Interactions of Long-Chain Aldehydes with Luciferase. A Carbon-13 Nuclear Magnetic Resonance Study†

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ABSTRACT: The interaction of long-chain aldehydes with bacterial luciferase has been studied by ^{13}C NMR spectroscopy of natural-abundance and ^{13}C-enriched 1-dodecanal. At high substrate/enzyme ratios, the spin–spin relaxation rates of C(1)–C(3) are faster than for the other carbons and are in the order C(1) > C(2) > C(3). The aldehyde is strongly bound in the active site along the entire length of the alkyl chain with the strongest interaction at the CHO group. At low substrate/enzyme ratios, interactions are apparent at C(10), which are removed upon denaturation of the enzyme. Spin–spin and spin–lattice relaxation rates were measured for odd-carbon ^{13}C-enriched 1-dodecanal in the presence of luciferase. From the ratios of T_1/T_2 a single value of $(1.8 \pm 0.7) \times 10^{-8}$ s was calculated for the rotational correlation time τ_c for the complex.

The bioluminescent enzyme luciferase, obtained from ATCC 7744 culture which is variously known as *Photobacterium fischeri*, *Vibrio fischeri*, or *Achromobacter fischeri*, catalyzes the oxidation of long, straight-chain aldehydes to fatty acids with the emission of a photon of light at λ_{max} = 490 nm. The enzyme also requires as cofactors $FMNH_2$[1] and molecular oxygen (Hastings, 1968; Shimomura et al., 1972; Dunn et al., 1973). There is a chain-length dependence for reaction of the aldehyde with enzyme. The 14-carbon aldehyde-1-tetradecanal reacts the fastest with the rate of reaction decreasing for longer or shorter chains (Hastings et al., 1963). Luciferase provides an ideal system for the study of substrate–enzyme binding interactions since, by the simple expedient of excluding molecular oxygen, turnover to product is prohibited.

We have chosen to study the interaction of the 12-carbon aldehyde 1-dodecanal with the enzyme. Due to the large dispersion of ^{13}C chemical shifts for alkanals, compared with ^1H chemical shifts, ^{13}C NMR is the most propitious method of studying the interaction of long-chain compounds. Besides providing visualization of many of the carbons along the entire chain, ^{13}C is a nonperturbing probe in the system.

Experimental Section

Luciferase from *V. fischeri* was obtained from the Sigma Chemical Co. and further purified. 1-Dodecanal was purchased from MC & B and distilled under reduced pressure before use. BSA and FMN were obtained from Sigma Chemical Co., and dithionite (sodium hydrosulfite) and 2-mercaptoethanol were purchased from MC & B and used without further purification. Other reagent-grade chemicals were obtained from various standard sources.

Proton noise-decoupled ^{13}C NMR spectra were obtained at 25.1 MHz by using a Varian XL-100-15 nuclear magnetic resonance spectrometer fitted with a Nicolet 24K 1080 computer and 600K Diablo disk. T_1 measurements were performed by the progressive saturation method with homospoil (McDonald & Leigh, 1973) by using a pulse sequence $(\pi/2|_{0°} - \tau - \pi/2|_{0°} - AT)_n$; i.e., the free induction decay signal is acquired (AT) following the second of a pulse pair sequence separated by a delay time τ. The field spoiling pulse, 50 μs, was applied during the delay time, τ. Usually, 10 different τ values were measured, with the smallest τ = 50 μs. The experimental error in the relaxation measurements is ±10%. The errors in T_1 listed in Table II are the calculated error bound, i.e., a three-point parabolic fit to the standard deviation minimum. Chemical shifts are given in parts per million downfield from internal HMDS.

Enzyme assays and kinetics were performed on a digital photon counting system composed of a Fluke Model 412B high-voltage power supply, operating at −1220 V, an EMI 9789 QA photomultiplier tube (dark current at maximum gain ≤ 10 count/s), an ORTEC 9315 amplifier/discriminator, and an ORTEC 9302 digital photon counter.

