alkylated Human Carbonic Anhydrase B.[a]

| Enzyme form[b] | pH[c] | Solvent[d] | $R_1$ ($s^{-1}$)[e] | $T_1$ (s) | NOE |
|---|---|---|---|---|---|
| CmHCAB | 8.00 | $D_2O$* | 0.37 ± 0.01 | 2.7 | 1.16 |
| CmHCAB | 8.14 | $D_2O$ | 0.41 ± 0.04 | 2.4 | 1.16 |
| CmHCAB + $N_3^-$ | 8.14 | $D_2O$ | 0.42 ± 0.06 | 2.3 | |
| CmHCAB | 5.72 | $D_2O$ | 0.66 ± 0.03 | 1.5 | 1.12 |
| CmHCAB | 5.42 | $D_2O$* | 0.60 ± 0.05 | 1.7 | |
| CmHCAB + $N_3^-$ | 5.72 | $D_2O$ | 0.47 ± 0.05 | 2.2 | |
| CmHCAB + $N_3^-$ | 5.42 | $D_2O$* | 0.37 ± 0.02 | 2.7 | |
| CmHCAB (15.0 MHz) | 8.00 | $D_2O$ | 0.98 ± 0.07 | 1.0 | |
| CmHCAB (15.0 MHz) | 5.55 | $D_2O$ | 1.65 ± 0.10 | 0.61 | |
| CmHCAB + $N_3^-$ (15.0 MHz) | 5.55 | $D_2O$ | 1.25 ± 0.08 | 0.80 | |
| ApoCmHCAB | 8.00 | $D_2O$ | 0.46 ± 0.10 | 2.2 | 1.06 |
| ApoCmHCAB | 5.91 | $D_2O$* | 0.47 ± 0.02 | 2.2 | |

[a] All measurements are at 25.15 MHz unless noted otherwise. [b] All samples containing the inhibitor azide (10 mM) had their $T_1$ values measured first in absence of the inhibitor and are listed in the table as having the identical pH in each case. [c] These are meter readings uncorrected for isotope effects. [d] Entries marked with an asterisk had the $D_2O$ extracted with dithizone. [e] The variations listed are the 95% confidence limits derived from the analysis of the data (Gerhards & Dietrich, 1976).

effect of the enriched carboxylate of CmHCAB and apoCmHCAB has been measured at 25.15 MHz over a pH range from 5.7 to 10.4 in both $H_2O$ and $D_2O$. The NOE is defined here as the ratio of the intensity in the presence of proton decoupling to that in its absence. For CmHCAB, eight independent determinations over this pH range have yielded an NOE of 1.16 ± 0.10, a value indistinguishable from the dipolar minimum value (Lyerla & Grant, 1972). Similar results were obtained for apoCmHCAB at pH 8 (1.06) and pH 9.5 (1.11) in $D_2O$. Some of these data are given in Table II. The experimental standard deviation of the intensity measurements in the NOE and relaxation studies was approximately 8%. In contrast to the NOE, the spin-lattice relaxation rate $R_1$ of *CmHCAB* shows a pH and inhibitor dependence (Table II). At 25.15 MHz, $R_1$ increases by about 50% in going from pH 8 (0.37–0.41 $s^{-1}$) to pH 5.4 (0.60–0.66 $s^{-1}$). A qualitatively similar change is also seen at 15.04 MHz where $R_1$ increases from 0.98 $s^{-1}$ to 1.6 $s^{-1}$. The addition of the inhibitor azide appears to reverse the increase in $R_1$ at low pH at either field and is without effect at pH 8. These effects, while not large, are not believed to be due to artifacts arising from the presence of paramagnetic impurities[4] in view of the precautions and controls (Table II) taken.

In addition to these studies, we have carried out a line-width determination on the enriched carboxyl resonance of CmHCAB at 90.5 MHz and pH 8. We have found a value of 5 Hz after correcting for inhomogeneity and digital filtering contributions. The line width at this field is expected to be dominated by the CSA mechanism (see Discussion) and provides a potential means of estimating CSA contributions at lower fields.

