by exposure to piperidine in aqueous methanol.[20] The ensuing compound, XV, in the form of the tritosylate salt is then coupled with the tosylate of N^{α}-Boc-Ser-Tyr-Ser-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (XVI) by the DCC/HOBt procedure[19] to afford protected [25-biocytin]-$ACTH_{1-25}$-amide (XVII) which is purified by chromatography on CMC. Finally, XVII is deprotected with 90% TFA, TFA ions are exchanged for acetate ions on acetate cycle IRA-400, and the product is exposed to aqueous thioglycolic acid[21] to remove trace quantities of the *S*-sulfoxides of Met and biotin. The biotin content of [25-biocytin]-$ACTH_{1-25}$-amide (XVIII) as determined by the dye assay[6] was 94% of theory based on the average amino acid recovery in acid hydrolysates of the peptide. [25-Biocytin]-$ACTH_{1-25}$-amide is as active as $ACTH_{1-24}$ in stimulating steroidogenesis in isolated bovine adrenal cortical cells and binds to avidin attached to Sepharose 4B.[1]

Acknowledgments. K.H. and F.M.F. Express their deep appreciation to Professor Dr. -Ing. Helmut Zahn for his hospitality, generous support, and encouragement. K.H. is indebted to the Alexander von Humboldt Stiftung, Bonn-Bad Godesberg, Federal German Republic, for a senior Scientist Award which enabled him to pursue a portion of this investigation at the Deutsches Wollforschungsinstitut in Aachen, Federal German Republic. We express our appreciation to Mrs. Michiko Kiso who performed the amino acid analyses and to William T. German for the optical rotations. This work was supported by a grant from the U.S. Public Health Service (AM-01128).

References and Notes

(1) K. Hofmann and Y. Kiso, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3516 (1976).
(2) K. Hofmann, F. M. Finn, H-J. Friesen, C. Diaconescu, and H. Zahn, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2697 (1977).
(3) The amino acids except glycine are of the L variety. The following abbreviations are used: Bct, biocytin; Boc, *tert*-butoxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DCHA, dicyclohexylamine; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; HOBt, 1-hydroxybenzotriazole; OBu^t, *tert*-butyl ester; OSU, *N*-hydroxysuccinimido ester; TEA, triethylamine; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.
(4) S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, and M. Weigele, *Science*, **178**, 871 (1972).
(5) Following exposure to hypochlorite the dried plates were sprayed with a 1:1 mixture of 0.4% KI and 1% starch in water.
(6) N. M. Green, *Methods Enzymol.*, **18A**, 414–424 (1970).
(7) D. S. Tarbell, Y. Yamamoto, and B.M. Pope, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 730 (1972).
(8) M. L. Jasiewicz, D. R. Schoenberg, and G. C. Mueller, *Exp. Cell. Res.*, **100**, 213 (1976).
(9) D. E. Brundish and R. Wade, *J. Chem. Soc., Perkin Trans. 1*, 2875 (1973).
(10) For a review, see J. Moss and M. D. Lane, *Adv. Enzymol.*, **35**, 321 (1971).
(11) For a review, see W. B. Jakoby and M. Wilchek, Ed., *Methods Enzymol.*, 34, 3 (1974).
(12) For a review, see K. Hofmann, *Handb. Physiol., Sect 7, Endocrinol.*, **4** (Part 2), 29 (1974).
(13) D. E. Wolf, J. Valiant, R. L. Peck, and K. Folkers, *J. Am. Chem. Soc.*, **74**, 2002 (1951).
(14) M. Bodanszky and D. T. Fagan, *J. Am. Chem. Soc.*, **99**, 235 (1977).
(15) K. Medzihradsky and H. Medzihradsky-Schweiger, *Acta Chim. Acad. Sci. Hung.*, **44**, 15 (1965).
(16) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).
(17) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
(18) E. E. Schallenberg and M. Calvin, *J. Am. Chem. Soc.*, **77**, 2779 (1955).
(19) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
(20) D. A. Ontjes and C. B. Anfinsen, *J. Biol. Chem.*, **244**, 6316 (1969).
(21) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).