The oxygen-initiated assay referred to as the dithionite assay was used to assay luciferase (Meighen & Hastings, 1971). To 0.8 mL of 0.025 M phosphate buffer (pH 7.0), 0.1 mL of 2% BSA, 0.35 μL of 2-mercaptoethanol, and 0.1 mL of 5×10^{-4} M FMN were added in a 4-mL sample vial. The enzyme preparation (5–25 μL) was then added, and the vial was flushed with nitrogen and sealed with a serum cap. Fifty microliters of 10 mg/mL dithionite was injected, and the reaction was initiated by the rapid injection of oxygen and 1-dodecanal by using 0.95 mL of a 0.01% (v/v) sonicated suspension of aldehyde. The initial light emission rate was determined by counting for a period of 2 s.

Results and Discussion

The "highest purity" commercial-grade enzyme contains at least 14 components as shown by $NaDodSO_4$ gel electrophoresis. The enzyme was further purified by the steps summarized in Table I. Since some nucleic acid is present, an initial protamine sulfate precipitation is performed. This is followed by ammonium sulfate precipitation and then a Sephadex G-150 column. The final specific activity of the purified enzyme is 19 times the original. $NaDodSO_4$ gel electrophoresis revealed the two luciferase bands at 42 000 and 37 000 and two weak bands at 40 000 and 65 000. Purified enzyme was subsequently used for all binding studies of aldehydes.

The natural-abundance ^{13}C NMR spectrum of 157 mM 1-dodecanal micelles in D_2O is shown in Figure 1. The use of the detergent Tween-20 is necessary to solubilize aldehyde to the concentrations required for this study. Of the large number of detergents tested, Tween-20 was shown to have no effect on the enzyme activity up to concentrations of 1% w/v.[2]

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[1] Abbreviations used: NMR, nuclear magnetic resonance; $FMNH_2$, reduced form of flavin mononucleotide; T_1, spin–lattice or longitudinal relaxation time; T_2, spin–spin or transverse relaxation time; FT, Fourier transform; ppm, parts per million; HMDS, hexamethyldisiloxane; BSA, bovine serum albumin; FMN, flavin mononucleotide; GLC, gas–liquid phase chromatography.

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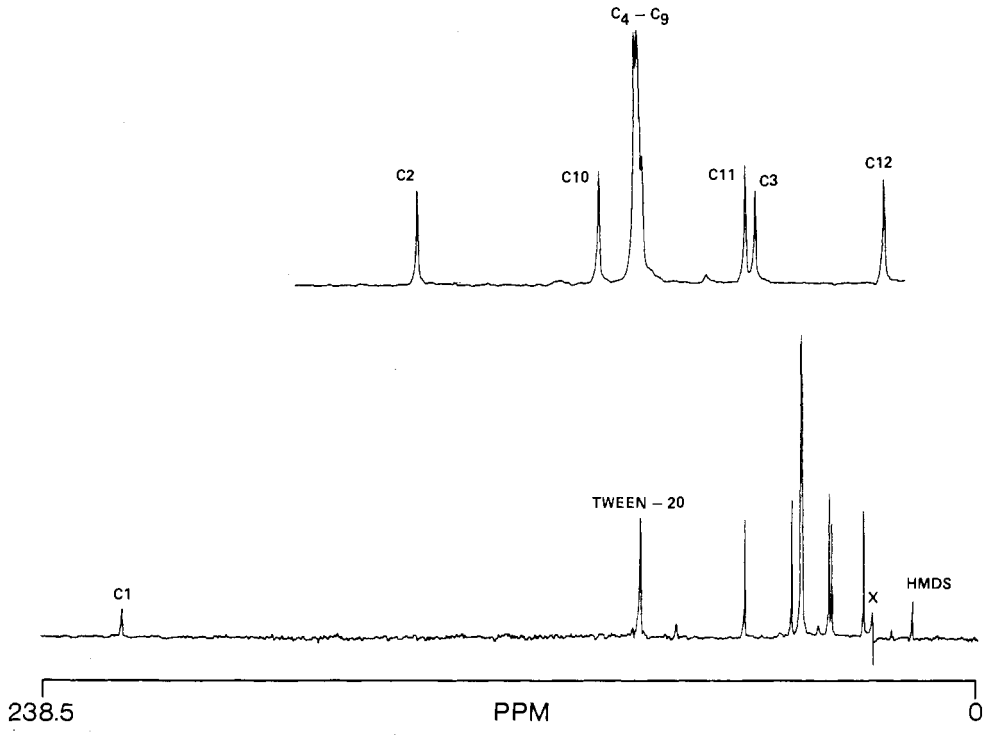