*$^{13}C$ NMR Determination of Protein Rotational Correlation Time.* We have determined the relaxation rate of the natural abundance $\alpha$-carbon resonances of *HCAB* at 25.15 MHz and have found it to be 9.3 ± 1.0 $s^{-1}$. Such a determination provides a good route to the estimation of the overall rotational correlation time of the protein using eq 1 above (Visscher & Gurd, 1975; Oldfield et al., 1975; Bauer et al., 1975). The $\alpha$ carbons can be safely assumed to be immobilized in the protein, with each one being exclusively relaxed by dipolar interaction with its single bonded proton 1.09 Å away. Using eq 1, the measured $R_1$ value results in two solutions for $\tau_C$, the larger one of (30 ± 4) × $10^{-9}$ s $rad^{-1}$ being the only one consistent with determinations on many other proteins (Visscher & Gurd, 1975; Oldfield et al., 1975; Bauer et al., 1975). We have consequently adopted the value of 30 ns $rad^{-1}$ for our theoretical calculations on *CmHCAB*, since no hydrodynamic or conformational changes have been found to occur on carboxymethylation of HCAB (Bradbury, 1969). In addition, the enzyme behaves as a spherical macromolecule by hydrodynamic criteria (Lindskog et al., 1971), so that no complications of the kind reported by Wilbur et al. (1976) are anticipated.

## Discussion

Detailed studies of the $^{13}C$ NMR titration behavior of the enriched carboxylate of CmHCAB have previously revealed three unusual features (Strader & Khalifah, 1976; Khalifah et al., 1977) that can be summarized as follows. (1) The carboxylate chemical shift is perturbed by the ionization of an active-site group of p$K$ = 9.0 that is probably the "catalytically essential" ionizing group frequently thought to be the aquo ligand of the zinc (Lindskog & Coleman, 1973; Whitney, 1970; Khalifah et al., 1977). (2) The titration shift experienced by the carboxylate upon ring ionization (p$K$ = 6.0) of the histidine to which it is attached is only 1.9 ppm, whereas a 3.1–3.2-ppm shift is seen in the model compounds $\tau$-CmHis and $\pi$-CmHis. (3) The absolute chemical shift of the carboxylate in CmHCAB appears normal relative to these model compounds at pH 5 and pH 10 but is shifted upfield in CmHCAB by a little over 1 ppm near pH 8. These differences can all be seen by comparing the titration curves for $\tau$-CmHis and CmHCAB shown in Figure 3.

The above anomalous features of the titration of CmHCAB can be most simply accounted for by assuming that the carboxylate is in an unusual upfield-shifting environment or conformation at pH 8 but not at pH 5 or pH 10. The chemical shift of carboxylate carbons is known to be sensitive to electric field effects, such that positive charges in their vicinity will usually produce an upfield shift (Batchelor, 1975). The active site of carbonic anhydrase in the vicinity of histidine-200 appears devoid of positively charged groups at pH 8 except for the zinc metal and its protonated aquo ligand (Notstrand et al., 1975; Kannan et al., 1975; Campbell et al., 1974). We have consequently proposed that the $^{13}C$ NMR titration data anomalies result from the possible interaction or coordination of the carboxylate with either the zinc metal (inner sphere) or its protonated aquo ligand (outer sphere) at pH 8 (Khalifah

[4] Paramagnetic ion contaminants have plagued studies of carboxyl relaxation in small molecules ($T_1 \sim$ 50–100 s), especially since their effects are pH dependent (Gust et al., 1976; Cohen et al., 1975).

et al., 1977). This interaction is then presumably disrupted by competition with a hydroxyl at high pH, leading to the p$K$ = 9.0 inflection, or by protonation and enforced rotation of the imidazole ring of histidine-200 at lower pH with an inflection at pH 6.0. It is noteworthy that the enzyme is inhibited by carboxylates (Lindskog et al., 1971; Taylor et al., 1971) with the site of attachment presumably being the metal as with other monovalent anionic inhibitors (Bertini et al., 1976; Taylor et al., 1971; Bergstén et al., 1972; Riepe & Wang, 1968). It is reasonable thus to also expect that the proposed carboxylate interaction with the metal or its ligand be abolished in complexes of CmHCAB with inhibitors. This indeed appears to be the case, since the $^{13}$C NMR titration of the carboxylate in such complexes shows both an absence of the p$K$ = 9.0 inflection and a normalization of the magnitude of the titration shift accompanying ring ionization (Khalifah, 1977). All the above hypotheses are shown schematically in simplified form in Figure 4.