Protein Rotational Correlation Times Determined in Aqueous Solution by Carbon-13 Rotating Frame Spin-Lattice Relaxation in the Presence of an Off-Resonance Radiofrequency Field

Thomas L. James,* Gerald B. Matson, and Irwin D. Kuntz

Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143. *Received October 11, 1977*

Abstract: An NMR off-resonance rotating frame relaxation technique is presented and applied to the study of rotational tumbling of protein molecules in solution. The experimental observable consists of the ^{13}C resonance peaks in the carbonyl region of the NMR spectrum for the protein. The ratio of the peak intensities in the presence and absence of the off-resonance rf field is related to both the Zeeman spin-lattice relaxation rate ($1/T_1$) and to a relaxation rate (designated $1/T_{1\rho}^{off}$) which is produced by the off-resonance irradiation. It is shown that the assumption of random isotropic rotation of the protein molecules allows this ratio of peak intensities to be interpreted in terms of a rotational correlation time. To illustrate the $T_{1\rho}^{off}$ technique, rotational correlation times for lysozyme, chymotrypsinogen A, concanavalin A, human methemoglobin A, bovine serum albumin, and immunoglobulin A were determined. The correlation time values obtained via the off-resonance $T_{1\rho}$ technique compare favorably in all cases with values determined by other experimental methods.

Several techniques have been applied to study rotational motions of proteins in aqueous solution including dielectric relaxation,[1] polarized light scattering,[2] fluorescence depolarization,[3a] electric birefringence,[3b] water proton NMR[4] spin-lattice relaxation dispersion,[5] and ^{13}C NMR relaxation.[6,7] We have recently developed an NMR technique which entails rotating frame spin-lattice relaxation in the presence of an off-resonance radiofrequency field;[8] this technique is useful for investigating rotational motions of macromolecules and appears to have potential for studying internal motions in macromolecules.

The work presented here utilizes our off-resonance $T_{1\rho}$ technique to study the rotational reorientation of a series of proteins in aqueous solution. The ^{13}C resonances of the envelope composed of carbonyls predominately on the protein backbone are observed. These carbons are expected to be relaxed via the dipole-dipole mechanism with nearby protons in a 23.5 kG magnetic field.[9] The backbone carbonyls were

0002-7863/78/1500-3590$01.00/0

chosen because they provide a strong resonance signal and their rotational motion should approximately mirror the overall tumbling motion of the protein.

There are a number of salient features which make the off-resonance $T_{1\rho}$ method particularly advantageous for this study. (i) The motional regime of applicability lies with motions having correlation times longer than the inverse Larmor frequency, thus encompassing the region of protein molecular tumbling. (ii) The time-consuming generation of relaxation data per se is avoided; instead, the rotational correlation time information is contained in a single ratio of signal intensities obtained in the presence and absence of the off-resonance irradiation. (iii) The technique is readily applicable to commercial Fourier transform NMR spectrometers, allowing utilization of the advantages of Fourier transform NMR spectroscopy. It may be noted that while the ability to circumvent relaxation measurements is advantageous in the study presented here, direct measurements of relaxation rates in the presence and absence of the off-resonance irradiation could be obtained through straightforward modification of the method presented.

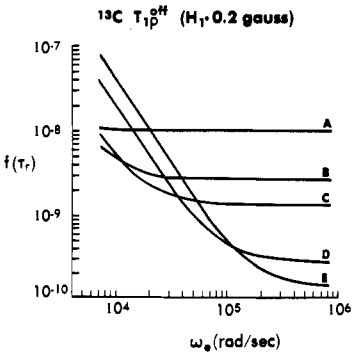

Figure 1. Theoretical dependence of the off-resonance rotating frame spin-lattice relaxation rate, $1/T_{1\rho}^{off} = \hbar^2\gamma_H^2\gamma_c^2 f(\tau_r)/10r^6$, for ^{13}C on the angular frequency of the effective field produced when an rf field is applied off-resonance according to eq 6 of the text. An applied rf field H_1 of 0.2 G and a stationary magnetic field of 23 487 G were assumed for the calculations. The individual curves were calculated for correlation time values of: (A) 1×10^{-8} s; (B) 5×10^{-8} sec; (C) 1×10^{-7} s; (D) 5×10^{-7} s; and (E) 1×10^{-6} s.

Theory

The theoretical basis for the investigation of molecular motions via rotating frame relaxation phenomena in the presence of an off-resonance rf field has been described in our previous paper[8] for nuclear dipole-dipole spin-lattice relaxation of like spins. The basis for ^{13}C spin-lattice relaxation due to dipole-dipole coupling to protons in the presence of an off-resonance rf field is presented in this section.

