Paramagnetic ^1H and ^{13}C NMR Studies on Cobalt-Substituted Human Carbonic Anhydrase I Carboxymethylated at Active Site Histidine-200: Molecular Basis for the Changes in Catalytic Properties Induced by the Modification†

Raja G. Khalifah,* Janice I. Rogers, Patricia Harmon, Pamela Jeffers Morely,‡ and Steven B. Carroll

ABSTRACT: Using bromo[1-^{13}C]acetate to modify N^{τ} of His-200 of human carbonic anhydrase isozyme I leads to the introduction of a useful ^{13}C NMR probe into the active site. To complement our previous diamagnetic NMR studies with this probe, we have now succeeded in directly observing the paramagnetically perturbed resonance of the carboxylate in the cobalt-substituted modified enzyme above pH 8. In the pH range 8–10, the resonance undergoes a pH-dependent slow-exchange process, with the more alkaline form having a much smaller pseudocontact shift and a narrower line width. Below pH 8, the resonance apparently undergoes a very large paramagnetic downfield shift that was estimated by extrapolation. An ionization of approximate pK of 6 appears to control this process. Paramagnetic spin-relaxation studies on the resonance under conditions where it was directly observed yielded distance measurements between the carboxylate carbon and the active site cobalt ion. In inhibitor complexes, this distance was in the range of 5–7 Å. In the absence of inhibitors, the distance was approximately 3.0–3.2 Å at pH 7.9, consistent with the coordination of the carboxylate to the metal. However, at pH 10, the distance was increased to 4.8 Å. These distance determinations were aided by relaxation measurements of a paramagnetically shifted proton resonance at 60–65 ppm downfield assigned by others to a proton of a ligand histidine of metal and confirmed by us to be 5.2 ± 0.1 Å from the metal. Our findings provide a molecular basis for the observed changes in catalytic properties that accompany the carboxymethylation.

Carbonic anhydrase (EC 4.2.1.1) is a zinc metalloenzyme known to occur in humans in the form of three distinct gene products referred to as isozymes I (formerly HCAB), II (formerly HCAC), and III (the skeletal muscle enzyme) (Lindskog et al., 1971; Lindskog, 1982). The erythrocyte isozyme I contains a unique active site histidine-200 that occurs close to the essential zinc in the active site and is highly reactive toward haloacetates (Bradbury, 1969; Whitney et al., 1967). Carboxymethylating CA I[1] at His-200 with bromoacetate or iodoacetate produces marked changes in the catalytic activity of the enzyme, the pH dependence of the residual activity, and the affinity toward sulfonamides and other inhibitors (Whitney, 1970; Khalifah & Edsall, 1972; Taylor et al., 1971). However, the molecular basis for these changes is not well understood (Khalifah, 1980). We consequently labeled His-200 with ^{13}C-enriched bromoacetate, thereby introducing a potentially useful ^{13}C NMR carboxylate probe into the inner active site. Our extensive diamagnetic NMR studies on this derivative (CmCA I) (Khalifah et al., 1977; Khalifah, 1977; Jeffers et al., 1978; Khalifah & Jeffers, 1978; Khalifah & Morley, 1980) suggested that the carboxymethyl carboxylate interacts with the essential zinc under specific pH conditions [see also Coleman (1975)], possibly providing a molecular explanation for the catalytic activity and active site ionization changes that result from the carboxymethylation.

As part of our efforts to understand the catalytic mechanism of this enzyme and to elucidate the possible carboxylate–metal interaction in CmCA I, we attempted ^{13}C NMR paramagnetic perturbation studies on the cobalt-substituted (Bertini et al., 1981a; Bertini & Luchinat, 1983) and ^{13}C-labeled CmCA I. Such studies have the potential for measurement of carboxylate–metal distances (Mildvan & Gupta, 1978; Burton et al., 1979) and can thus critically shed light on the interaction hypothesis. Our efforts were initially thwarted, as the resonance of the labeled carboxylate apparently broadened and/or shifted beyond detection (Jeffers et al., 1978) due to the large paramagnetic effect of the nearby cobalt ion. We subsequently found that the carboxylate resonance is detectable under conditions of complete inhibition of the enzyme (Khalifah & Morley, 1980) where the carboxylate is predicted to be displaced from the metal by competing external inhibitors. As reported in our preliminary paper (Morely et al., 1984) and in full detail here, we have utilized these observations to deduce the properties of the *uninhibited* CmCA I enzyme, first by extrapolation and then by *directly* locating its paramagnetically shifted resonance under favorable conditions. Thus, our studies have now permitted direct paramagnetic relaxation measurements that have led to the determination of carboxylate–metal distances in CmCA I in the presence and absence of inhibitors. Our previous hypothesis is reexamined in light of these new and more definitive observations.

Experimental Procedures

Enzymes and Chemicals. Human carbonic anhydrase I was prepared in gram quantities from freshly outdated erythrocytes by the affinity chromatography procedure previously described (Khalifah et al., 1977). Specific carboxymethylation of N^{τ}

† From the Biochemistry Department, University of Kansas Medical School (R.G.K.) and the Veterans Administration Medical Center (R.G.K., J.I.R., and P.H.), Kansas City, Missouri 64128, and the Chemistry Department, University of Virginia, Charlottesville, Virginia 22901 (P.J.M. and S.B.C.). *Received December 6, 1983.* This work was supported by the Medical Research Service of the Veterans Administration and by the U.S. Public Health Service (NIH Grants HL-17963 and HL-26348-02). Portions of the work by P.J.M. were carried out in partial fulfillment of the requirements for the Ph.D. in chemistry at the University of Virginia. A preliminary account of part of this work was presented at the New York Academy of Sciences Conference on the Biology and Chemistry of the Carbonic Anhydrases, Sept 28–30, 1983.

* Address all correspondence to this author at Research Service, Veterans Administration Medical Center, Kansas City, MO 64128.

‡ Present address: Spectra-Physics, Inc., San Jose, CA 95134.

[1] Abbreviations: CA, carbonic anhydrase; CmCA I, human carbonic anhydrase isozyme I carboxymethylated at N^{τ} of histidine-200; EDTA, ethylenediaminetetraacetic acid.

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of His-200 was accomplished with 90% enriched bromo[1-^{13}C]acetate (KOR Isotopes) by following well-known procedures [cf. Strader & Khalifah (1976) and references therein]. For kinetic studies of inhibition, unmodified enzyme was removed by ion-exchange chromatography on a Bio-Rex 70 column (Laurent et al., 1965). Metal-free apoCmCA I was prepared by dialysis of CmCA I against pyridine-2,6-dicarboxylic acid (Kidani et al, 1976; Hunt et al., 1977). Co(II)CmCA I was prepared by the stoichiometric addition of Specpure cobalt sulfate (Johnson-Matthey). The extent of cobalt incorporation was monitored by acquisition of the characteristic visible spectrum (Bertini et al., 1978, 1982a; Bertini & Luchinat, 1983) and by the increase in catalytic activity. All samples for NMR measurements contained sulfate to bring the ionic strength to 0.2.