FIGURE 1: The natural-abundance, proton noise-decoupled ^{13}C NMR spectrum of 0.157 M 1-dodecanal in 0.1 M potassium phosphate (pH 6.9) and 0.5% w/v Tween-20 at 21 °C. The peak "X" is a computer-generated artifact. Spectral parameters: pulse width = 15 μs (60° flip angle); pulse delay = 4.32 s; number of accumulations = 5740; number of data points = 8192; line broadening = 2.0 Hz. Inset: expansion of the region between 11 and 50 ppm.

Table I: Purification Scheme

purification step	total A_{280}	A_{280}/A_{260}	sp[a] act. (s^{-1} A_{280}^{-1})	total act. (s^{-1} A_{280}^{-1})
V. fischeri luciferase, Sigma L-2379	1590	0.64	5.79×10^6	9.21×10^9
protamine sulfate precipitation	758	1.00	1.55×10^7	1.18×10^{10}
ammonium sulfate precipitation, 40–75% saturation	271	1.45	3.85×10^7	1.04×10^{10}
Sephadex G-150 gel filtration, fraction 24–32 pool	237	1.74	1.10×10^8	2.61×10^{10}

[a] The phototube was not standardized to yield specific activity in units of quanta seconds^{-1} absorbance$_{280}^{-1}$.

The alkyl region is shown in expanded form in the inset to Figure 1, and attention should be drawn to the assignment of C(3) and C(11) at 20.51 and 21.15 ppm, respectively, which has been reversed from the previous assignments of C(3) and the ω-1 carbon in alkanals (Stothers, 1972).

Relaxation Measurements. For a carbon relaxed completely by dipolar interaction with N directly bonded protons, the spin–spin relaxation rate is given by (Abragam, 1961a,b)

$$\frac{1}{T_2} = N\gamma_C^2\gamma_H^2\hbar r^{-6}S(S+1)\left[\frac{1}{6}J^{(0)}(0) + \frac{1}{24}J^{(0)}(\omega_C - \omega_H) + \frac{3}{4}J^{(1)}(\omega_C) + \frac{3}{2}J^{(1)}(\omega_H) + \frac{3}{8}J^{(2)}(\omega_C + \omega_H)\right] \quad (1)$$

where S = the spin quantum number of the proton, the γ's are the gyromagnetic ratios, and the ω's are the larmor frequencies of carbon and protons. Substituting for the spectral densities (Abragam, 1961a,b)

$$J^{(0)}(\omega) = \frac{24}{15}\frac{1}{1+\omega^2\tau_c^2} \quad (2)$$

$$J^{(1)}(\omega) = \frac{4}{15}\frac{1}{1+\omega^2\tau_c^2} \quad (3)$$

$$J^{(2)}(\omega) = \frac{16}{15}\frac{1}{1+\omega^2\tau_c^2} \quad (4)$$

one obtains for the relaxation rate

$$\frac{1}{T_2} = \frac{N}{20}\gamma_C^2\gamma_H^2\hbar^2 r^{-6}\left[4\tau_c + \frac{\tau_c}{1+(\omega_C-\omega_H)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_C^2\tau_c^2} + \frac{6\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_C+\omega_H)^2\tau_c^2}\right] \quad (5)$$

A similar expression can be obtained for the spin–lattice relaxation rate, $1/T_1$, and is

$$\frac{1}{T_1} = \frac{N}{10}\gamma^2{}_C\gamma^2{}_H\hbar^2 r^{-6}\left[\frac{\tau_c}{1+(\omega_C-\omega_H)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_C^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_C+\omega_H)^2\tau_c^2}\right] \quad (6)$$