The effective field H_e in the rotating coordinate system is given by:

$$\vec{H}_e = \vec{\omega}_e/\gamma_c = [H_0 - (\omega/\gamma_c)]\vec{k} + H_1\vec{i} \quad (1)$$

for a ^{13}C spin system with gyromagnetic ratio γ_c in a stationary magnetic field H_0 and an rf field H_1 which is off-resonance by:

$$\Delta = 2\pi\nu_{off} = -\gamma_c[H_0 - (\omega/\gamma_c)] \quad (2)$$

The effective field H_e forms an angle θ with the stationary magnetic field H_0 such that:

$$\tan\theta = \gamma_c H_1/\Delta \quad (3)$$

The angular precession frequency around the effective field vector is then:

$$\omega_e = \Delta/\cos\theta \quad (4)$$

With the high-temperature approximation $kT \gg \gamma\hbar H_0$, and the high frequency approximation, $H_0 \gg H_e$, the rotating frame relaxation rate for ^{13}C spins in a far off-resonance rf field relaxed by dipolar coupling to protons may be obtained from the expressions of Blicharski[10] yielding:[11]

$$\frac{1}{T_{1\rho}^{off}} = \frac{1}{10}\frac{\hbar^2\gamma_H^2\gamma_c^2}{r^6}\left\{\sin^2\theta\left[\frac{2\tau_r}{1+\omega_e^2\tau_r^2} + \frac{3}{2}\frac{\tau_r}{1+(\omega_e+\omega_H)^2\tau_r^2} + \frac{3}{2}\frac{\tau_r}{1+(\omega_e-\omega_H)^2\tau_r^2}\right] + \cos^4\left(\frac{\theta}{2}\right)\left[\frac{\tau_r}{1+(\omega_c+\omega_e-\omega_H)^2\tau_r^2} + \frac{3\tau_r}{1+(\omega_c+\omega_e)^2\tau_r^2} + \frac{6\tau_r}{1+(\omega_c+\omega_H+\omega_e)^2\tau_r^2}\right]\right\} \quad (5)$$

$$\approx \frac{1}{10}\frac{\hbar^2\gamma_H^2\gamma_c^2}{r^6}\left\{\sin^2\theta\left[\frac{2\tau_r}{1+\omega_e^2\tau_r^2}\right] + \left[\frac{\tau_r}{1+(\omega_c-\omega_H)^2\tau_r^2} + \frac{3\tau_r}{1+\omega_c^2\tau_r^2} + \frac{6\tau_r}{1+(\omega_c+\omega_H)^2\tau_r^2}\right]\right\} \quad (6)$$

where $\hbar$ is Planck's constant divided by 2π, γ_H is the gyromagnetic ratio for a proton, r is the carbon-proton internuclear distance, τ_r is the rotational correlation time, and ω_c and ω_H are the Larmor precessional frequencies for ^{13}C and ^1H, respectively. Terms involving the fourth power of $\sin\theta$ have been deleted from eq 5 since the criterion of being far off-resonance is achieved for our purposes when:

$$|\Delta| \geq 5\gamma_c H_1 \quad (7)$$

In the absence of an off-resonance rf field, eq 6 reduces to the usual Zeeman spin-lattice relaxation rate given by:

$$\frac{1}{T_1} = \frac{1}{10}\frac{\hbar^2\gamma_H^2\gamma_c^2}{r^6}\left[\frac{\tau_r}{1+(\omega_H-\omega_c)^2\tau_r^2} + \frac{3\tau_r}{1+\omega_c^2\tau_r^2} + \frac{6\tau_r}{1+(\omega_H+\omega_c)^2\tau_r^2}\right] \quad (8)$$

in agreement with expressions obtained by Doddrell et al.[12] and Noggle and Schirmer[13] for ^{13}C spin-lattice relaxation in the stationary magnetic field.

The equations for off-resonance $T_{1\rho}^{off}$ relaxation and T_1 relaxation can be combined to yield:

$$\frac{1}{T_{1\rho}^{off}} = \frac{1}{10}\frac{\hbar^2\gamma_H^2\gamma_c^2}{r^6}\left[\sin^2\theta\left(\frac{2\tau_r}{1+\omega_e^2\tau_r^2}\right)\right] + \frac{1}{T_1} \quad (9)$$

$$= \frac{1}{T_{eff}} + \frac{1}{T_1} \quad (10)$$

The frequency dependence of $T_{1\rho}^{off}$ is shown in Figure 1. Plotted is the term in braces in eq 6, $f(\tau_r) = [\hbar^2\gamma_H^2\gamma_c^2 T_{1\rho}^{off}/10r^6]^{-1}$, as a function of the effective angular frequency ω_e and the rotational correlation time τ_r. The smallest value for ω_e was chosen to meet the far off-resonance condition, eq 7, for an assumed H_0 field of 23 487 G and an assumed H_1 field of 0.2 G.