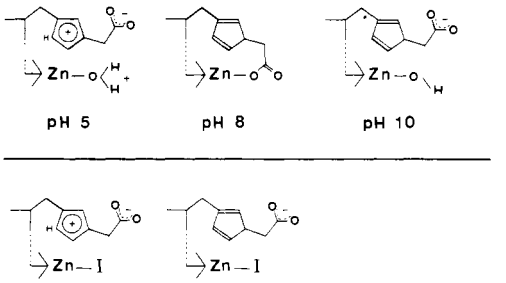


FIGURE 4: Simplified schematic diagram illustrating one type of interaction of the carboxymethyl carboxylate of histidine-200 with the essential zinc according to the hypothesis discussed in the text. Top structures show the effects of active-site ionizations on the interaction, while the lower structures illustrate the effects of adding inhibitors (I) that bind to the metal. *The data do not distinguish between inner-sphere (as shown) or outer-sphere coordination, nor do they exclude pentacoordination.* The form at pH 8 in which the carboxylate is not coordinated and a water ligand binds to the zinc is implicitly assumed to occur in low concentrations.

The mechanistically important hypothesis of a zinc–carboxylate interaction discussed above, which was first advanced by Coleman (1975) on different grounds, is difficult to prove unequivocally short of a high-resolution x-ray crystallographic determination.[5] However, we have sought in the present work to test some of its consequences and predictions. Major inconsistencies, if found, would still be valuable in its evaluation or modification. We have thus pursued three types of investigation here: metal removal, metal replacement with a paramagnetic metal, and studies of the motion of the carboxylate by spin–lattice relaxation measurements. Removal of the zinc around pH 8 should, according to the above, lead to a downfield shift of about 1 ppm, since this would abolish any interaction with the positively charged metal or its ligand. This is indeed experimentally found (Figure 3), with the chemical shift of the carboxylate of apoCmHCAB becoming nearly identical with its chemical shift in CmHCAB when the latter is at pH 10 and with the shift in CmHCAB complexes with inhibitors, also at high pH (Khalifah, 1977). For comparison, we have found that removal of $Zn^{2+}$ from chelation with the ionized carboxylates of EDTA and EGTA causes downfield shifts of 1.4 and 1.8 ppm, respectively. A full pH titration of the carboxylate of apoCmHCAB indicates an abnormally low p$K$ for histidine-200 in this derivative. This is contrary to what would be expected on the sole basis of removing a positive charge (the metal) from the vicinity of the histidine. However, we do not feel that this invalidates our chemical shift conclusion, as the site of protonation on the ring ($N^{\pi}$) is about 5 Å from the carboxyl and may be subject to different environmental influences. In addition, it is not possible to predict the charge distribution around the histidine upon removal of the metal. Inspection of a molecular model of the enzyme[6] shows histidine-200 to be located in the inner part of the active-site cavity and would be closely surrounded by four other histidines (residue 64 and Zn ligands 94, 96, and 119), all of which are potentially capable of protonation in absence of Zn.

The general proximity of the carboxylate to the metal is also indicated by our inability to detect the enriched carboxylate resonance in $Co^{2+}$-substituted CmHCAB (Figure 2C). The cobalt in $Co^{2+}$-HCAB is known to be paramagnetic (Lindskog & Ehrenberg, 1967; Aasa et al., 1976) and the visible spectra of $Co^{2+}$-substituted HCAB and CmHCAB are virtually identical except for a p$K$ shift of the aquo ligand of the metal (Whitney, 1970; Taylor et al., 1970). Paramagnetic ions are expected to exert dipolar and/or contact effects on the resonances of nearby nuclei (LaMar et al., 1973) and such effects have been recently reported in $^{13}$C NMR studies of carboxylates of inhibitors in $Co^{2+}$-substituted bovine carbonic anhydrase (Bertini et al., 1976, 1977; Yeagle et al., 1975). We are unable to detect changes in the metal–carboxylate distance by these measurements at present, since the shifted and/or broadened resonance was not observed over the pH range of 5.8 to 9.8 and in the presence and absence of sulfonamide inhibitors. It may be noted that the disappearance of the enriched resonance in $Co^{2+}$-substituted CmHCAB is *not* due to broadening by nonspecifically bound $Co^{2+}$, since the resonance of uncomplexed apoCmHCAB was only diminished in intensity by the addition of the metal (Figure 2D).

