Electron Paramagnetic Resonance of Spin-Labeled Aequorin†

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ABSTRACT: Aequorin is a Ca-activated bioluminescent protein from jellyfish. This protein contains two sulfhydryl groups, one of which is essential for its bioluminescence. Little information concerning the structure of and relationship between the metal binding sites of aequorin and the sulfhydryl group(s) is known. Aequorin was modified by attachment of either a maleimide spin-label [studied by electron paramagnetic resonance (EPR)] or the fluorescent label Acrylodan at the essential sulfhydryl in order to gain such information. These modifications caused destabilization of the chromophore of aequorin. Both of the attached labels showed considerable freedom of motion. The spin-label was quite accessible to the solvent, and the fluorescent label was less so. In addition the metal binding properties of the spin-labeled aequorin were studied by Mn(II) EPR. One tight Mn(II) binding site per spin-labeled aequorin was found. The distance between the Mn(II) binding site and the spin-label is at least 20 Å. Furthermore, the relative affinity of spin-labeled aequorin for various metal ions was found to be in the order Pr(III) > Mn(II) > Ca(II) > Mg(II).

Aequorin is a M_r 20 000, bioluminescent protein isolated from the jellyfish *Aequorea forskålea* [Shimomura et al., 1962, 1963; for a review see Blinks et al. (1976)]. The light emission from aequorin occurs at 469 nm and is induced by the binding of Ca(II) or other metal ions (Shimomura et al., 1963; Shimomura & Johnson, 1972; Izutsu et al., 1972, 1974). Luminescence is inhibited by the binding of Mg(II) and has no requirement for exogenous oxygen or other cofactors. Emission of light occurs as a consequence of oxidation of a tightly bound chromophore, an imidazolopyrazinone (Shimomura & Johnson, 1972; Cormier et al., 1973a,b); the actual emitter is the excited state of a 2,3,6-substituted pyrazine (Cormier et al., 1973a,b), and the light emitted is blue with a λ_{max} of 469 nm. Subsequent to bioluminescence, the protein exhibits a bright blue fluorescence which has the same spectral characteristics as the bioluminescence (Shimomura & Johnson, 1969). An individual protein molecule can emit light only once; aequorin does not therefore "turn over" as an enzyme does, although the protein does catalyze the reactions leading to bioluminescence. The binding of Ca(II) appears to trigger the catalytic process. In the absence of Ca(II), light *is* emitted (so-called calcium-independent light emission) but at a very low rate (Allen et al., 1977). The identity of the "active site" and of the functional amino acid side chains has not yet been established, but it is known that aequorin contains at least one sulfhydryl group which is essential for its Ca-activated luminescence; modification of the SH group with any of several chemical agents causes a complete loss of Ca-activated bioluminescence (Shimomura et al., 1974; Shimomura & Johnson, 1978; Prendergast & Mann, 1978).

Very few structural details of aequorin are known. Determination of its amino acid sequence is under way. Efforts at obtaining the crystal structure from X-ray crystallography have not yet been successful. However, magnetic resonance techniques (EPR and NMR)[1] offer an opportunity to gain useful structural information. In this work EPR methods were used to examine the structure of, and metal ion binding to, aequorin with special reference to the stoichiometry of cation binding. To achieve this, aequorin was chemically labeled at a thiol residue with the spin-label 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy. The EPR spectrum of the spin-label allows monitoring of the accessibility of the thiol group to the solvent and of the effects produced on these signals by metal ion binding to the protein. EPR techniques are especially useful for following the interaction of paramagnetic ions such as Mn(II) with the protein; furthermore, a measurement of the perturbation of the spin-label signal by the bound metal ion is capable of providing information on the distance between the metal ion and the spin-label. In the studies reported here, Mn(II) was used as the paramagnetic ion. Some of the experiments related to metal ion binding were performed by monitoring the EPR spectrum of Mn(II) free in solution and using the signal intensity as a measure of its concentration in the sample (Cohn & Townsend, 1954). Fluorescence measurements on aequorin labeled with a sulfhydryl-specific fluorophore were used to corroborate the EPR data.

For ease of description we refer to protein that bioluminesces in response to Ca(II) as native aequorin, protein which has bioluminesced as discharged protein, and discharged protein without the chromophore as apoprotein. The meaning of the term "spin-labeled" protein is self-evident.