One of the advantageous features of the off-resonance $T_{1\rho}$ technique is that instead of monitoring a relaxation process as a function of time, only a single ratio of resonance peak intensities is measured. The basis for this feature lies in the observation of Jacquinot and Goldman[14] that, for exponential relaxation, the steady-state value of the magnetization aligned with the effective field H_e in the presence of a far off-resonance rf field is:

$$M_{eff} = \frac{M_0}{1 + T_1/T_{eff}} \quad (11)$$

where M_0 is the thermal equilibrium value of the magnetiza-

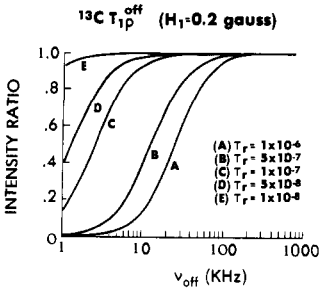

Figure 2. Dependence of the ratio of ^{13}C resonance peak intensities in the presence and in the absence of an rf field of strength H_1 = 0.2 G on the off-resonance frequency of the rf field according to the mathematical model comprising eq 2, 6, 10, and 11. It was assumed that the stationary magnetic field strength H_0 is 23 487 G. The curves were calculated for correlation time values of: (A) 1×10^{-8} s; (B) 5×10^{-8} s; (C) 1×10^{-7} s; (D) 5×10^{-7} s; and (E) 1×10^{-6} s.

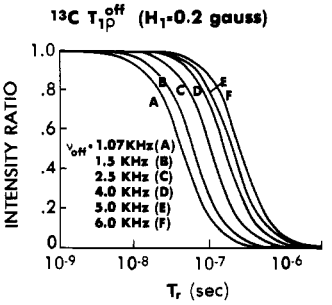

Figure 3. Theoretical dependence of the ratio of ^{13}C resonance peak intensities in the presence and absence of a 0.2-G rf field on the rotational correlation time according to the mathematical model described in the Theory section. A stationary magnetic field H_0 of 23 487 G was assumed for the calculations. The curves were generated for off-resonance frequencies ν_{off} of (A) 1.07 kHz, i.e., just achieving the far off-resonance condition of eq 7; (B) 1.5 kHz; (C) 2.5 kHz; (D) 4.0 kHz; (E) 5.0 kHz; and (F) 6.0 kHz.

tion aligned along the axis defined by the stationary magnetic field H_0.

Use of the mathematical model resulting from a combination of eq 2, 6, 10, and 11 leads to generation of the curves shown in Figure 2 where R (= M_{eff}/M_0) is plotted as a function of off-resonance frequency for several values of the rotational correlation time. The parameter R is easily measured as the ratio of the intensity of a resonance peak in the presence of an off-resonance rf field to the intensity in the absence of the rf field. The curves in Figure 2 were generated assuming an H_0 field of 23 487 G and an H_1 field of 0.2 G. The values of the off-resonance frequency ν_{off} were chosen to conform to the far off-resonance criterion.

For the purposes of this paper, it is perhaps useful to consider the value of the observable parameter R as a function of rotational correlation time τ_r. A depiction of this relationship is given in Figure 3 for a series of off-resonance frequencies. Again it is assumed that H_0 is 23 487 G and H_1 is 0.2 G.

Experimental Section

Materials. Hen egg-white lysozyme (Grade I, Sigma), bovine serum albumin (crystallized and lyophilized, Sigma), α-immunoglobulin (Miles), γ-chymotrypsinogen A (Type II from bovine pancreas, Sigma), and concanavalin A (Grade IV from jack beans, Sigma) were obtained commercially and used without further purification. Human hemoglobin A was prepared from whole blood obtained from normal individuals by venipuncture. The erythrocytes were washed with 0.9% sterile NaCl solution and hemolyzed by freeze-thawing and osmotic shock. The lysate was centrifuged at 12 000g to remove stroma, leaving purified hemoglobin solution. Methemoglobin A was prepared