Enzymatic Assays and Inhibition. Routine CA activity measurements were carried out with the Wilbur–Anderson carbon dioxide hydration assay (Rickli et al., 1964). However, for studies of metal incorporation into apoCmCA I, precautions were necessary to prevent metal ion contamination. Consequently, the veronal assay buffer was made 0.1 mM in EDTA. For the determination of the inhibition constant K_i for azide, we followed the *p*-nitrophenyl acetate hydrolysis activity of the enzyme [cf. Whitney et al. (1967)], as the buffers could be chosen such that they were identical with the buffers in the NMR experiments. Rates were spectrophotometrically determined from the initial slope method and a 348-nm isosbestic point. Noncompetitive inhibition was assumed, as the substrate concentration was below its K_m.

NMR Spectroscopy. ^{13}C NMR spectra were obtained at three frequencies: 20.1 MHz (1.88 T) on a Bruker WP-80 spectrometer, 25.15 MHz (2.35 T) on a JEOL PFT 100/EC 100 spectrometer, and 50.32 MHz (4.70 T) on a Bruker WP-200 spectrometer. Samples were contained in 10-mm tubes and spectra were taken under full proton broad-band decoupling conditions, ambient probe temperatures being 298–304 K. Chemical shifts are referenced to $(CH_3)_4Si$ and were measured relative to internal dioxane (1–2 μL/mL), whose shift was taken as 67.40 ppm. About 10% D_2O was added for internal lock. Precautions were taken to remove metal ions from glassware and D_2O in relaxation experiments. Spin–lattice relaxation times were obtained by the inversion–recovery method and were calculated from three-parameter nonlinear regression methods. T_2 was measured from the line width after correction for instrumental and digital contributions by subtracting the dioxane line width. Additionally, ^{1}H NMR experiments were carried out at 200.13 MHz with the Bruker instrument. For these experiments, the enzyme was lyophilized twice from D_2O to remove exchangeable protons and was then redissolved in 99.96% D_2O and run in 5-mm tubes. Proton chemical shifts are reported relative to $(CH_3)_4Si$, using the HOD resonance at 4.78 ppm as the internal standard.

Results

Chemical Shift Studies of Enzyme–Inhibitor Complexes. The enriched carboxylate resonance of Co(II)CmCA I is readily detectable in the 175–185 ppm range but only when the enzyme is fully complexed with inhibitors. The resonance is easily assigned by its intensity and by its absence in enzyme modified with nonenriched bromoacetate. Decreasing the extent of inhibition leads in cases of fast exchange to the progressive downfield shifting and extreme broadening of the resonance. Figure 1 illustrates this behavior with the case of the powerful inhibitor azide. The resonance occurs at close to 181 ppm with a line width of about 15 Hz when the enzyme is saturated with azide, while in the absence of inhibitor at

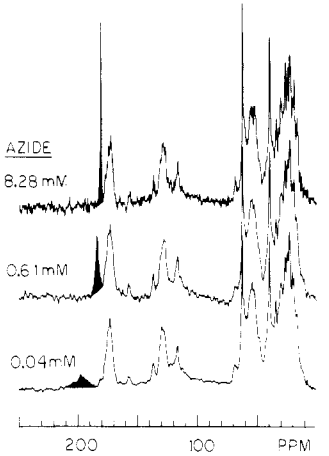

FIGURE 1: ^{13}C NMR spectra (50.32 MHz) of cobalt-substituted and ^{13}C-labeled CmCA I in the presence of different concentrations of the inhibitor azide at pH 8.6. The enriched carboxylate resonance is shaded and is seen to undergo progressive upfield shifting and narrowing in the presence of increasing concentration of azide. The limiting shift values are approximately 207 and 181 ppm for the uninhibited and the complexed enzyme, respectively, as obtained by a nonlinear regression analysis of the dependence of the chemical shift on inhibitor concentration (see text). The indicated values on the spectra are the estimated free-azide concentrations. Protein concentration was initially 6 mM, and spectra were acquired with a 25-kHz sweep width.

this pH the resonance shifts to about 200 ppm and has a line width of about 1000 Hz. The chemical shift of the inhibited enzyme varies with the nature of the inhibitor, as the following representative values indicate: acetazolamide, 176.6 ppm; cyanide (1 equiv), 179.3 ppm; cyanate, 178.3 ppm; iodide, 185 ppm; thiocyanate, 185 ppm; bromide, 186.7 ppm. Corresponding shifts for the zinc enzyme occur in the range of 172–176 ppm, depending on pH, as previously discussed (Strader & Khalifah, 1976; Khalifah, 1977). The pH dependence of the shifts in the inhibitor complexes of the cobalt enzyme was not specifically determined. In the case of the extensively studied azide complexes where titrations were carried out (see below), the chemical shift of the complex was pH invariant from about pH 9 to pH 6 and decreased below that, suggestive of a pK_a below 6. In general, it appears that compared to complexes with the zinc enzyme, pseudocontact shifts of approximately 2–10 ppm are experienced by the carboxylate in the cobalt derivatives. It is noteworthy that the addition of a large excess of cyanide leads to a bleaching of the deep purple color and to the appearance of carboxylate intensity at the position of the apoenzyme [cf. Jeffers et al. (1978)], suggestive of the removal of the metal from the active site.

Paramagnetic ^{13}C Relaxation Studies of Inhibitor Complexes. Spin–lattice relaxation measurements were carried out on the enriched carboxylate in several inhibitor complexes of Co(II)CmCA I. The paramagnetic contributions to these relaxation rates were obtained after subtracting the estimated (small) diamagnetic contributions of the zinc CmCA I [cf. Jeffers et al. (1978)]. Similar estimates of T_2 relaxation times were obtained from the observed line widths. The results are given in Table I. Diamagnetic contributions varied from 2 to 20% for T_1 and from 20 to 60% for T_2. In the case of the sulfonamide inhibitor acetazolamide, the carboxylate spin–lattice relaxation rate was determined at 20.1, 25.2, and 50.32 MHz.