We have attempted to probe the carboxylate environment through another $^{13}$C NMR approach, namely, by the study of relaxation properties that are known to be sensitive to the *motion* of the observed nucleus (Lyerla & Grant, 1972). More specifically, we have measured the NOE and spin–lattice relaxation rate of the enriched carboxylate at different conditions and inquired whether the data are consistent with immobilization of the carboxylate or with the presence of some internal motion. While immobilization does not prove coordination to the metal, the finding of any internal motion under some conditions would be clearly inconsistent with coordination or strong interactions involving active-site groups. The interpretation of the relaxation data has been carried out by comparison with theoretical calculations on the expected relaxation parameters for various models of motion. Two main relaxation mechanisms have been considered. These are dipolar interactions of the carboxylate carbon with the two adjacent methylene protons 2.09 Å away[7] (Figure 5) and chemical shift anisotropy contributions assuming typical carboxylate shielding tensor parameters (see Theoretical Considerations above). The latter source may be important for carbonyl car-

[5] NMR studies of $^{113}$Cd- and $^{199}$Hg-substituted, $^{13}$C-enriched CmHCAB have the potential for observing a coupling constant between the enriched carbon and these spin ½ metals if coordination exists (our unpublished work). Preparation of these derivatives is in progress.

[6] A Nicholson (Labquip) "push-fit" atomic model of HCAB was built in our laboratory based on the atomic coordinates deposited by Dr. K. K. Kannan at the Protein Data Bank of the Brookhaven National Laboratory.

[7] The C–H distance of 2.09 Å and the $\theta$ value of 96.7° (angle between the C–H vector and the $N^{\tau}$–$C_M$ axis) are based on our analysis of the crystal structure data of the $NCH_2COOH$ grouping in glycylglycine hydrochloride (Parasarathy, 1969) and perdeuterio-$\alpha$-glycylglycine (Freeman et al., 1970).

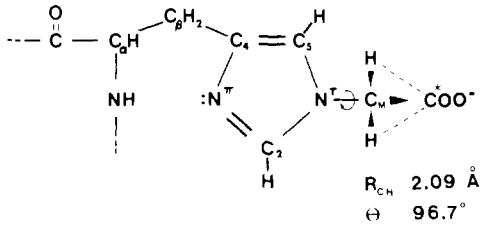


FIGURE 5: $N^{\tau}$-Carboxymethylhistidyl unit showing nomenclature and the enriched carboxyl (asterisked). The angle between the C-H vectors (- - -) and the $N^{\tau}$-$C_M$ axis is denoted by $\theta$. Note that rotation of the carboxyl around the $C_M$-C* axis does not affect the C*-H vectors.

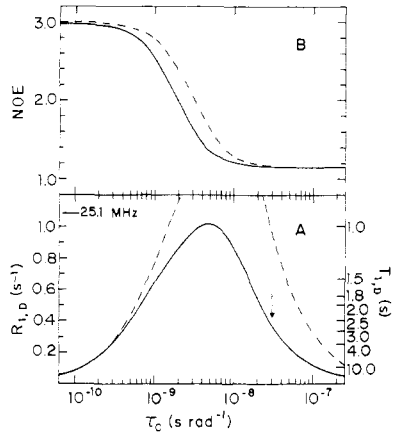


FIGURE 6: Calculated spin-lattice relaxation rates (A) and nuclear Overhauser enhancements (B) for a $^{13}C$ nucleus relaxed by dipolar interaction with *two* protons 2.09 Å away and undergoing rigid isotropic rotation characterized by a correlation time $\tau_C$. The solid curves are for a resonance frequency of 25.15 MHz and the dashed curves are for 15.04 MHz.

bons of proteins (Norton et al., 1977), although not in small carboxylates (Farrar et al., 1972). Figure 6 presents $R_1^D$ and NOE calculations[8] for an immobilized carboxymethyl carboxylate in $D_2O$ solutions at 15.04 and 25.15 MHz as a function of the overall correlation time $\tau_C$ for isotropic rotation of the protein. Figure 7 shows the effects of internal rotation around the $N^{\tau}$-$C_M$ bond (Figure 5), characterized by a correlation time $\tau_G$, for a protein with 30 ns rad$^{-1}$ overall rotational correlation time. Figure 8 shows the CSA contributions to the relaxation of an immobilized carboxylate (Figure 8A) and a carboxylate capable of internal rotation around the $C_M$-$C_O$ bond in a protein with a $\tau_C$ of 30 ns rad$^{-1}$ (Figure 8B). The correlation time for this rotation ($\tau_G'$) should be distinguished from that relevant to the dipolar contribution ($\tau_G$), since different bonds are involved.