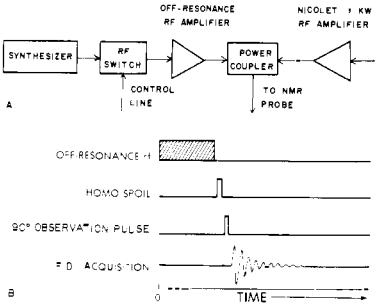

Figure 4. (A) Block diagram of the method used to gate the off-resonance irradiation to the transmitter coils of the probe. The off-resonance frequency is generated by the Fluke 6160B frequency synthesizer, and the duration of the off-resonance irradiation is controlled through the Watkins-Johnson S7 rf switch as discussed in the text. An rf amplifier capable of delivering up to several watts supplies the off-resonance power to the Electronic Navigation Industries PM40-2 power coupler (rated at 12 W internal load dissipation) which directs it to the transmitter coils of the probe. The power coupler also serves to protect the off-resonance rf amplifier from the observed rf pulses generated by the high-power Nicolet rf amplifier. (B) Timing sequence (not drawn to scale) for performing the $T_{1\rho}^{off}$ experiment on the Fourier transform NMR spectrometer.

by air oxidation, and the solution was concentrated by ultrafiltration with an Amicon P30 membrane.

NMR Experiments. The ^{13}C off-resonance experiments were performed at 25 MHz on a Varian XL-100 spectrometer equipped with a Varian cross-coil probe and a Nicolet TT-100 Fourier transform accessory. Generation of the off-resonance radiation was accomplished by a separate frequency synthesizer whose reference frequency was locked to the master oscillator of the XL-100. The duration of the off-resonance radiation was controlled by an rf switch, and the radiation was introduced to the transmitter coils of the probe via an amplifier and power coupler as depicted in Figure 4A. While the power coupler transmits only half of the applied power to the probe, its presence does provide adequate protection of the off-resonance amplifier from the high-power Nicolet amplifier.

Control of the rf switch to gate the off-resonance irradiation was accomplished by external circuitry which interrupted the computer's initiation signal for an experiment and gated on the off-resonance irradiation for the required duration, then reestablished the initiation signal to continue the experiment under control of the Nicolet 293 programmer. However, it should be noted that, with suitable software changes, the timing control for the off-resonance gate could have been generated through the 293 programmer.

The timing diagram for the experiments is given in Figure 4B, which depicts a period of off-resonance irradiation followed by a gradient pulse to destroy any transverse magnetization due to nonzero θ values, and then an on-resonance 90° observe pulse followed by accumulation of the resulting free induction decay (FID). The preamplifier was not gated off during the off-resonance period; careful paddle adjustment to maximize the orthogonality between the probe transmitter and receiver coils was found to be adequate to prevent receiver overload due to the off-resonance irradiation. Effects on the ratio measurements due to instrumental drift were minimized by accumulating the FID's in sets of four, with the off-resonance radiation present in alternate sets. The total number of accumulations ranged up to 3400, depending upon the sample. Consequently, a typical run to obtain a pair of spectra required 4 to 12 h.

Control runs demonstrated no observable impairment of the signal-to-noise ratio due to the presence of the rf irradiation and showed that, as expected, the off-resonance irradiation did not significantly diminish signal amplitudes for a small molecule whose correlation times for relaxation were expected to be in the extreme narrowing limit.

Gated proton decoupling during acquisition of the ^{13}C FID was utilized on all runs. A probe temperature of 25 ± 1 °C obtained for all experiments reported here.

Results and Discussion

The peak intensities for the carbonyl ^{13}C resonances of the proteins listed in the Experimental Section were monitored in

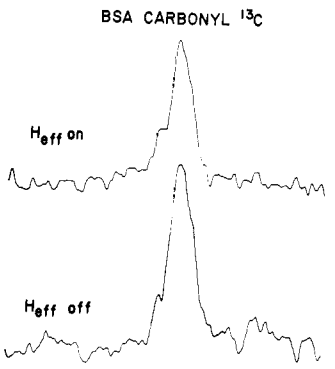

Figure 5. ^{13}C NMR (25.144 MHz) of the carbonyl groups in bovine serum albumin (25%) in the presence (top) and the absence (bottom) of an rf field H_1 of strength 0.20 G applied 4.0 kHz off-resonance; T = 25 °C.