Chemical Shift of Uninhibited Co(II)CmCA I. The enriched carboxylate resonance of the uninhibited cobalt-substituted enzyme initially proved undetectable in our studies

Table I: ^{13}C Paramagnetic Relaxation of the Enriched Carboxylate of Co(II)CmCA I

inhibitor	pH	field (T)	T_{1M} (s)[a]	πT_2 (s)
N_3^-	6.9	2.35	0.43[b]	0.5
N_3^-	8.0	2.35	0.51	0.3
I^-	8.5	2.35	0.12	0.04
I^-	9.2	2.35	0.14	0.04
CN^-	6.6	2.35	0.037	0.05
CN^-	6.9	2.35	0.041	0.06
NCO^-	7.0	2.35	0.12	0.1
acetazolamide	7.9	2.35	0.12	0.2
acetazolamide	8.2	1.88	0.094	
acetazolamide	8.2	4.70	0.103	
none	7.9	1.88	0.0046 ± 0.0011	
none	7.9	4.70	0.0098 ± 0.0017	
none	10.3	4.70	0.046 ± 0.005	

[a] Obtained by subtracting diamagnetic contributions from observed relaxation rates. Diamagnetic contributions for an immobilized carboxylate relaxed by two protons 2.09 Å away yield $1/T_1$ values of 0.63, 0.44, and 0.16 at 1.88, 2.35, and 4.70 T, respectively, for an immobilized carboxylate on a protein with a τ_R value of 30 ns rad^{-1}. Calculations included dipolar and chemical shift anisotropy contributions [cf. Jeffers et al. (1978)]. [b] Estimated standard deviations are 10% for inhibitor complexes.

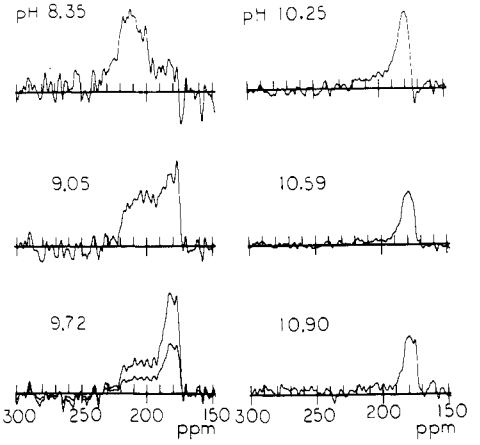

FIGURE 2: Dependence of the enriched carboxylate resonance of Co(II)CmCA I on pH in the alkaline range. Due to the overlap of the natural abundance carbonyl carbon region of the protein, the difference between the spectra of ^{13}C-enriched Co(II)CmCA I and nonenriched ZnCmCA I is shown at each pH. Spectra were taken at 50.32 MHz with 25-kHz sweep widths at the indicated pH values. The spectra have been taken with different numbers of scans and have not been normalized to give the same total intensity.

at 25 MHz with limited sweep-width capabilities (Jeffers et al., 1978). However, using a "reverse inhibitor titration" method that we recently described (Morley et al., 1984), we have been able to predict and subsequently directly verify that the resonance is extremely broad (approximately 1000 Hz) and occurs near 200 ppm near pH 8 (cf. bottom spectrum in Figure 1). We have extended these initial findings and now report that the resonance can be directly observed in the alkaline range (pH 8–11). However, it undergoes significant pH-dependent changes, as illustrated in the difference spectra of Figure 2 where the natural abundance signals of the protein have been subtracted. The resonance appears to be in slow exchange between a neutral-pH and a high-pH form, with the more alkaline spectrum being much narrower and exhibiting a chemical shift (180 ppm) only about 5 ppm from the diamagnetic position. It is important to recall that the carboxylate resonance in the zinc CmCA I also undergoes a significant pH perturbation with a midpoint near pH 9.2 (Khalifah et al., 1977) that has been linked to the ionization of the water ligand of the active site zinc.

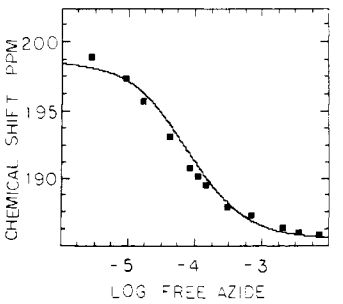

FIGURE 3: Dependence of the enriched carboxylate chemical shift on the concentration of the free inhibitor azide. Data were taken from the experiment of Figure 1 at 50.32 MHz and pH 8.6 where the resonance could be observed throughout. The solid line represents a weighted nonlinear regression fit to eq 1 and 2 of the text.

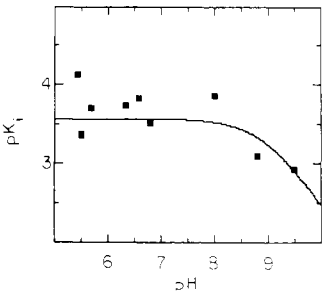

FIGURE 4: Dependence of the azide inhibition constant of Co(II)-CmCA I on pH. The K_i values were obtained by inhibition of the esterase activity of the enzyme. The curve represents a nonlinear regression fit of the apparent pK_i to eq 3 of the text where binding was assumed to be controlled by a single active site ionization on the enzyme.

In contrast to the above results, we have been unable to directly observe the carboxylate resonance below about pH 7.9 and extending down to pH 6.4. Consequently, we have relied on the indirect approach (Morley et al., 1984), where we use the reverse inhibitor titration method. The chemical shift in the presence of the inhibitor azide appears to follow fast-exchange behavior; i.e., it is the weighted average of the (unknown) chemical shift of the free enzyme and the fully inhibited complex, according to

$$\delta_{obsd} = f_{EI}\delta_{EI} + (1 - f_{EI})\delta_E \quad (1)$$

where the subscripts E and EI refer to the free enzyme and to the inhibitor complex, respectively. Figure 3 demonstrates conformity with this behavior under conditions of pH where the resonance could be observed throughout. The fraction of complexed enzyme can be expressed in terms of the total enzyme and total inhibitor concentrations (E_0 and I_0) as follows:

$$f_{EI} = [(E_0 + I_0 + K_i) - [(E_0 + I_0 + K_i)^2 - 4E_0I_0]^{1/2}]/(2E_0) \quad (2)$$

where K_i is the dissociation constant for azide inhibition. Insertion of eq 2 into eq 1 leads to an expression for the observed chemical shift in terms of the three unknown parameters (δ_E, δ_{EI}, and K_i), so that nonlinear regression analysis of a series of δ_{obsd} measurements at different azide concentrations at a given pH can lead to the calculation of the desired chemical shift δ_E of the free enzyme. In practice, it was observed that as the pH was lowered below pH 8, the line width increased more rapidly and prevented observation of the resonance at low fractions of inhibition. As a result of this, curve fitting of the increasingly limited data led to computed K_i and δ_E parameters that were highly correlated. Consequently, we chose to increase the meaningfulness of the analysis by independently determining the K_i for azide inhibition of Co(II)CmCA I using inhibition of the esterase activity of the enzyme (Verpoorte et al., 1967) in buffers identical with those