Carbon-13 relaxation times, both T_1 and T_2* [T_2* = $(\pi\Delta\nu_{1/2})^{-1}$ where $\Delta\nu_{1/2}$ is the line width at half-height], for 75 mM 1-dodecanal in the presence of 0.094 μM *V. fischeri* luciferase are given in Table II. At these concentrations we have a very high substrate to enzyme ratio ([S]/[E] = 8×10^5). In spite of this the spin–lattice relaxation times indicate a substantial decrease in magnitude along the entire chain, even for the addition of a very small amount of enzyme, i.e., for a minimal increase in solution viscosity. The free aldehyde shows a variation of T_1 along the chain of ~5-fold [from 0.53

[2] The exchange of 1-dodecanal between micelles and enzyme appears to be extremely rapid. There is no detectable difference in rate, for instance, between 1-dodecanal in micelles and 1-dodecanal dissolved in buffer (low concentrations).

Table II: ^{13}C Relaxation Data for 1-Dodecanal and 1-Dodecanal-Luciferase (21 °C; pH 6.9)

	T_1 (s)		T_2* (s)	
	75 mM dodecanal (sonicated)	[S]/[E][a] = 8×10^5	75 mM dodecanal	[S]/[E] = 8×10^5
C(1) (CHO)	3.70 ± 0.40	0.47 ± 0.14	0.1	0.07
C(2)	1.68 ± 0.07		0.25	0.13
C(3)	0.93 ± 0.07	0.20 ± 0.06	0.25	0.14
$(CH_2)_n$	0.58 ± 0.03 0.53 ± 0.05 0.60 ± 0.06	0.30 ± 0.02	~0.20–0.22	~0.14
C(10)	1.08 ± 0.08	0.35 ± 0.05	0.25	0.2
C(11)	1.46 ± 0.11	0.45 ± 0.05	0.25	0.15
C(12) (CH_3)	2.67 ± 0.18	0.52 ± 0.08	0.22	0.22
Tween	0.23 ± 0.01	0.19 ± 0.03	~0.06	~0.06

[a] [S] = 66.7 mM; [E] = 0.083 μM.

s for $(CH_2)_n$ to 2.67 s for CH_3], and this decreases substantially, to 2.5-fold, upon addition of luciferase. The spin–spin relaxation times, columns 4 and 5, are determined from the line widths of the proton noise-decoupled ^{13}C NMR spectra, and the difference (column 5 − column 4) is a measure of the binding of 1-dodecanal to the enzyme. The constancy of both T_1 and T_2* from C(2) to C(11) indicates strong binding, probably through London dispersion forces, along the entire length of the alkyl chain. The very short value of T_2* for C(1) is indicative of a stronger anchoring of the aldehyde group in the active site. As more enzyme is added to the system, the first three carbons show a progressively greater decrease in T_2*, in the order C(1) > C(2) > C(3), than do the remaining alkyl carbons which remain about the same. The addition of enzyme did not increase the line width of the Tween-20 resonance, which remained constant at ~5 Hz. The T_1 of this resonance also changed very little, from 0.23 ± 0.01 s in enzyme-free solutions to 0.19 ± 0.03 s in the presence of 0.094 μM luciferase.

For studies involving low substrate/enzyme ratios we have prepared odd-carbon ^{13}C-labeled aldehyde. Feeding of sodium acetate-*1*-^{13}C (90% ^{13}C) to the CY2 mutant of *Escherichia coli* (Cronon & Batchelor, 1973) produced phospholipids containing fatty acids, alternately labeled in the alkyl chain, which, upon methanolysis, were purified by GLC (Cushley, Grover, and Treleaven, unpublished experiments). Enriched lauric acid was reduced to give odd-carbon labeled 1-dodecanal with 50% enrichment at each carbon site as shown by NMR and mass spectrometry.