Table I. Carbonyl ^{13}C Resonance Peak Intensity Ratio Obtained in the Presence and Absence of an Off-Resonance rf Field (0.20–0.22 G) and the Rotational Correlation Times for Several Proteins in Aqueous Solution at 25 °C

Protein	Mol wt	Concn, mM	ν_{off}, kHz	R^a	τ_r,[b] ns
LY	14 600	11.6	1.5	0.90	18
CHY	25 000	6.0	2.5	0.87	35
CON A	53 000	3.2	2.5	0.62	68
MetHbA	64 500	2.7	5.0	0.84	83
BSA	69 000	3.6	4.0	0.64	105
		2.9	4.0	0.67	95
		1.8	4.0	0.74	81
γG	150 000	1.0	6.0	0.32	338

[a] The estimated error is ±0.02. [b] The rotational correlation time was calculated using the mathematical model rendered by eq 2, 6, 10, and 11 with the tabulated values of ν_{off} and R, and with H_0 = 23 487 G and H_1 appropriate for the particular experiment. It was assumed that the carbonyl resonances experience a random isotropic reorientation; consequently, the values of τ_r should be considered as "effective" correlation times.

the presence and the absence of an off-resonance rf field. Figure 5 shows an example of the results illustrated with the carbonyl resonances of bovine serum albumin (BSA) in the presence and the absence of an rf field of strength H_1 = 0.2 G applied 4.0 kHz off-resonance. The peak intensity is diminished in the presence of the off-resonance rf field in accord with the discussion in the theory section when the $\sin^2\theta$ term in eq 6 is significant.

The off-resonance results for the proteins lysozyme, chymotrypsinogen A, concanavalin A, methemoglobin A, bovine serum albumin, and immunoglobulin are given in Table I. Values for the rotational correlation time, τ_r, were determined using eq 2, 6, 10, and 11 assuming isotropic rotational motion. This leads to an "effective" rotational correlation time since the shapes of most of the proteins studied are anisotropic to a varying degree.

It should also be pointed out that the calculated correlation times are possibly on the low side. This results from a small contribution to the signal from side-chain carbonyls which may have a smaller correlation time due to internal motion. Some error could also be introduced by the possibility of a small amount of paramagnetic ion binding to the proteins. The paramagnetic ions could contribute to T_1 relaxation but probably not to T_{eff} relaxation.

Although the full suite of eq 2, 6, 10, and 11 was employed for the correlation time calculations presented here, it may be shown that as long as $\tau_r \gg 1/\omega_c$, an estimate of the correlation time to within 10% can be obtained from:

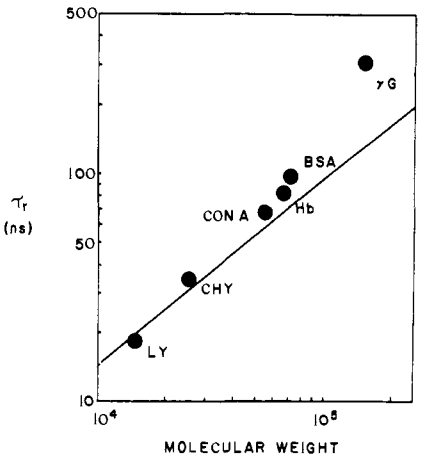

Figure 6. Rotational correlation times determined from the off-resonance $T_{1\rho}$ technique with the carbonyl ^{13}C resonance of six proteins at 25 °C as a function of protein molecular weight. Protein concentrations are from 15 to 25% (w/v). Abbreviations used in the figure are: LY, lysozyme; CHY, chymotrypsinogen A; CON A, concanavalin A; Hb, human adult methemoglobin; BSA, bovine serum albumin; and γG, immunoglobulin G. The line was calculated using eq 13 and a partial specific volume of 0.8 for proteins.

$$\tau_r = \sqrt{\frac{3}{2}\frac{(1-R)}{R}}\left(\frac{\nu_{off}}{\gamma_c H_1 \nu_c}\right) \tag{12}$$

where ν_c (= $\omega_c/2\pi$) is 25.144 MHz for the present set of experiments.

Although no quantitative discussion will be attempted, it is evident from the results in Table I for bovine serum albumin that the calculated values for τ_r decrease with decreasing protein concentration (hence, decreasing viscosity) as might be expected for concentrated protein solutions.

The calculated correlation times for protein rotational reorientation are also plotted in Figure 6 as a function of molecular weight. For purposes of comparison, a line was drawn in Figure 6 using the Debye–Stokes theory for calculating the rotational correlation time of a spherical molecule of radius a in a continuous fluid of viscosity η at temperature T:[15]

$$\tau_r = 4\pi a^3 \eta / 3kT \tag{13}$$

where k is Boltzmann's constant. The line in Figure 6 was calculated using the viscosity of pure water and a temperature of 25 °C. It is apparent that most calculated values of the correlation time are larger than would be predicted on the basis of the Debye-Stokes equation. A major reason for this is the anisotropic shape of most of the proteins studied. The greater the ellipticity of the molecule, the greater the "effective" correlation time,[16] i.e., the heavier it acts. Another contributing factor is that the viscosity is certainly greater than that of pure water which was used to calculate the line in the figure.