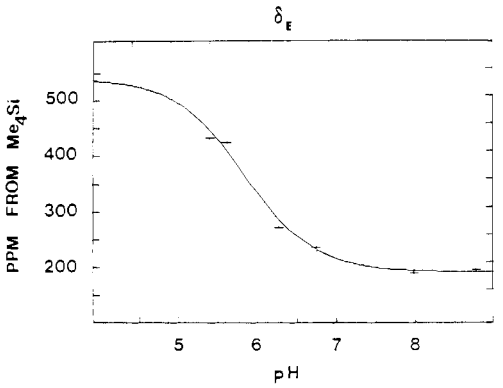

FIGURE 5: Dependence of the chemical shift of the carboxylate of uninhibited Co(II)CmCA I on pH. The values of δ_E were obtained by extrapolation of the chemical shift of the azide-inhibited enzyme to zero azide concentration at each pH with the constrained nonlinear regression procedure described in the text. The K_i value for azide at each pH used in the fitting was independently determined by activity inhibition (cf. Figure 4). All spectra were taken at 25.15 MHz.

for the NMR measurements. Figure 4 indicates the K_i values we obtained. Since the inhibitor binding is linked to the active site ionization of the enzyme with a pK_a of about 9.2 in Co(II)CmCA I (Whitney, 1970; Whitney & Brandt, 1976), we smoothed these pK_i vs. pH data by fitting them with nonlinear regression to the following form:

$$pK_i^{app} = pK_i^* + \log [[H^+]/(K_a + [H^+])] \quad (3)$$

The pK_a value so obtained was 8.9 ± 0.2, in agreement with expectations.

Using these independent K_i values, we than applied constrained two-parameter nonlinear regression to fit the dependence of the δ_{obsd} on azide concentration at each pH (combined eq 1 and 2) to obtain the estimated δ_E of the uninhibited Co(II)CmCA I along with δ_{EI} of the complexes. A typical plot has been previously given (Morley et al., 1984) under the more favorable conditions of high pH. The extrapolated chemical shifts of the uninhibited enzyme obtained in this way are shown in Figure 5. It is readily apparent that, despite the indirectness and uncertainties of this approach, a definite trend is seen where the chemical shift of the carboxylate of the uninhibited enzyme undergoes extremely large pH-dependent changes in its chemical shift that approach a few hundred ppm at the low pH limit. An analysis of this pH dependence indicates that a process of approximate pK_a of 5.9 is involved. These findings may explain the inability to directly observe the carboxylate resonance at low pH, particularly since a similar analysis of the line-width changes would also indicate extremely large broadening as the pH is decreased. The pK_a = 5.9 process can be assigned to the ionization of the CmHis-200 ring and is virtually identical with the value obtained in studies on the zinc CmCA I (Khalifah et al., 1977).

^{13}C Paramagnetic Relaxation of Uninhibited Co(II)CmCA I. We undertook ^{13}C NMR spin–lattice relaxation measurements on the enriched carboxylate of Co(II)CmCA I at the pH limits where the resonance could be directly observed (cf. Figure 2). Parts A and B of Figure 6 show representative inversion–recovery measurements at 50.32 MHz at pH 7.9 and 10.3. As discussed above for Figure 2, the spectra at pH 10.3 are shown in the difference mode where the spectrum of unenriched Zn(II)CmCA I was taken under identical conditions and was then subtracted from the spectra of the cobalt enzyme to remove natural abundance overlapping resonances of the carbonyl region of the protein. The relaxation rates were determined by three-parameter nonlinear regression analysis and are also given in Table I along with their standard deviations from the fit. The experiment at pH 7.9 was repeated on the same sample at a lower field of 1.88 T, and the results are included in the table. Due to the more complicated nature of the experiment at high pH and the limited access to the lower field instrument, a second field experiment could not be run for the pH 10.3 sample. Diamagnetic corrections to the observed relaxation rates were a negligble 1% or less [cf. Jeffers et al. (1978)].

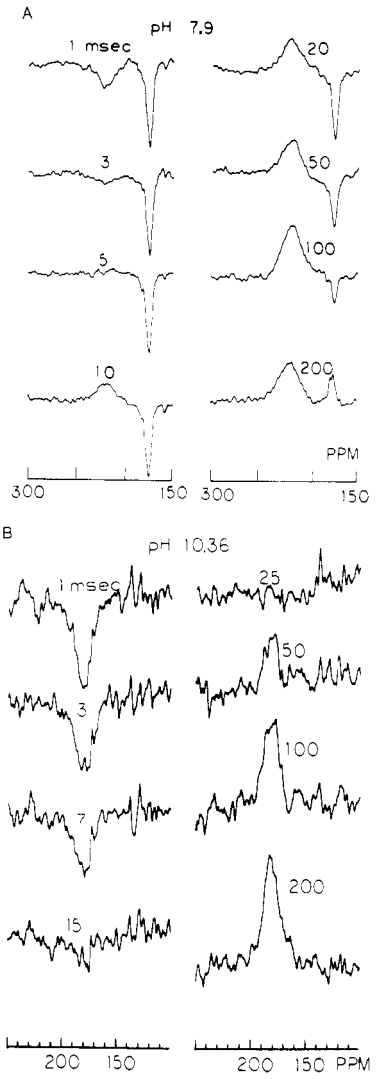

FIGURE 6: ^{13}C NMR inversion–recovery spin–lattice relaxation of the enriched carboxylate of Co(II)CmCA I taken at 50.32 MHz with 25 kHz sweep widths. The waiting times between the 180 and 90° pulses in milliseconds are indicated in the figure. (A) Sample at pH 7.9. The resonance of the carboxylate is the broad signal below 200 ppm, while the more slowly relaxing natural abundance envelope of the carbonyl region is seen near 173 ppm. (B) Sample at pH 10.3. Due to the overlap of the carboxylate resonance near 180 ppm with the natural abundance carbonyl envelope of the enzyme, the latter has been removed by subtraction of a sample of CmCA I prepared with nonenriched bromoacetate.