The proton noise-decoupled ^{13}C NMR spectrum of the alkyl region (11–50 ppm) for odd-carbon ^{13}C-enriched 1-dodecanal in phosphate buffer is given in Figure 2a. Figure 2b and 2c shows the effects on the spectrum of increasing concentration of luciferase. Carbon 1 has disappeared completely by addition of 0.25 mL of 165 μM luciferase (Figure 2a) due to the strong binding discussed above. The selective increase in $1/T_2$* due to increasing enzyme concentration is apparent from Figure 2. For a concentration bringing [S]/[E] = 6.3×10^2, the resonance signal for C(3) has essentially disappeared (Figure 2c). In natural-abundance ^{13}C spectra, C(2) has broadened out at even higher [S]/[E] ratios (~1×10^4). It is the clear, progressive effect of luciferase on the relaxation rates of C(1), C(2), and C(3) which prompts us to reverse the previous assignments of the closely spaced resonance signals due to C(3) and C(11).

The spin–lattice relaxation time, T_1, was found to increase with temperature; i.e., the relaxation rate decreased with increasing temperature, hence the system is found to be in the fast exchange regime where $T_{1,bd} \gg \tau_{bd}$, where τ_{bd} is the lifetime of the bound species.

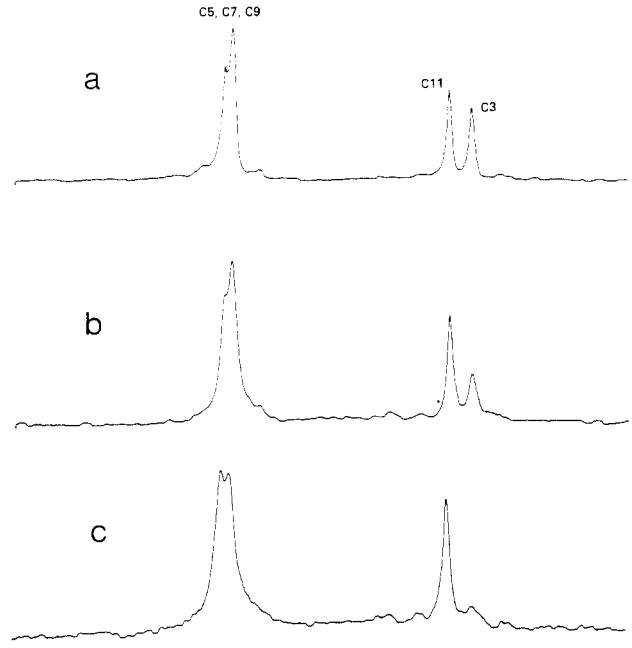

FIGURE 2: (a) Region, between 15.5 and 34.7 ppm, of the proton noise-decoupled ^{13}C NMR spectrum of 0.079 M odd-carbon ^{13}C-enriched 1-dodecanal in 0.1 M potassium phosphate (pH 6.9), 0.5% w/v Tween-20, and 0.05% v/v 2-mercaptoethanol, at 15 °C. Spectral parameters: pulse width = 15 μs; pulse delay = 1.32 s; number of accumulations = 500; number of data points = 8192; line broadening = 2.0 Hz. (b) Solution (a) plus luciferase, yielding [S]/[E] = 1.9×10^3; number of accumulations = 150. (c) More luciferase added, giving [S]/[E] = 6.3×10^2; number of accumulations = 500.

For fast exchange the expression for both $T_{1,bd}$ and $T_{2,bd}$ has the simple form (Dwek, 1973)

$$\frac{1}{T_{1,bd}} = \frac{1}{nP_{bd}}\left(\frac{1}{T_{1,obsd}} - \frac{1}{T_{1,f}}\right) \quad (7)$$

and

$$\frac{1}{T_{2,bd}} = \frac{1}{nP_{bd}}\left(\frac{1}{T_{2,obsd}} - \frac{1}{T_{2,f}}\right) \quad (8)$$

respectively, where n = the number of moles, P_{bd} = the mole fraction of bound species, and T_{obsd} and T_f are the measured relaxation times in the presence and absence of enzyme, respectively.