The rotational correlation time values determined for the proteins in this study by the off-resonance $T_{1\rho}$ technique are compared with values obtained by other experimental methods in Table II. Although some variability is to be expected for reorientation times determined from different techniques and under different solution conditions, the agreement with previous work is quite good.

It is evident from this investigation that the off-resonance $T_{1\rho}$ technique can be usefully employed to study macromolecule tumbling in solution. The simple treatment in the present study assumed isotropic rotations of the protein molecules. Nonspherical proteins undergoing anisotropic tumbling could be considered from the viewpoint of Woessner's treatment of random anisotropic rotations.[6,23] A quantitative measurement of the different correlation times associated with anisotropic

Table II. Comparison of Rotational Correlation Times for Some Proteins Determined from Carbonyl Off-Resonance $T_{1\rho}$ Experiments with Other Literature Values

Protein	τ_r,ns: $T_{1\rho}^{off}$ technique[a]	Other techniques
LY	18	19,[b] 25,[c] 50[d]
CHY	35	35[e]
CON A	68	93,[f] 110[d]
MetHb A	83	47,[g] 61,[h] (84, 130),[i] 102[d]
BSA	105	78,[b] 118,[j] 125[k]
γG	338	220,[j] 420[d]

[a] At 25 °C. [b] By NMR ^{13}C relaxation at 43 °C.[6] [c] By fluorescence depolarization.[17] [d] By water proton NMR dispersion at 25 °C.[5] [e] By electric birefringence.[3b] [f] By fluorescence depolarization.[18] [g] By ^{13}C NMR relaxation of carbon monoxide hemoglobin at 36 °C.[7] [h] By fluorescence depolarization.[19] [i] By dielectric relaxation.[20] [j] By dielectric relaxation.[21] [k] By fluorescence depolarization.[22]

rotational motions, however, would require that individual resonances from nuclei on different parts of the protein backbone be observed. Similarly, the investigation of internal motions would require observation of resonances from nuclei on other protein moieties as well as knowledge of the overall tumbling motion of the protein.

Acknowledgments. Financial support for this work was received from the Division of Research Resources, National Institutes of Health, for maintenance of the UCSF Magnetic Resonance Laboratory through Grant No. RR 00892-01A1, and from Research Grants PCM-18156 from the National Science Foundation and AM 19047 from the National Institutes of Health.

References and Notes

(1) P. Schlecht, H. Vogel, and A. Mayer, *Biopolymers*, **6**, 1717 (1968).
(2) S. B. Dubln, N. A. Clark, and J. Benedek, *J. Chem. Phys.*, **54**, 5158 (1971).
(3) (a) J. Yguerabide, H. F. Epstein, and L. Stryer, *J. Mol. Biol.*, **51**, 573 (1970); (b) S. Krause and C. T. O'Konski, *Biopolymers*, **1**, 503 (1963).
(4) Abbreviations used are: rf, radiofrequency; NMR, nuclear magnetic resonance; FID, free induction decay; LY, lysozyme; CHY, chymotrypsinogen A; CON A, concanavalin A; Hb, human adult hemoglobin; BSA, bovine serum albumin; γG, immunoglobulin G.
(5) K. Hallenga and S. H. Koenig, *Biochemistry*, **15**, 4255 (1976).
(6) D. J. Wilbur, R. S. Norton, A. D. Clouse, R. Addleman, and A. Allerhand, *J. Am. Chem. Soc.*, **98**, 8250 (1976).
(7) E. Oldfield, R. S. Norton, and A. Allerhand, *J. Biol. Chem.*, **250**, 6368 (1975).
(8) T. L. James, G. B. Matson, I. D. Kuntz, R. W. Fisher, and D. H. Buttlaire, *J. Magn. Reson.*, **28**, 417 (1977).
(9) R. S. Norton, A. O. Clouse, R. Addleman, and A. Allerhand, *J. Am. Chem. Soc.*, **99**, 79 (1977).
(10) J. A. Blicharski, *Acta Phys. Pol. A*, **41**, 223 (1971); *Z. Naturforsch. A*, **27**, 1355 (1972).
(11) G. B. Matson and T. L. James, manuscript in preparation.
(12) D. Doddrell, V. Glushko, and A. Allerhand, *J. Chem. Phys.*, **56**, 3683 (1972).
(13) J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect", Academic Press, New York, N.Y., 1971, pp 15 and 25.
(14) J. F. Jacquinot and M. Goldman, *Phys. Rev. B*, **8**, 1944 (1973).
(15) T. L. James, "Nuclear Magnetic Resonance in Biochemistry: Principles and Applications", Academic Press, New York, N.Y., p 39.
(16) C. Tanford, "Physical Chemistry of Macromolecules", Wiley, New York, N.Y., 1961.
(17) R. Irwin and J. E. Churchich, *J. Biol. Chem.*, **246**, 5329 (1971).
(18) D. C. H. Yang, W. E. Gall, and G. M. Edelman, *J. Biol. Chem.*, **249**, 7018 (1974).
(19) S. R. Anderson, M. Bruneri, and G. Weber, *Biochemistry*, **9**, 4723 (1970).
(20) J. L. Oncley, *Chem. Rev.*, **30**, 433 (1942).
(21) P. Ingram and H. G. Jerrard, *Nature (London)*, **198**, 57 (1962).
(22) P. G. Squire, R. Moser, and C. T. O'Konski, *Biochemistry*, **7**, 4261 (1968).
(23) D. E. Woessner, *J. Chem. Phys.*, **37**, 647 (1962).