Paramagnetic ^1H NMR Studies of Co(II)CmCA I. It has been recently shown (Bertini et al., 1981a, 1983; Bertini & Luchinat, 1983) that useful information about the paramagnetism of the cobalt ion in the active site of carbonic anhydrase can be obtained by observing isotropically shifted proton resonances of ligand histidines. In particular, a resonance in the neighborhood of 60 ppm downfield has been assigned to the C_4-H of the coordinated His-119 imidazole ring. We have found a 400-Hz broad line near 65 ppm in the proton spectrum of Co(II)CmCA I at pH 5.6–10. Since this resonance has been assigned by Bertini and co-workers and was used by them to determine the cobalt electronic spin correlation time, we have

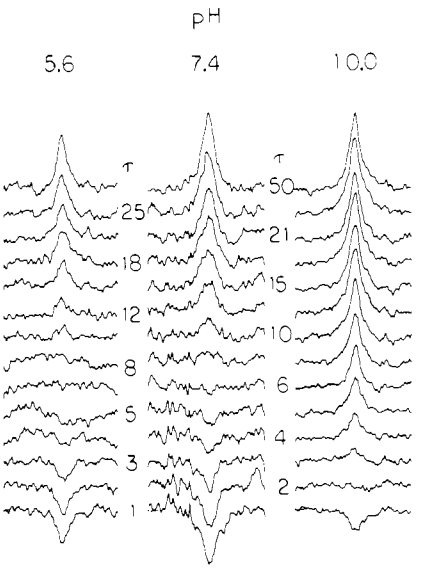

FIGURE 7: ^{1}H NMR inversion–recovery spin–lattice relaxation of the paramagnetically shifted proton resonance seen near 65 ppm downfield and assigned to a C_4-H of His-119, a ligand of the active site metal (see text). Experiments were carried out at 200.13 MHz. The pH of each experiment was 5.6 (left spectra), 7.4 (middle), and 10.0 (right), and the spectral regions shown are 56.5–78.0, 51.0–72.5, and 52.5–75.0 ppm, respectively. The waiting times (τ values) between inversion and sampling are shown in milliseconds.

Table II: Chemical Shift and T_1 Relaxation of the Downfield Proton of Co(II)CmCA I

pH	δ (ppm)	T_1 (ms)	R (Å)	τ_s ($\times 10^{11}$ s rad^{-1})
5.57	65.5	14.8 ± 2.3	(5.2)[a]	0.16 ± 0.05
7.43	61.3	12.0 ± 0.8	5.2 ± 0.1	(0.2–0.3)[b]
10.0	63.4	5.0 ± 0.3	(5.2)[a]	0.83 ± 0.05

[a] Assumed distance used in calculating correlation time in next column. [b] Assumed correlation time, determined from ^{13}C relaxation of enriched carboxylate (see text), used in determining distance in previous column.

carried out proton inversion–recovery spin–lattice relaxation measurements at the three pH regions of interest to our ^{13}C studies. Figure 7 demonstrates that the relaxation of the resonance is considerably faster at pH 10 than at the two lower pH values. Table II lists the chemical shifts and the T_1 rates of this resonance at the pH regions of interest, the latter being obtained by three-parameter nonlinear regression analysis. It is of interest that the chemical shift of this resonance showed pH dependence in the region below pH 7.4, but a complete titration experiment was not carried out.

Discussion

The results on the enriched carboxylate of the cobalt enzyme can best be understood by keeping the previous findings on the zinc enzyme in mind. The latter can be briefly summarized as follows. The carboxylate in CmCA I showed chemical shift perturbations by a group with pK_a of 6.0, assigned to the ring of CmHis-200 to which the carboxylate is attached, and a group with pK_a of 9.2, assigned to the water–hydroxide ligand of the metal. The latter perturbation is abolished upon removal of the metal or upon binding of inhibitors. These results, along with supporting spin–lattice relaxation measurements on the carboxylate of the diamagnetic CmCA I (Jeffers et al., 1978) and chemical shift titrations of a model compound (Khalifah et al., 1977), led to the following hypotheses that we wished to examine in the present work: (1) the carboxymethyl carboxylate coordinates to metal at pH 8; (2) the carboxylate is displaced from the metal at high pH (10 or above), presumably by solvent hydroxyls; (3) the carboxylate coordination is disrupted at low pH (5.5) upon protonation of the modified CmHis-200; (4) anionic inhibitors known to bind at the zinc displace the carboxylate from the metal sphere. As noted above, these conclusions have mechanistic implications and provide a particularly specific explanation for the apparent increase of the critical active site ionization, believed to be the water ligand of the metal, from a pK_a of 7.6 in CA I to a pK_a of 9.2 in CmCA I. The lower activity of the modified enzyme at high pH is unaccounted for.

In view of the strong similarity of the cobalt and zinc carbonic anhydrases (Lindskog, 1982) and the enormous potential of paramagnetic NMR studies for providing detailed geometric information [cf. Mildvan & Gupta (1978), Burton et al. (1979), and Lee & Sykes (1980)], we undertook the present study. The most direct means of determining cobalt–carboxylate distances is through the study of the spin–lattice relaxation effects of the cobalt ion on the carboxylate resonance. For the case of carbonic anhydrase with a rotational correlation time τ_R of 30 ns rad^{-1} and the cobalt ion with expected electronic relaxation time in the range of (1–10) $\times$ 10^{-12} s rad^{-1}, only the dipolar interaction (Solomon, 1955) between the metal ion and the carboxylate carbon need be considered. The observed paramagnetic shifts rule out significant hyperfine scalar interactions to T_1 (Solomon & Bloembergen, 1956). The g-tensor anisotropy is not expected to be large (Aasa et al., 1976), so that the contribution from this source (Sternlicht, 1965) will be ignored. The possible contributions from the so-called Curie spin (Gueron, 1975) or susceptibility (Vega & Fiat, 1976) mechanism to T_1 can be shown to contribute less than 5% of the dipolar one, although it will make a significant contribution to T_2. Consequently, we write for the paramagnetic dipolar T_{1M} relaxation [cf. Burton et al. (1979)]

$$T_{1M}^{-1} = [2\gamma_c^2 g^2 \beta^2 S(S+1)/(15R^6)] \times [3\tau_s/(1+\omega_c^2\tau_s^2) + 7\tau_s/(1+\omega_s^2\tau_s^2)] \quad (4)$$

In the above, γ_c is the magnetogyric ratio of ^{13}C, g is the electronic g factor taken as 2.23 (Aasa et al. 1976), S is the spin quantum number taken as $^3/_2$ (Aasa et al., 1976), ω_c and ω_s are the carbon and electron Larmor frequencies at the field of study, and R is the distance between the metal ion and the carbon nucleus whose resonance is being observed.