Materials and Methods

Aequorin was isolated and purified from the jellyfish *Aequorea forskålea* by methods described elsewhere (Blinks et al., 1978). In view of the extreme sensitivity of aequorin to even minute contamination of activating metal ions such as Ca(II), the solvent H_2O and buffered solutions used in the experiments were passed through a Chelex-100 column for the removal of such contamination. For the EPR experiments the

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene; 2-ME, 2-mercaptoethanol.

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protein was dissolved in pH 7.0 aqueous solution containing 20 mM MOPS buffer and 150 mM KCl. Protein concentrations were established by using $\epsilon_{280}^{0.1\%} = 1.95\ cm^{-1}$ for the native and discharged protein and $\epsilon_{280}^{0.1\%} = 1.0\ cm^{-1}$ for the spin-labeled (apo-) protein (see below). The spin-label 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy, obtained from Syva (Palo Alto, CA), was used as a solution in DMF. All other chemicals were of analytical reagent grade.

The labeling of the protein was normally carried out at room temperature with a 4–10-fold excess of spin-label. A typical example is a pH 7.0 solution that had 2 mM maleimide spin-label, 200 μM aequorin, 20 mM MOPS, 150 mM KCl, 1 mM EDTA, and 1 mM KCN (the last mentioned being a scavenger for Ag ion contamination from pH electrodes). The maximum percentage of DMF (v/v) was about 13; this amount of DMF has no significant effect on aequorin activity (Prendergast, 1977). Since chemical modification of a thiol residue in aequorin results in loss of Ca-sensitive bioluminescence which is irreversible if the reagent cannot be removed from the thiol (Prendergast et al., 1977), the reaction of the maleimide was easily followed by assay of Ca-triggered bioluminescence. The rate of inactivation of aequorin by NEM (at the same concentration as the spin-label maleimide) was also determined by measurement of the loss of aequorin bioluminescence and was found to parallel that due to the spin-label. Labeling of the protein was monitored by removing aliquots of the reaction mixture, separating the excess spin-label from the protein by elution through a Sephadex G-25 column, and observing the EPR signal of the labeled protein. Once the reaction was complete, the excess spin-label and the EDTA were removed by extensive dialysis at 4 °C against four changes of a 100-fold excess of 20 mM MOPS–150 mM KCl solution for a total time of 48 h. By comparing the EPR signal of free label with that from the labeled protein, we could readily ascertain that no free label was present in the final solutions. There was no indication of a free label component to the EPR signals from those solutions. Experiments with iodo[^{14}C]acetamide which also reacts with the thiol verified that the spin-label interacts with the thiol residue. In addition, titrations with DTNB indicated that the spin-labeled protein had no readily available sulfhydryl group.

Metal ion solutions were prepared in 20 mM MOPS–KCl buffer by using the respective chlorides. In the case of Mn(II), the concentrations were checked by EDTA titration exploiting the fact that EDTA-bound Mn gives no definable EPR signal under the conditions of our experiments. Ultrapure-grade $MgCl_2$ and $MnCl_2$ were obtained from Puratronic (Alfa), and Ultrapure $PrCl_3$ was from Aldrich. $CaCl_2$ used was of analytical reagent grade obtained from Fisher.

EPR Measurements. The EPR signals were mostly observed with a home-built X-band EPR spectrometer comparable to a Varian V4502. A large sample access cylindrical cavity (Varian V4535) fitted with a fused silica variable temperature Dewar was used. Phase-sensitive detection at 50 kHz was employed with the result that signals characteristic of the derivative of the absorption were detected. The EPR samples of volume about 40 μL were contained in 0.9 mm × 1.21 mm × 110 mm long flint glass tubes obtained from Drummond Scientific Co. (Broomall, PA). Typical EPR parameters for observation of the spin-label signals were the following: microwave frequency 9.1 GHz; microwave power 33 mW; central magnetic field 3240 G; magnetic field modulation 0.45 G; field sweep 50 G in 5 min with a time constant of 0.2 s. For the observation of Mn(II) signals the EPR parameters which were set different from those given above were the following: microwave power 103 mW; field modulation 13.5 G; field sweep 1000 G in 5 min with a time constant of 1 s. Under these conditions it was possible to detect a minimum of 2 μM free Mn(II). A few Mn(II) EPR experiments were also done with a Varian E109 spectrometer (at Methodist Hospital, Indianapolis) in order to detect lower Mn(II) concentrations (~0.2 μM). For those experiments Wilmad fused silica tubes of dimension 1.0 mm × 1.2 mm × 100 mm containing 50 μL of sample were used. The Mn(II) concentrations were measured from the amplitudes of the EPR signals. The uncertainty is 10% for concentrations above 10 μM and is higher for lower concentrations, becoming nearly 50% for the lowest concentrations. Unless otherwise noted, the sample temperature for the EPR experiments was 20 ± 0.5 °C.