The examination of the results of the calculations reveals that the measured values of $R_1$ and the NOE of *CmHCAB* at pH 8 are very compatible with immobilization. Thus at 25.15 MHz, the observed NOE is 1.2 and the observed $R_1$ is 0.37-0.41 s$^{-1}$. The calculated values for a $\tau_C$ of 30 ns rad$^{-1}$ are 1.16 for the NOE and 0.37 s$^{-1}$ ($R_1^D$) plus 0.065 s$^{-1}$ ($R_1^{CSA}$). Similar agreement is found at 15.04 MHz where $R_1$ was measured at 0.98 s$^{-1}$ while the calculated value was 0.97 s$^{-1}$ ($R_1^D$) plus 0.06 s$^{-1}$ ($R_1^{CSA}$). The calculated CSA contribution is rather negligible at 15.04 MHz but amounts to about 20% of the dipolar value at 25.15 MHz. This is insufficient to significantly alter the dipolar NOE. A confirmation of the probable magnitude of the CSA contributions comes

[8] The calculated $R_1^D$ values are especially sensitive to the assumed C-H distance, so that a change of 0.05 Å leads to a 15% change in $R_1^D$.

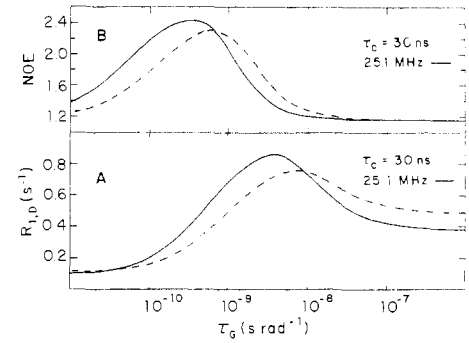


FIGURE 7: Effect of internal rotation around the $N^{\tau}$-$C_M$ bond (correlation time $\tau_G$) on the dipolar contributions to the spin-lattice relaxation rate (A) and nuclear Overhauser enhancement (B) for a carboxymethyl carboxylate located on a macromolecule with an overall isotropic rotation characterized by a correlation time $\tau_C$ of 30 ns rad$^{-1}$. Calculations are given for frequencies of 25.15 MHz (solid curves) and 15.04 MHz (dashed curves).

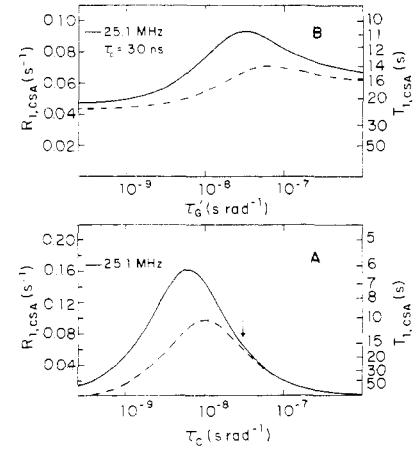


FIGURE 8: Chemical shift anisotropy contributions to the spin-lattice relaxation rate of an immobilized (A) and rotating (B) carboxylate located on a macromolecule with an overall isotropic correlation time $\tau_C$. In B, $\tau_C$ is fixed at 30 ns rad$^{-1}$, while $\tau_G'$ is allowed to vary and refers to rotation around the $C_M$-$C_O$ axis. Calculations are for frequencies of 25.15 MHz (solid curves) and 15.04 MHz (dashed curves).

from the measured line width at 90.5 MHz, where the CSA mechanism can be shown to dominate (Norton et al., 1977). The observed value of 5 Hz is in good agreement with the calculated value of 4 Hz obtained by assuming the carboxylate is immobilized (eq 5, using $\tau_C$ = 30 ns rad$^{-1}$). Despite the small magnitude of the CSA contributions at 25.15 MHz, they were fully considered since *changes* in $R_1$ were found (see below), and both $R_1^D$ and $R_1^{CSA}$ are known to be very sensitive to effects of internal rotation (Doddrell et al, 1972; Hull & Sykes, 1975) that could only be predicted by calculation.