The successful utilization of the T_1 relaxation to the determination of the internuclear distance R is predicated on knowing the value for the electronic correlation time τ_s. Previous paramagnetic studies on cobalt carbonic anhydrase have utilized the τ_s value of about 1×10^{-11} determined by Fabry et al. (1970), but Bertini and co-workers have recently suggested that this correlation time is highly dependent on the geometry of metal coordination sphere (Bertini et al., 1981a,b, 1982b, 1983; Bertini & Luchinat, 1983). Moreover, they have discovered that the low-pH form (water ligand on the metal instead of hydroxide) of CA I has a correlation time that differs significantly from that at high pH, the latter being similar to CA II at either low or high pH. In view of the above, it was imperative that the electronic relaxation time be reliably and independently determined for our system. We have used two approaches in this regard, the first being the field dependence of the relaxation rates [cf. Mildvan & Gupta (1978)] and the second being the measurement of the relaxation time of a proton at a known distance from the metal. Figure 8 demonstrates the predicted dependence of the relaxation rate ratio for two magnetic fields used (4.70 and 1.88 T) on the electronic correlation time. Correlation times of 1×10^{-11} s rad^{-1} or greater should produce changes of about 10% or less

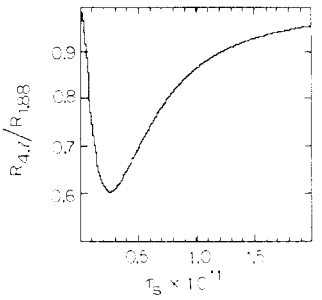

FIGURE 8: Predicted dependence of the ratio of the ^{13}C NMR spin–lattice relaxation rate ($R = 1/T_1$) at 4.7 T to that at 1.88 T on the electronic relaxation correlation time. It is assumed that a ^{13}C resonance is being observed and that the electronic relaxation dominates the overall correlation time for the interaction, as would be the case with cobalt ions and a protein the size of carbonic anhydrase. See eq 4 of the text for details.

Table III: Estimated Carboxylate Carbon Distances from Metal in Co(II)CmCA I

inhibitor	pH	field (T)	coordination[a]	τ_s ($\times 10^{11}$ s rad^{-1})	R (Å)[b]
acetazolamide	8.2	1.88	4	1–2	5.7–6.2
acetazolamide	7.9	2.35	4	1–2	5.8–6.4
acetazolamide	8.2	4.70	4	1–2	5.6–6.3
CN^-	6.6	2.35	4	1–2	4.8–5.3
CN^-	6.9	2.35	4	1–2	4.9–5.4
NCO^-	7.0	2.35	4	1–2	5.8–6.4
N_3^-	6.9	2.35	4–5[c]	0.25–1	6.4–7.2
N_3^-	8.0	2.35	4–5	0.25–1	6.5–7.4
I^-	8.5	2.35	5	0.25	5.1
I^-	9.2	2.35	5	0.25	5.3
none	10.3	4.70	4	0.83	4.8 ± 0.1
none	7.9	1.88	5	0.2–0.3	3.03 ± 0.18
none	7.9	4.70	5	0.2–0.3	3.18 ± 0.15

[a] Assignments of Bertini & Luchinat (1983). [b] Range of distances reflects range of correlation times used in previous column and/or, in the case of noninhibited samples, the experimental uncertainty in determining the relaxation times. [c] Assigned as mixed 4–5-coordination (Bertini & Luchinat, 1983), but no independent correlation time is available for this coordination. Hence, a range is selected for distance determination.

in T_1, while shorter correlation times in the vicinity of (0.2–0.3) $\times 10^{-11}$ s rad^{-1} should lead to changes by a factor of nearly 2 for these two fields. The results we obtained on the acetazolamide complex (Table I) are clearly independent of the magnetic field and are compatible only with long correlation times. This agrees with Bertini's findings that tetrahedral complexes such as the acetazolamide complex have long τ_s while pentacoordinate ones have short τ_s (Bertini et al., 1981a,b, 1982b, 1983; Bertini & Luchinat, 1983). Table III gives the computed metal–carboxylate distances for acetazolamide and other inhibitors with different assumed correlation times. Of these, CN^- and NCO^- are presumed to be 4-coordinate according to Bertini's analysis, while azide is mixed 4- and 5-coordinate and I^- is 5-coordinate. Using the shorter correlation times for these latter two inhibitors, we see that the internuclear distance is not less than about 5 Å. It is thus apparent that the carboxylate in all these inhibitor complexes is sufficiently distant from the metal ion such as to rule out coordination to the metal. The small pseudocontact shifts seen in these complexes and their narrow line widths are also consistent with the relaxation findings. The magnitude of the pseudocontact shifts is dependent on a number of factors such as the anisotropy of the metal, the distance of the nucleus from the metal, and its orientation with respect to the symmetry axis of the coordination sphere (LaMar et al., 1973). Consequently, the small differences in the chemical shifts are not possible to interpret in a definitive way, and the relaxation measurements are much more informative at present.

Of greatest interest, of course, is the carboxylate–metal distance in uninhibited enzyme. The ability to observe the carboxylate resonance (Figure 2) allowed us to carry out similar relaxation studies. At pH 7.9, we were able to obtain measurements at two magnetic fields (1.88 and 4.70 T). In contrast to the acetazolamide complex data, the paramagnetic relaxation rate was highly dependent on the field strength, with the rate at 4.70 T being 47 ± 14% of that at 1.88 T. Within experimental error, this is at the minimum in the plot of Figure 8 and indicates that the correlation time should be in the vicinity of (2–3) $\times 10^{-12}$ s rad^{-1}. This is in excellent agreement with the value (2.50×10^{-12} s rad^{-1}) that Bertini et al. (1983) recently determined for the corresponding low-pH form of the unmodified Co(II)CA I from their paramagnetic NMR study of a proton resonance assigned to C_4-H of the His-119 ligand of the metal and taken to be 5.3 Å from the metal. With this range of correlation times, we can now compute the distance of the carboxylate carbon from the metal at pH 7.9 from the ^{13}C relaxation data and eq 4. We thus find a distance of 3.18 ± 0.15 Å for the data at 4.70 T and 3.03 ± 0.18 Å for the data at 1.88 T. These distances are precisely those expected between zinc and a ligand carboxylate carbon in model complexes. For example, distances of 3.0 ± 0.1 Å are found between zinc and the carbons of two coordinated carboxyls of a zinc complex of aspartate (Freeman, 1967). A distance of 3.0 Å has also been reported for the corresponding carbon of acetate bound to bovine CA II (Bertini et al., 1977) from similar measurements.[2]

Since we also wished to measure the correlation time at high pH where we did not possess variable-field ^{13}C data, we studied the relaxation of the paramagnetically shifted proton resonance found near 60 ppm downfield (Bertini et al., 1981a, 1983) at the three pH regions of interest (Figure 7 and Table II). We first measured the distance of the proton giving rise to this resonance at pH 7.4, close to the pH where we determined the correlation time from our ^{13}C data. Using eq 4 expressed for protons, we computed the proton–metal distance to be 5.2 ± 0.1 Å, in excellent agreement with Bertini's estimate from model compounds. For our purposes, it is immaterial whether the assignment of Bertini and co-workers for this resonance is correct, since only its distance from the metal is needed. However, our data independently establish that the distance is compatible with a C_4-H of a coordinated histidine. Using this distance in combination with the proton T_1 value of this resonance obtained at pH 10.0 (Table II), we compute a correlation time of (0.83 ± 0.05) $\times 10^{-11}$ s rad^{-1}. With this value in hand, we find that the distance between the carboxylate carbon and the metal to be 4.8 ± 0.1 Å on the basis of the ^{13}C T_1 value we previously obtained at pH 10.3 (Table I). This distance is incompatible with coordination of the carboxylate to the metal, and it is quite similar to the values obtained above in some complexes of the enzyme with inhibitors. The increase in the carboxylate–metal distance at high pH accounts fully for the smaller paramagnetic shift and narrower line width seen for the carboxylate at high pH (Figure 2). The latter results alone were insufficient to prove this.