Fluorescence Measurements. Aequorin was labeled with Acrylodan [2-(dimethylamino)-6-acryloylnaphthalene, a reagent which reacts preferentially at thiol groups when labeling is carried out at pH 7.0] by the methods described by Prendergast et al. (1983). Briefly the procedure was as follows: the protein was incubated at 4 °C with a 4-fold molar excess of Acrylodan (added from a stock solution prepared in DMF) until no Ca-activated bioluminescence remained. The reaction was carried out in 125 mM KCl, 25 mM MOPS, and 1 mM EDTA at pH 7.0. When all Ca-activated bioluminescence had been lost, the protein was dialyzed extensively at 4 °C against the same buffered solution as that used for labeling.

Fluorescence emission spectra were measured in a thermostated SLM 4800 spectrofluorometer. All emission spectra were corrected for instrumental artifacts by use of correction factors generated on the SLM 4800 with an NBS standard lamp.

Fluorescence lifetimes τ were measured by the phase-modulation method of Spencer & Weber (1969). In general, modulation frequencies of 18 and 30 MHz were employed. For these measurements unpolarized excitation was used (λ_{ex} = 375–390 nm), but a polarizer was placed in the emission path and oriented to 33.5° ("magic angle") to eliminate the effects of Brownian rotation on τ. Emission wavelengths were selected by use either of appropriate interference filters or of sharp "cut-on" filters. Solutions of *p*-terphenyl and POPOP in ethanol (τ = 0.93 and 1.35 ns, respectively) were used as reference solutions for all lifetime measurements (Lakowicz et al., 1981).

Steady-state fluorescence anisotropy (r_{ss}) was measured as described by Weber & Bablouzian (1966) in the SLM 4800. For measurements of r_{ss} of Acrylodan fluorescence, a Schott KV 450 nm "cutoff" filter was employed in the emission path; for these measurements an excitation wavelength of 390 nm was used on the basis of the excitation polarization spectrum of Acrylodan which shows a plateau value of r_{ss} over the range 360–410 nm. For oxygen quenching, experiments were done with a high-pressure cell designed and constructed at Mayo and fashioned after the cell described by Lakowicz & Weber (1973). Lifetime-resolved anisotropy measurements were made with the SLM unit oriented in the "L" format. The excitation light was vertically polarized. Either an interference filter (10 nm band-pass) or a Schott KV 450 cutoff filter was employed in the emission path to select the desired emission wavelength region. With this optical arrangement the simple relation

$$r_{ss} = (I_V - I_H)/(I_V + 2I_H)$$

(where I_V and I_H are the vertically and horizontally polarized components of the emission) could be used to calculate the anisotropy r_{ss} without need for correction. For calculation of

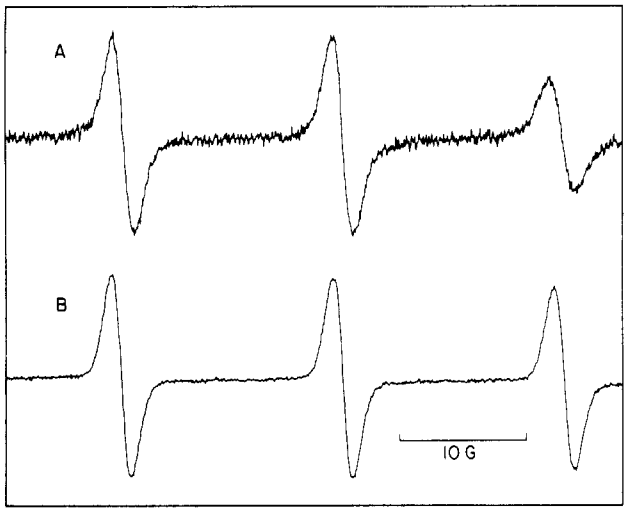

FIGURE 1: X-band EPR signals from maleimide spin-label attached to aequorin (A) and free in buffered solution (B) at pH 7.0 and 20 °C. Sample conditions and EPR parameters are given in the text. The concentrations of spin-label were 43 and 100 μM for (A) and (B), respectively.