The spin–lattice and spin–spin relaxation rates for the bound form of odd-carbon ^{13}C-enriched 1-dodecanal at two different enzyme concentrations at 15 °C are presented in Table III. The values of $1/T_{1,bd}$ and $1/T_{2,bd}$* were determined by using eq 7 and 8. For a K_m value[3] as low as that for 1-dodecanal ($K_m \sim 1.6 \times 10^{-6}$ M; Hastings et al., 1973), the fraction of bound species, nP_{bd}, = [E]/[S] (Lanir & Navon, 1971).

From eq 5 and 6 a correlation time, τ_c, can be calculated for the motion. However, it is apparent from eq 6 that, due to the nature of the term in the square brackets passing through a minimum at $\omega_C\tau_c \sim 0.6$, for any other value of $1/T_1$ there are two values for τ_c. The common practice is, therefore, to determine the relaxation times at different frequencies to arrive at a unique value for τ_c.

Another method for determining the value of τ_c utilizing the values contained in Table III is to determine the ratio of

[3] The Michaelis constant is calculated from the solubility of 1-dodecanal by the relationship $K_m \simeq$ solubility (mol/L)/20 (Hastings et al., 1963).

Table III: Relaxation Rates for Enriched 1-Dodecanal Plus Luciferase (15 °C)

carbon no.	[S]/[E]^a = 1.9 × 10^3	[S]/[E]^b = 6.3 × 10^2
	$nP_{bd}/T_{1,bd}$	
C(1)		
C(3)	1.41 ± 0.10	
CH_2 (l)	2.71 ± 0.16	3.86 ± 0.20
CH_2 (h)	1.97 ± 0.16	3.48 ± 0.20
C(11)	0.96 ± 0.11	
	$nP_{bd}/T_{2,bd}$*	
C(1)	26	133
C(3)	10	27
CH_2	11^c	23^c
C(11)	3	8

^{a} [S] = 63.2 mM; [E] = 33.7 μM. ^{b} [S] = 45.14 mM; [E] = 101 μM. ^{c} Obtained from total width of high-field (h) and low-field (l) signals.

T_1/T_2. This method has a distinct advantage over the frequency variation method in that the number of interacting dipoles, and their separation in a highly substituted compound, does not enter into the calculation. By use of eq 5 and 6 a theoretical curve for the ratio of T_1 to T_2 vs. τ_c for ^{13}C nuclei at 25.1 MHz is given in Figure 3. From the figure the effective range of ^{13}C correlation times leading to useful ratios is $\sim 5 \times 10^{-10}$ to $\sim 1 \times 10^{-7}$ s. This is even greater than the comparable range of τ_c from a proton system, at the same field strength, which gives $\tau_c \sim 3 \times 10^{-10}$ to $\sim 2 \times 10^{-8}$ s. Thus, ^{13}C is a better nucleus than ^{1}H for studying the reorientational characteristics of macromolecules through ligand binding interactions. From the values in Table III five separate ratios of $T_{1,bd}/T_{2,bd}$ can be obtained, giving a high statistical weight to the calculated correlation time. The average value of $\tau_c = (1.8 \pm 0.7) \times 10^{-8}$ s is obtained, meaning, since τ_c is calculated from a comparison of carbon relaxation behavior along the entire chain length, that the motion of 1-dodecanal bound to luciferase can be described by a single correlation time which must be due to the rotational diffusion of the macromolecule.

The shape of intact luciferase from *V. fischeri* is not known, but, assuming a completely globular protein of 79 000 molecular weight with maximum solvation, a radius, $r \sim 40$ Å is found (Tanford, 1961). Since the buffer solution used in these studies (45 mM 1-dodecanal, 0.5% v/v Tween-20, 0.1 M potassium phosphate, and 0.05% v/v 2-mercaptoethanol) has a viscosity, at 15 °C, of $\eta = 1.427$ cp, one can calculate a rotational correlation time for a protein from the expression

$$\tau_c = \frac{4\pi\eta r^3}{3kT} \qquad (9)$$

which gives $\tau_c = 9.8 \times 10^{-8}$ s at 15 °C. This correlation time is related to the rotational correlation time of Debye, τ, for oriented rigid spheres of radius r with permanent dipole moments and in a medium of viscosity η by the relationship $\tau_c = \tau/3$ (Debye, 1929).