The most striking difference between our expectations from the earlier work and the present paramagnetic results occurs at the low pH limit. On the basis of chemical shift compar-

[2] This distance calculation was based on an assumed correlation time of 1×10^{-12} s rad^{-1} that has not been experimentally verified.

isons between model compounds [(carboxymethyl)histidines] and CmCA I, it was suggested (Jeffers et al., 1978) that protonation of the modified CmHis-200, which occurs with a pK_a of 6.0 (Khalifah et al., 1977), leads to a more normal environment for the carboxylate similar to that of the model compound, i.e., different from being coordinated with the metal. The paramagnetic shift of the uninhibited Co(II)-CmCA I (Figure 5) does not decrease as the pH is lowered from 8 to 5.5 but increases dramatically instead. Clearly, significant changes in the metal coordination sphere or the carboxylate environment occur as a result of protonation of the CmHis-200 ring that are not obviously compatible with a significant increase in the carboxylate–metal distance. The inability to directly observe the resonance at low pH precludes at present the measurement of this distance, although it may eventually be possible to estimate T_1 by extrapolation in the presence of decreasing inhibitor concentrations, as was done with the chemical shift. The change seen in the proton NMR resonance of the C_4-H of presumably His-119 suggests possible small changes in the coordination environment of the metal at low pH. However, the relaxation of this resonance is not too different at pH 5.6 than at pH 7.4, and the estimated electronic relaxation correlation time from the proton T_1 is 0.16×10^{-11} s rad^{-1}. In addition, there is only a small change in the cobalt visible spectrum of the enzyme at low pH (Whitney, 1970). Thus, on the basis of the criteria of Bertini & Luchinat (1983), the metal must still be pentacoordinate at pH near 8. Evidently, protonation of the ring of CmHis-200 produces alterations that lead to differences in the positioning of the carboxylate carbon with respect to the symmetry axis of the metal, to changes in the covalency of the interaction of the carboxylate with the metal, or, less likely, to changes in the magnetic anisotropy of the metal. Either of the first two factors can produce large changes in the isotropic shift of the carboxylate without greatly perturbing the coordination geometry of the metal.

It should also be kept in mind that these differences in conclusions between the studies on the zinc and cobalt enzymes at low pH, which are of little mechanistic consequence, may reflect a real difference in structure or properties between these derivatives. Precedent for this exists in the study of Whitney & Brandt (1976) on CmCA I itself, who found that the nature of the linkage of iodide inhibition with a pK_a = 6.2 group (presumably CmHis-200) differs between the zinc and the cobalt enzyme. Our own results on the pH dependence of the carboxylate resonance of the azide complex are consistent with such a difference, as we found that the pK_a of CmHis-200 is increased from 6.0 to 7.3 in the zinc enzyme (Khalifah, 1977) and decreased to around 5.9 in the cobalt enzyme (see above). In addition, the spin–lattice relaxation studies on the carboxylate in the zinc CmCA I (Jeffers et al., 1978) did reveal measureable changes at low pH indicative of increased internal mobility, such as might result from the breaking of the metal–carboxylate coordination.

Conclusions

The paramagnetic studies reported here have quantitatively defined the nature of the conformational and structural changes that occur upon the modification of active site His-200 of CA I. The carboxymethyl carboxylate coordinates to the active site metal at pH 8 and below and is displaced either by an increase of the pH or by addition of inhibitors that bind to the metal. The major conclusions from the previous diamagnetic studies have thus been gratifyingly confirmed by the paramagnetic findings. The difficulties associated with interpreting small changes in diamagnetic shifts and relaxation rates to yield unequivocal structural information are well recognized. The information obtained on Co(II)CmCA I and Zn(II)CmCA I by the combined NMR approach provides a detailed description of the molecular changes that result from modification of an inner active site residue. The catalytic activity and other properties that result from this modification can now be confidently ascribed to the interactions of the carboxymethyl carboxylate with the critical metal and its water–hydroxide ligand. The increase in the pK_a of the catalytically essential active site group (water–hydroxide ligand of the zinc) and the decrease in affinity toward anionic substrates and inhibitors must largely arise from competition of the intramolecular carboxylate with solvent hydroxyls and anions for binding at the zinc. This decrease in catalytic activity of CmCA I at high pH where the carboxylate has been displaced by a hydroxyl can now be tentatively attributed to the continued close proximity of the carboxylate to the metal (4.8 ± 0.1 Å on the average). This distance is compatible with hydrogen bonding of the carboxylate with a hydroxide ligand of the metal, although energetically there is no reason to expect that such an interaction would be favorable. Substrates and ligands of the zinc can thus experience hydrogen bonding or electrostatic interactions with the carboxylate, especially considering that the latter lies in the direction of the opening of the active site cavity. Although the modified His-200 residue, which is not conserved among the isozymes, is unlikely to be directly involved in the catalytic mechanism, its presence may well account for the major differences in catalytic properties between CA I and II isozymes. In general, our results are fully consistent with and provide support for a zinc–hydroxide mechanism (Lindskog & Coleman, 1973; Lindkog, 1982) for this enzyme.

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Registry No. N_3, 14343-69-2; I, 20461-54-5; CN, 57-12-5; NCO, 661-20-1; acetazolamide, 59-66-5; L-histidine, 71-00-1.

References

Aasa, R., Hanson, M., & Lindskog, S. (1976) *Biochim. Biophys. Acta 453*, 211–217.

Bertini, I., & Luchinat, C. (1983) *Acc. Chem. Res. 16*, 272–279.

Bertini, I., Luchinat, C., & Scozzafava, A. (1977) *J. Chem. Soc., Dalton Trans.*, 1962–1965.

Bertini, I., Canti, G., Luchinat, C., & Scozzafava, A. (1978) *J. Am. Chem. Soc. 100*, 4873–4877.