motional parameters from lifetime-resolved anisotropies, the following formula was used:

$$\frac{1}{r_{ss}} = \frac{v(v+\kappa)}{r_0 v + r_\infty \kappa} = \frac{(1+\gamma_p\tau)(1+\gamma_p\tau+\gamma\tau)}{r_0(1+\gamma_p\tau)+r_\infty\gamma\tau} \quad (1)$$

where $\kappa = \gamma\tau$, $v = 1 + \gamma_p\tau$, $\gamma = \phi_i^{-1}$, $\gamma_p = \phi_p^{-1}$, ϕ_i is the internal rotational correlation time, ϕ_p is the protein rotational correlation time, and r_0 and r_∞ are appropriate limiting values of the anisotropy corresponding to real-time δ-function responses $r(t) = [(r_0 - r_\infty)e^{-t/\phi_i} + r_\infty]e^{-t/\phi_p}$ and $I(t) \propto e^{-t/\tau}$. To analyze the data, the reciprocal of the anisotropy is plotted vs. the fluorescence lifetime (Perrin plot). This formula (L. W. Engel and F. G. Prendergast, unpublished results) is akin to that employed by Lakowicz et al. (1983) to calculate the rotational correlation time of proteins from measurements of lifetime-resolved anisotropy. In our analysis, however, no assumptions were made regarding the relative values of ϕ_p and ϕ_i when we interpreted the $1/r_{ss}$ vs. τ plot. Rather the data were mathematically fit and the values of ϕ_p and ϕ_i estimated from the fit parameters. In principle, the intercept of the $1/r_{ss}$ vs. τ plot on the y axis should yield $1/r_0$ where r_0 is the limiting anisotropy. However, if there are picosecond depolarizing rotations, there will be a wide disparity between the real limiting anisotropy (r_0) and the apparent "zero time" anisotropy [denoted r_0' by Ichiye & Karplus (1983)] obtained by extrapolation. The difference ($\Delta r_0 = r_0 - r_0'$) provides a good indicator of fluorophore motion independent of motion of the whole protein. We may term Δr_0 the limiting anisotropy defect. As Δr_0 increases, so also does the contribution of local depolarizing motions to the measured anisotropy.

Results

Figure 1 shows a comparison of the EPR signals obtained for the maleimide nitroxyl spin-label attached to the protein and for the spin-label free in solution. The three-line pattern is due to the hyperfine interaction between the nitroxyl electron magnetic moment and the magnetic moment of the ^{14}N nucleus in the nitroxyl group. The signal from the attached label (Figure 1A) shows a characteristic broadening of the high-field line along with a concomitant loss in amplitude as compared with the other two hyperfine lines. This effect is due to the influence of the protein on the rotational motion of the attached spin-label and can be used to calculate a rotational correlation time. The rotational correlation time was calculated from two different expressions

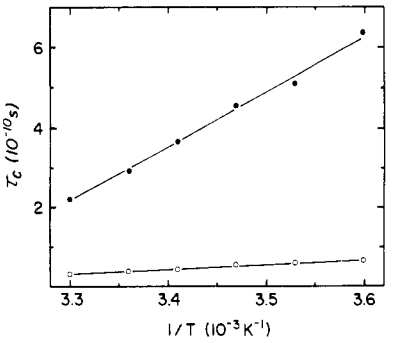

FIGURE 2: Temperature dependence of the rotational correlation time τ_c (see text) for maleimide spin-label attached to aequorin (●) and free in buffered solution (○) at pH 7.0 as deduced from EPR spectra. The concentration of the spin-label was 100 μM in both cases.