Communications to the Editor

The Primary Water Splitting Light Reaction. Mass Spectrometric Determination of Gaseous Hydrogen and Oxygen Evolution from Water Photolysis by Platinized Chlorophyll a Dihydrate Polycrystals[1,2]

Sir:

In earlier work we attributed Chl a photogalvanic effects to water splitting reactions that result from illumination of the chlorophyll a dehydrate aggregate $(\text{Chl a}\cdot 2H_2O)_n$.[3] The photooxidation of $(\text{Chl a}\cdot 2H_2O)_{n\geq 2}$ by water was subsequently observed in ESR experiments.[4] These observations were corroborated by the finding[5] that the midpoint reduction potential of $(\text{Chl a}\cdot 2H_2O)_n$ is 0.92 V, exceeding that, 0.81 V, required for the water oxidation (O_2/H_2O) half-reaction at pH 7. The demonstration of water splitting by $(\text{Chl a}\cdot 2H_2O)_{n\geq 2}$ is of current interest. It has been suggested[3] that the water splitting reaction in vivo results directly from the primary light reaction of $(\text{Chl a}\cdot 2H_2O)_2$. The water splitting question is topical in view of the current search for a direct process for harvesting solar energy to produce gaseous hydrogen for fuel. Considerable attention has been focused on n-type semiconducting photoanodes such as TiO_2[6] and $SrTiO_3$.[7,8] However, these materials operate in the near-ultraviolet wavelength region where the solar radiant energy is low. In contrast, the action spectrum of the photoreactivity of $(\text{Chl a}\cdot 2H_2O)_n$ with water spans the visible and far-red wavelength regions.[3] In this paper we describe the mass spectrometric determinations of H_2 and O_2 evolution due to water photolysis by platinized $(\text{Chl a}\cdot 2H_2O)_n$.

The splitting of water by the chlorophyll in photogalvanic application[3] may be given in terms of the half-reactions:

$$\text{Chl a-Pt photocathode:}\quad 2H_2O + 2e \rightarrow H_2 + 2OH^- \quad (1)$$

$$\text{Chl a free anode:}\quad 2H_2O \rightarrow 4H^+ + O_2 + 4e^- \quad (2)$$

A Pt foil was platinized by passing a 30-mA current for 10 min through a 7×10^{-2} M chloroplatinic acid solution containing 6×10^{-4} M lead acetate. A layer of polycrystalline chlorophyll, containing 1.5×10^{17} Chl a molecules, was deposited on the platinized electrode surface in the usual manner.[3,9] The Chl a plated electrode was then platinized again in the same chloroplatinic acid solution by passing the 30-mA current for 15 s. The resulting sample electrode was baked at 60 °C under atmospheric pressure for several hours in order to be rid of any adventitious gaseous occlusion during the platinization procedure.

The action spectrum of the photogalvanic response of the platinized Chl a electrode at pH 7, measured in a cell[3] employing as the second half-cell a platinized electrode not

0002-7863/78/1500-3594$01.00/0