Bertini, I., Canti, G., Luchinat, C., & Fabrizio, M. (1981a) *J. Am. Chem. Soc. 103*, 7784–7788.

Bertini, I., Canti, G., & Luchinat, C. (1981b) *Inorg. Chim. Acta 56*, 99–107.

Bertini, I., Luchinat, C., & Scozzafava, A. (1982a) *Struct. Bonding (Berlin) 48*, 45–92.

Bertini, I., Canti, G., Luchinat, C., & Messori, L. (1982b) *Inorg. Chem. 21*, 3426–3429.

Bertini, I., Lanini, G., & Luchinat, C. (1983) *J. Am. Chem. Soc. 105*, 5116–5118.

Bradbury, S. L. (1969) *J. Biol. Chem. 244*, 2002–2009.

Burton, D. R., Forsen, S., Karlstrom, G., & Dwek, R. (1979) *Prog. Nucl. Magn. Reson. Spectrosc. 13*, 1–45.

Coleman, J. E. (1975) *Annu. Rev. Pharmacol. 15*, 221–242.

Fabry, M. E., Koenig, S. H., & Shillinger, W. E. (1970) *J. Biol. Chem. 245*, 4256–4262.

Freeman, H. C. (1967) *Adv. Protein Chem. 22*, 258–424.

Gueron, M. (1975) *J. Magn. Reson. 19*, 58-66.
Hunt, J. B., Rhee, M.-J., & Storm, C. B. (1977) *Anal. Biochem. 55*, 614-617.
Jeffers, P. K., Sutherland, W. M., & Khalifah, R. G. (1978) *Biochemistry 17*, 1305-1312.
Khalifah, R. G. (1977) *Biochemistry 16*, 2236-2240.
Khalifah, R. G. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 206-215, Springer-Verlag, Berlin.
Khalifah, R. G., & Edsall, J. T. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 172-176.
Khalifah, R. G., & Jeffers, P. K. (1978) in *Biomolecular Structure and Function* (Agris, P. F., Ed.) pp 363-368, Academic Press, New York.
Khalifah, R. G., & Morley, P. J. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 226-229, Springer-Verlag, Berlin.
Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241-2247.
Kidani, Y., Hirose, J., & Koike, H. (1976) *J. Biochem. (Tokyo) 79*, 43-51.
La Mar, G. N., Horrocks, W. DeW., & Holm, R. H., Eds. (1973) in *NMR of Paramagnetic Molecules. Principles and Applications*, Academic Press, New York.
Laurent, G., Charrel, M., Luccioni, F., Autran, M. F., & Derrien, Y. (1965) *Bull. Soc. Chim. Biol 47*, 1101-1124.
Lee, L., & Sykes, B. D. (1980) *Adv. Inorg. Biochem. 2*, 183-210.
Lindskog, S. (1982) *Adv. Inorg. Biochem. 4*, 115-170.
Linkdskog, S., & Coleman, J. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2505-2508.
Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O., & Strandberg, B. (1971) *Enzymes, 3rd Ed. 5*, 587-665.
Mildvan, A. S., & Gupta, R. K. (1978) *Methods Enzymol. 49*, 322-359.
Morley, P. J., Carroll, S. B., Harmon, P., & Khalifah, R. G. (1984) *J. Am. Chem. Soc. 106* 1830-1832.
Rickli, E. E., Ghazanfar, S. A. S., Gibbons, B. H., & Edsall, J. T. (1964) *J. Biol. Chem. 239*, 1065-1078.
Solomon, I. (1955) *Phys. Rev. 99*, 559-565.
Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261-266.
Sternlicht, H. (1965) *J. Chem. Phys. 42*, 2250-2251.
Strader, D. J., & Khalifah, R. G. (1976) *J. Am. Chem. Soc. 98*, 5043-5044.
Taylor, P. W., & Burgen, A. S. V. (1971) *Biochemistry 10*, 3859-3866.
Vega, A. J., & Fiat, D. (1976) *Mol. Phys. 31*, 347-355.
Verpoorte, J. A., Mehta, S., & Edsall, J. T. (1967) *J. Biol. Chem. 242*, 4221-4229.
Whitney, P. L. (1970) *Eur. J. Biochem. 16*, 126-135.
Whitney, P. L., & Brandt, H. (1976) *J. Biol. Chem. 251*, 3862-3867.
Whitney, P. L., Nyman, P. O., & Malmstrom, B. G. (1967) *J. Biol. Chem. 242*, 4212-4220.

Elementary Steps in the Reaction Mechanism of the α-Ketoglutarate Dehydrogenase Multienzyme Complex from *Escherichia coli*: Kinetics of Succinylation and Desuccinylation†

David E. Waskiewicz‡ and Gordon G. Hammes*

ABSTRACT: The kinetics of the succinylation and the desuccinylation of the α-ketoglutarate dehydrogenase complex from *Escherichia coli* have been studied at 4 °C in 2 mM thiamin pyrophosphate, 2 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0) by steady-state and quenched-flow techniques. The initial steady-state velocity for the reaction of the complex is inhibited by high concentrations of α-ketoglutarate. The data are consistent either with cooperative interactions between two catalytic sites or with the existence of an α-ketoglutarate regulatory site. The time course of the succinylation by α-ketoglutarate of the unmodified complex or the complex in which a fraction of the α-ketoglutarate decarboxylase subunits (E_1) has been inhibited with *N*-ethylmaleimide reveals a complex kinetic process. A mechanism consistent with the kinetic data is proposed in which some E_1 subunits succinylate one lipoic acid per E_1 and other E_1 subunits succinylate two lipoic acids per E_1. Furthermore, each succinylation reaction occurs via a two-step process with rate constants of 49 and 89 s^{-1} at saturating concentrations of α-ketoglutarate for the first and second steps, respectively. At long times, 13-16 mol of succinate binds per mol of unmodified complex. The stoichiometry of binding obtained with *N*-ethylmaleimide-treated complex is initially lower but approaches the same values as for the unmodified complex over the course of minutes. Coenzyme A removes the succinyl groups on the unmodified enzyme with a rate constant ≥200 s^{-1}. The results obtained suggest a limited accessibility between sites on the complex.

The α-ketoglutarate dehydrogenase complex from *Escherichia coli* contains three enzymes, α-ketoglutarate decarboxylase (E_1),[1] dihydrolipoyl transsuccinylase (E_2), and dihydrolipoyl dehydrogenase (E_3), and catalyzes the following sequence of reactions:

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[1] Abbreviations: E_1, α-ketoglutarate decarboxylase; E_2, dihydrolipoyl transsuccinylase; E_3, dihydrolipoyl dehydrogenase; CoA, coenzyme A; MalNEt, *N*-ethylmaleimide.

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