$$\tau_c = (6.1 \times 10^{-10}\ \text{s})W_{+1}[(h_{+1}/h_{-1})^{1/2} - 1] \quad (2)$$

and

$$\tau_c = (5.1 \times 10^{-10}\ \text{s})W_{+1}[1 + (h_{+1}/h_{-1})^{1/2} - 2(h_{+1}/h_0)^{1/2}] \quad (3)$$

where W_{+1} is the width in gauss of the low-field line (corresponds to a nuclear spin component $I_z = +1$ for ^{14}N) and h_{+1}, h_0, and h_{-1}, respectively, represent peak-to-peak amplitudes of the low-field, middle-field, and high-field lines (Stone et al., 1965; Haak et al., 1976). Magnetic resonance parameters for the maleimide spin-label given by Thomas et al. (1976) were used to obtain the numerical coefficients in eq 2 and 3. These expressions are generally valid provided $\tau_c \leq 3 \times 10^{-9}$ s and depend on motional averaging of anisotropies in both the Zeeman interaction (g values) and the hyperfine interaction (A values) in the case of eq 2 and hyperfine interaction alone in the case of eq 3.

From the data in Figure 1 and other similar spectra obtained at 20 °C, we find $\tau_c = 3.8 \times 10^{-10}$ s for the label attached to aequorin and $\tau_c = 4.6 \times 10^{-11}$ s for label free in the buffered solution from eq 2 while from eq 3 we find $\tau_c = 3.4 \times 10^{-10}$ s for spin-labeled aequorin and $\tau_c = 4.1 \times 10^{-11}$ s for free spin-label. The uncertainty in the τ_c values is $\simeq$10%, indicating that eq 2 and 3 yield nearly the same values for τ_c. Significant differences between the τ_c values calculated from these equations could arise from anisotropic motion (Marsh, 1981). However, the near equality in τ_c values does not necessarily imply that the label motion is isotropic. In addition the temperature dependence of τ_c (eq 2) was determined for the free and attached label and is shown in Figure 2. The distinct difference in the slopes of these lines is a clear indication that the label is attached to the protein.

The hyperfine interaction constant characterizing the triplet of the spin-label EPR spectrum was found to be 17.0 ± 0.1 G for the label attached to the protein (Figure 1A) and for the label free in the same buffered solution (Figure 1B) and to be 15.8 ± 0.1 G for the label in DMF. This result indicates that the environment of the label on the protein is accessible to the solvent. Further confirmation of this conjecture comes from the fact that the spin-label on the protein was readily reduced when ascorbate was added to a solution of spin-labeled aequorin; i.e., the label EPR signal disappeared.

The amplitude of the EPR signal of the spin-labeled aequorin was a reliable measure of the concentration of the labeled protein. The concentrations were determined by comparison of signals of the labeled protein with standards

of known concentration of free maleimide spin-label under identical sample conditions after appropriate allowance for line-width differences. These measurements are used to establish the stoichiometry of sulfhydryl labeling of aequorin by the maleimide spin-label. The EPR signals from the labeled protein gave no indication of a protein molecule containing more than a single spin-label. Only one type of signal was observed, and that signal showed no unusual line broadening or splitting which could occur from paramagnetic interactions between two labels attached to the same protein. For a given preparation of spin-labeled aequorin, the initial protein concentration was measured on the basis of optical absorbance at 280 nm. After labeling and dialysis the concentration of the bound spin-label as determined by EPR was the same as the initial aequorin concentration except for a slight (<5%) loss of protein. On the other hand, when the protein concentration in this sample (after labeling) was determined by using the optical absorbance of native aequorin at 280 nm, a value approximately 50% of the initial protein concentration (before labeling) was obtained. There was no evidence of protein loss of the order of 50% due either to precipitation or to dialysis. (In any dialysis experiment with aequorin, the protein loss has never been over 5%.) Futhermore, bioluminescence assay showed no measurable activity of the labeled aequorin, indicating that all of the protein was modified and thus was inactivated by the label. The reduction by about 50% in the optical absorbance of the spin-labeled protein after dialysis compared with the absorbance prior to labeling can be accounted for if the chromophore dissociated during the labeling process and was subsequently removed during dialysis since a substantial part ($\sim$50%) of the optical absorbance of aequorin at 280 nm is contributed by the chromophore. Evidence of chromophore dissociation upon sulfhydryl modification was also found in the labeling of aequorin with Acrylodan. In particular aequorin was labeled with Acrylodan and the protein subjected to gel filtration chromatography on Sephadex G-25 with 125 mM KCl, 25 mM MOPS, and 1 mM EDTA, pH 7.0, as eluent. Unmodified aequorin is unaffected by such treatment. However chromatography of Acrylodan-labeled aequorin resulted in appearance of a distinct blue fluorescent band adsorbed to the gel matrix. This fluorescent band eluted readily with a 50% ethanol/water mixture, and the fluorescence properties of the eluted material were apparently those of a mixture of etioluciferin and oxyluciferin (Blinks et al., 1976). This demonstrates that thiol modification causes structural alteration in the protein which results in release of the chromophore. Presumably, the released chromophore free in solution undergoes spontaneous oxidation to form fluorescent derivatives which adsorb to Sephadex G-25. The loss of the chromophore upon sulfhydryl labeling and dialysis is also confirmed by proton NMR measurements (B. D. Ray et al., unpublished results). We thus conclude that the protein studied was in fact spin-labeled apoprotein with one spin-label per aequorin molecule.