The slight discrepancy between the value of τ_c calculated from ^{13}C relaxation measurements and that predicted from eq 9 can be explained by (1) a hydration sphere much less than the maximum [McCalley et al. (1972) state that 30% of the radius of the macromolecule is usually attributed to the hydration sphere] and (2) an anisotropy in the motion of the ^{13}C spins either by (a) an extra, faster motion of the ^{13}C nuclei bound to the active site or by (b) a nonspherical shape for the enzyme.

The thickness of a hydration sphere about a macromolecule is most difficult to calculate since the water layer, and the

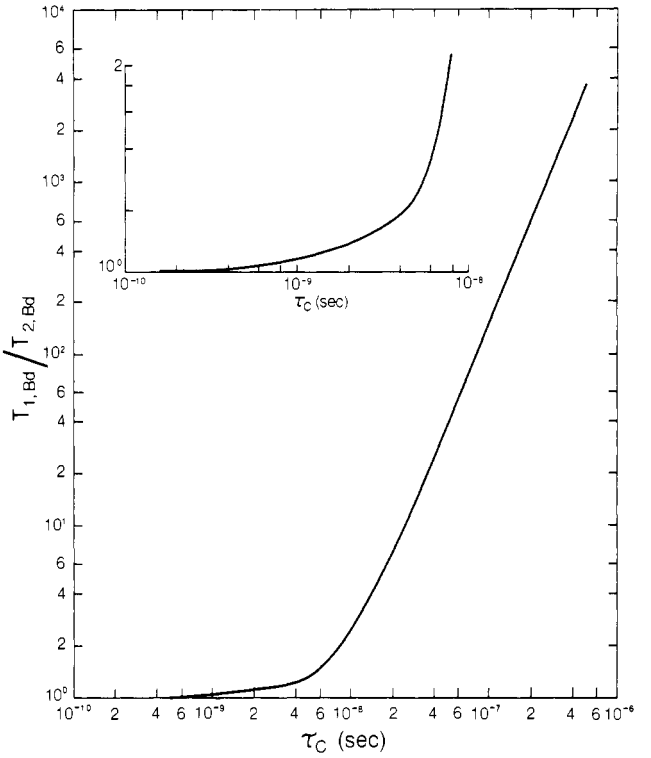

FIGURE 3: Theoretical plot of T_1/T_2 vs. correlation time, τ_c, for ^{13}C nuclei relaxed by dipolar interaction with protons at an absorption frequency of 25.16 MHz. Inset: expansion of the region for T_1/T_2 between 1 and 2.

mobile ion distribution of counterions around the protein, can be diffuse and/or anisotropic. An extra, fast motion of the ^{13}C spins is contraindicated from the relaxation data which indicate tight binding along the entire alkyl chain. Also, the K_m for 1-dodecanal yields a free energy of −7800 cal. The calculated hydrophobic free energy, $\Delta U°_c$, for a dodecyl compound in a *restricted* hydrophobic pocket, which is a good analogy for the present case, is $\Delta U°_c = -9500 \pm 500$ cal/mol (Tanford, 1973, 1974). In this treatment groups at the polar end, C(1) and C(2), are not treated as hydrophobic. The ~2000-cal increase in free energy found for luciferase is consistent with entropy changes associated with a more tightly bound 1-dodecanal molecule.

The most likely explanation for the discrepancy between measured and predicted correlation times for the macromolecule is a slight anisotropy in the shape of the protein. Until we have firm data on the axial ratios for luciferase, we prefer not to apply more sophisticated treatments of anisotropic rotation (Woessner, 1962) to measure the reorientational correlation times. Experiments to determine the molecular anisotropy of *V. fischeri* luciferase are in progress.