The decrease of pH from 8 to 5.4-5.7 causes a significant increase in the observed $R_1$ of CmHCAB (but not apoCmHCAB), with the NOE remaining constant. This is clearly suggestive of the onset of some internal motion in the carboxylate. At 25.15 MHz the observed change in $R_1$ is approximately 0.24 s$^{-1}$. This *cannot* be accounted for by the sole rotation of the carboxyl group around the $C_M$-$C_O$ axis, a motion that does not affect the C-H dipolar vector and can only contribute to $R_1^{CSA}$. Figure 8 shows that no amount of such rotation can increase the $R_1^{CSA}$ of a previously immobilized carboxyl by more than 0.03 s$^{-1}$ at this field. It is thus necessary to invoke motion or rotation around at least the $N^{\tau}$-$C_M$ bond. Figure 7 shows that motion around this bond with a $\tau_G$ of either $7.6 \times 10^{-9}$ or $1.7 \times 10^{-8}$ s rad$^{-1}$ could

produce an increase in $R_1$ of the desired magnitude. However, only the latter value is consistent with the NOE value of 1.2. A similar analysis of the data at 15.04 MHz, where $R_1$ increases by 0.7 $s^{-1}$ on lowering the pH, leads to the requirement of a $\tau_G$ of $1.3 \times 10^{-8}$ s $rad^{-1}$, consistent with the other field. These results do not exclude, of course, the possible rotation of the imidazole ring itself, although it is unnecessary to invoke it at present.

Internal rotation of the above magnitude represents a highly restricted rotation, indicative of considerable steric or energetic barriers in the active site. This is in contrast with a $\tau_G$ of $5 \times 10^{-11}$ s $rad^{-1}$ that has been reported for carboxymethyl groups attached nonspecifically to presumably exposed histidines of cytochrome *c* (Eakin et al., 1975). Inspection of a molecular model of HCAB[6] confirms that motion of the carboxymethyl group could bring the carboxylate into contact with or in close proximity to a number of active-site groups, depending on the orientation of the imidazole ring to which it is attached. These include side-chain groups of histidines-64 and -94, tryptophan-5, proline-201 (carbonyl oxygen), as well as the zinc metal and its solvent ligand. Indeed, the limited relaxation data on apoCmHCAB also show considerably hindered internal motion of the carboxymethyl group, even though the possibility of coordination to the metal does not occur. It is perhaps not so unreasonable in this light to find that the introduction of even a small inhibitor, such as azide which is known to bind at the zinc (Riepe & Wang, 1968), is sufficient to eliminate whatever motion was present in CmHCAB at low pH. This observation would have been contrary to expectations based solely on the displacement of the carboxylate, if coordinated, by the inhibitor.

In conclusion, the chemical shift data reported in this study and elsewhere (Khalifah, 1977) are consistent with, but do not prove, an interaction of the carboxylate with the zinc or its aquo ligand, as shown in Figure 4 for the case of inner sphere coordination. The relaxation data on CmHCAB seem to show an immobilization at pH 8 where the chemical shift is abnormal, and they indicate an acquired mobility at low pH where the chemical shift is "normalized". Immobilization by itself, however, is not a sufficient indication of coordination or interaction with the metal, especially since there is apparently considerable hindrance to the motion of the carboxymethyl group in absence of the metal. The hypothesis becomes a reasonable, working one when it is realized that the metal and its protonated aquo ligand are the only potentially positively charged groups in the active site at pH 8. It is also pertinent that histidine-200 is within 6 Å of the zinc (Notstrand et al., 1975; Kannan et al., 1975) and that model building[6] shows the carboxyl to be within coordination distance to the zinc. In addition, carboxylates are well-known inhibitors of the enzyme and are believed to bind at the zinc, as mentioned above.

The mechanistic implications of this coordination hypothesis have been briefly touched upon before (Coleman, 1975; Khalifah et al., 1977). The hypothesis provides an excellent molecular explanation of the catalytic changes that result from carboxymethylation. Most obvious in this regard is the significant increase in the pK of the "catalytically essential" ionizing group (Whitney, 1970) frequently identified as the metal-coordinated hydroxide at high pH (Lindskog & Coleman, 1973; see also Kannan et al., 1977). Competition between the carboxylate and hydroxyls for binding to the metal will necessarily raise the apparent p*K* of the catalytic activity in the zinc-hydroxide mechanism. One can similarly account for the decrease in affinity for monovalent anionic inhibitors that are believed to bind at the zinc (Bergstén et al., 1972; Taylor et al., 1970; Taylor & Burgen, 1971; Whitney & Brandt, 1976). Perhaps most important is the recognition that the changes in catalytic properties that result from carboxymethylation probably do not result from the disruption of the role, if any, that histidine-200 plays in the mechanism. The activity changes could result entirely or partially from the effects that the introduced carboxylate exerts through its interaction with the essential metal. Our results thus serve as a strong reminder that the effects of chemical modification of active-site groups should be subjected to searching investigation before conclusions can be drawn regarding their mechanistic role in catalysis.

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