The stoichiometry of Acrylodan labeling is difficult to determine for three reasons. First, the extinction coefficient of the ligand varies rather markedly with solvent (Prendergast et al., 1983). Second, the ligand absorbs light significantly at 280 nm where the protein also absorbs. Third, thiol adducts have a different extinction from the parent compound. Nevertheless, the following method was used to *estimate* the stoichiometry. The molar extinction coefficient ϵ_M of the 2-mercaptoethanol adduct (2-ME–Acrylodan) in ethanol is 14 000 cm^{-1} at 365 nm. The fluorescence of the aequorin adduct of Acrylodan is reasonably similar to that of the 2-mercaptoethanol adduct in ethanol, and it may therefore be reasoned that the molar extinction coefficient of the Acrylodan bound to the protein would be 14 000 cm^{-1}. By measurement of the ratio of the absorbance at 280 nm to that at 365 nm for the 2-ME–Acrylodan in ethanol and by use of the extinction coefficients of aequorin and of the Acrylodan moiety bound to the protein, the concentrations of protein and ligand were determined. Under the conditions of the experiments described in this paper, the labeling ratio (ligand:protein) was 1.1; i.e., one ligand is bound per molecule of protein. Even with the intrinsic uncertainty regarding the extinction coefficient of the bound fluorophore, it is improbable that the stoichiometry is greater than one since the molar extinction is minimal in water (ϵ_M = 12 900 cm^{-1}), and at this value the calculated stoichiometry would be 1.19, i.e., still effectively 1.

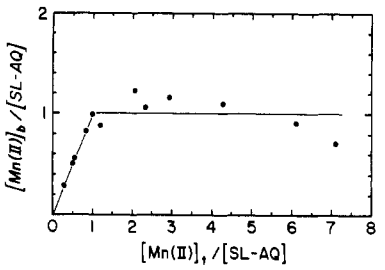

FIGURE 3: Plot of the ratio of Mn(II) bound to spin-labeled aequorin to the total concentration of spin-labeled aequorin ($[Mn(II)]_b$/[SL-AQ]) vs. the ratio of total Mn(II) concentration to total spin-labeled aequorin concentration ($[Mn(II)]_t$/[SL-AQ]). The measurements were made on MOPS-buffered solutions at pH 7.0 and 20 °C. The range of protein concentration employed is 18–178 μM, and the range of total Mn(II) concentration is 20–500 μM.

The influence of metal ions on the EPR signals of the spin-labeled protein was also examined. It was found that neither Ca(II) nor Mg(II) had an effect on the EPR signal of spin-labeled aequorin. Futhermore, the paramagnetic ions Mn(II) and Pr(III) were ineffective in perturbing the spin-label spectrum. No effect whatsoever was seen on the spin-label EPR signals in the presence of these metal ions. The line widths, heights, and τ_c values were unchanged. EPR signals from Mn(II) do not interfere with the spin-label signals since the spin-label signals fall between the third and fourth hyperfine lines of the characteristic six-line EPR pattern observed from free Mn(II) while the bound Mn(II) gave no EPR signal under our experimental conditions.