Finally, in Figure 4 we present the natural-abundance, proton noise-decoupled ^{13}C spectrum of 1-dodecanal in the region between 9 and 38 ppm. When sufficient luciferase was added to make the substrate to enzyme ratio low ([S]/[E] = 78), C(10), the antipenultimate or ω-2 carbon on the chain, appeared to broaden more in proportion to the other chain resonances (Figure 4a). Upon heat denaturation of the mixture (Figure 4b) C(10) and the other alkyl signals sharpened. This establishes the fact that the increase in relaxation rate was due to selective binding of 1-dodecanal in the enzyme active site. Upon further decrease to [S]/[E] = 42 (Figure 4c) C(10) was found to completely disappear. When the latter solution was heat-denatured, the peaks again sharpened (Figure 4d). Although the signal/noise ratio for

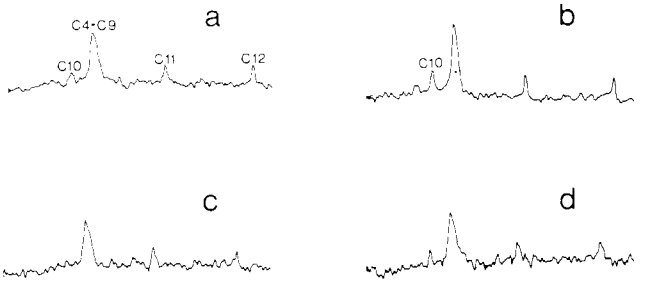

FIGURE 4: (a) Region, between 8.9 and 37.6 ppm, of the natural-abundance ^{13}C NMR spectrum of 0.0079 M 1-dodecanal in 0.1 M potassium phosphate (pH 6.9) and 1% w/v Tween-20 with luciferase added to bring [S]/[E] = 78. Spectral parameters: pulse width = 15 μs; pulse delay = 0.5 s; number of accumulations = 35000; number of data points = 8192; line broadening = 2.0 Hz. (b) Solution (a) after thermal denaturation; number of accumulations = 35000. (c) 1-Dodecanal/luciferase = 42; number of accumulations = 43000; (d) Solution (c) after thermal denaturation; number of accumulations = 60000.

these spectra is poor, the behavior of C(10) is interesting enough to point out a possible selective interaction at the ω-2 position, i.e., very remote from the active center of the molecule.

In order to confirm a selective interaction near the methyl terminal end of the substrate, we are currently elongating the odd-carbon ^{13}C-enriched-dodecanal chain by one carbon. This will yield a 13-carbon straight-chain aldehyde labeled at all of the even carbons. Together with further work planned we hope to completely delineate the interaction and rate of reorientation of long-chain aldehydes in the enzyme active site.

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Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor*. Electron Microscopy of Native and Reassembled Molecules†

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ABSTRACT: Examination and measurement of electron micrographs of negatively stained hemocyanin molecules from *Cherax destructor* show that the predominant aggregated forms, the 16S and 24S components, are typical structures for arthropod hexamers and dodecamers, respectively. In *Cherax* hemocyanin the hexamers are formed from the monomeric ($M_r \simeq 75000$) subunits, M_1 and M_2, while the dodecamers contain in addition a dimeric ($M_r \simeq 150000$) subunit, M_3'. Studies of the composition of solutions of the subunits M_1 and M_2 to which calcium ions have been added at pH 7.8 show that, under these conditions, reassembly occurs to particles indistinguishable from native hexamers. It is noteworthy that dodecamers are not seen since this confirms the previous suggestion that incorporation of the dimeric subunit in the assembly process is necessary for their formation. The results obtained from *Cherax* hemocyanin are related to those of previous structural studies of arthropod hemocyanins. In particular, the possible controlling role of certain specific subunits in arthropod hemocyanin oligomers containing more than one kind of subunit is illustrated with a model for the *Cherax* dodecamer, in which the dimeric subunit is shared between the two halves of the molecule.

The hemocyanin molecules observed in the hemolymphs of arthropod species belong to several different size classes. They are usually referred to as the 5S, 16S, 24S, 35S, and 60S components and are taken to consist of the equivalent of 1, 6, 12, 24, and 48 monomers, respectively, the monomer being of molecular weight about 75000 and containing two copper atoms. The distribution of the oligomers depends on the biological species. Thus, the higher molecular weight components tend to be emphasized in the hemolymphs of spiders and scorpions, both the 24S and 16S components in crabs and

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