There is clear evidence that all these cations bind to the spin-labeled protein. When a solution of Mn(II) is titrated with spin-labeled aequorin, the intensity of the Mn(II) EPR signal decreases progressively. The intensity of the observed signal is therefore a measure of free [Mn(II)]. The metal ion binding stoichiometry and the dissociation constant can, in principle, be determined by the measurement of the Mn(II) EPR signal intensity at various values of total Mn(II) and protein concentrations. Figure 3 shows the results of such an experiment. One tight binding site for Mn(II) per protein molecule was found.[2] However, the affinity constant for Mn binding is so high that it was not possible to measure, with

[2] The scatter of the experimental points in the plateau region of Figure 3 especially for $[Mn]_t/[SL\text{-}AQ] > 2.0$ is readily accounted for on the basis of 10% uncertainty in the concentrations. For example, consider the point corresponding to $[Mn]_t/[SL\text{-}AQ] = 2.06$ and $[Mn]_b/[SL\text{-}AQ] = 1.23$. The actual data were [SL-AQ] = 81.0 μM, $[Mn]_t$ = 167 μM, and $[Mn]_f$ = 67.5 μM. Considering an uncertainty of 10% in each of these measurements yields 2.40 and 1.68 as the upper and lower limits for $[Mn]_t/[SL\text{-}AQ]$ and 1.55 and 0.85 as the limits for $[Mn]_b/[SL\text{-}AQ]$.

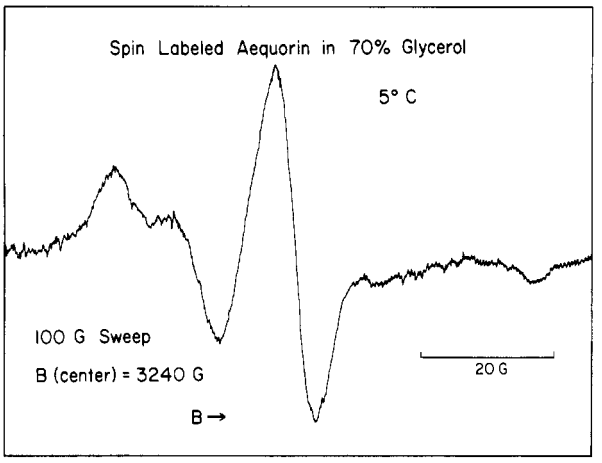

FIGURE 4: EPR signal of 40 μM maleimide spin-labeled aequorin at 5 °C in a pH 7.0 MOPS-buffered solution with 70% glycerol (v/v).

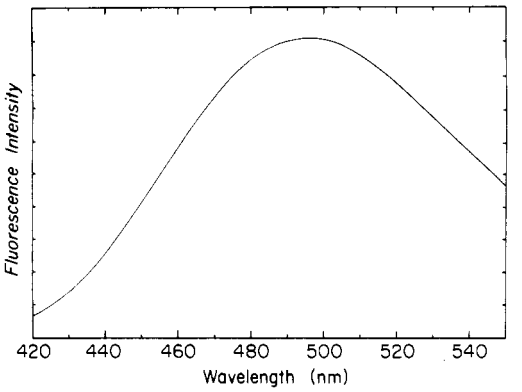

FIGURE 5: Corrected fluorescence emission spectrum of Acrylodan-labeled aequorin. The excitation wavelength was 375 nm; the excitation slits were set as 4 nm. The slits on the emission monochromator were 4 nm. The protein (5 μM) was dissolved in a solution containing 125 mM KCl, 10 mM MOPS, 1 mM EDTA, and 1 mM KCN. The spectrum was measured at 25 °C.

the sensitivity of the instrument available, the very low concentrations of free Mn(II) required for a precise determination of K_D. In our experiments, when the total Mn(II) concentration was less than the total concentration of spin-labeled aequorin, no free Mn(II) was detected. For greater [Mn(II)], only the excess was seen as free Mn(II). For the lowest spin-labeled aequorin concentration used ($\sim$15 μM), no free Mn(II) was observed within our experimental limits of detection ($\simeq$1 μM) when the total [Mn(II)] was equal to the spin-labeled aequorin concentration. Thus, we can only place a limiting value of $K_D \lesssim 10^{-6}$ M for Mn(II). Subsequently the extent to which Mn(II) is displaced from spin-labeled aequorin by different metal ions showed that K_D[Pr(III)]/K_D[Mn(II)] < 10, K_D[Ca(II)]/K_D[Mn(II)] > 10^3, and K_D-[Mg(II)]/K_D[Mn(II)] > 10^5.

For Mn(II) Leigh (1970) has given a theory for the effects expected upon the EPR signal of a nitroxyl radical due to magnetic dipolar interaction with a nearby paramagnetic Mn(II) ion. This theory has particular applicability where the rotational correlation time of the label is long compared with the spin–lattice relaxation time of Mn(II) (i.e., solidlike behavior) so that the vector joining the two paramagnetic centers does not change orientation appreciably on the time scale of the Mn(II) relaxation. This theory allows one to estimate distances between the paramagnetic metal and the nitroxyl. The procedure for calculation of the distance between the two paramagnetic centers is as follows: computer-simulated spectra calculated as a function of the parameter $C = g\beta\mu^2 T_1/(d^6\hbar)$ are matched with the experimental spectrum. In the expression for C, g is the g-value of the spin-label, β is the Bohr magneton, μ is the effective magnetic moment of the Mn(II) ion, T_1 is the spin–lattice relaxation time of the Mn(II) ion, d is the distance between the spin-label and the Mn(II), and $\hbar$ is Planck's constant divided by 2π. A value of C can then be obtained from the comparison and d calculated from that assuming a reasonable value for T_1.

At 20 °C the correlation time of the label attached to aequorin determined above (3.6×10^{-10} s) is short compared with the expected Mn(II) relaxation time of $\simeq 10^{-9}$ s. Since the signal of the spin-label was not affected by Mn(II) at 20 °C, in order to ascertain if the absence of perturbation was due to the short correlation time, measurements were made at 5 °C with the spin-labeled protein dissolved in a solution containing 70% glycerol. The spectrum obtained is shown in Figure 4. The motion of the spin-label is slowed to such an extent that eq 2 and 3 are no longer valid for calculation of τ_c. To determine the τ_c the models of McCalley et al. (1972) and Goldman et al. (1972), which apply to this slow motion regime, were used. Each method requires a reference spectrum in the rigid limit. Such a spectrum was obtained by cooling the sample to −98 °C. Both models yielded $\tau_c \simeq 7 \times 10^{-9}$ s for the spectrum of Figure 4. The addition of Mn(II) to the spin-labeled protein solution under these conditions (5 °C, 70% glycerol) again had no effect on the EPR signal of the spin-label.

If we assume that the effect on the spin-label EPR signal amplitude due to the binding of Mn(II) to the protein could have been as large as 10% but yet not observed because of signal to noise considerations, then we can calculate a lower limit for the distance between the spin-label and the Mn(II). From the spectral simulations given by Leigh (1970), it can be seen that such a situation corresponds to $C \simeq 0.2$. Thus, setting $C = 0.2$, $\mu^2 = S(S+1)\beta^2$ with $S = {}^5/_2$ for Mn(II), and $T_1 = 10^{-9}$ s gives $d \simeq 20$ Å as this lower limit. Exchange interaction effects are not expected to be significant in the solidlike region because the two bound paramagnetic species are different and such effects are usually of shorter range than dipolar effects (Villafranca & Raushel, 1982). Heisenberg exchange interactions in liquids can be important when there is an excess of Mn(II) over the spin-label and when collisions of the paramagnetic species are occurring (Hyde et al. 1979), neither of which should be important in this case. Continuous wave saturation measurements, which are generally sensitive to larger ion-label separations (Hyde et al., 1979), were not possible on this system at 20 °C—significant saturation of the spin-label EPR signal could not be accomplished with the microwave power available.

The fluorescence spectrum of Acrylodan-labeled aequorin is shown in Figure 5. Weber & Farris (1979) have shown that the fluorophore formed by the 2-(dimethylamino)-6-acylnaphthalene moiety shows markedly polarity-sensitive fluorescence, and since there are no groups attached to this system which can undergo torsional motion, the fluorescence spectrum of a particular derivative will provide a good indication of the polarity of the environment. Prendergast et al. (1983) have reinforced this notion of Weber and Farris and shown that, in contrast to the fluorescence of probes such as ANS (where torsional motions of the phenyl ring about the naphthyl ring contribute significantly to the fluorescence spectrum), Acrylodan fluorescence is indeed indicative of microenvironmental polarity. In the work of Prendergast et al. (1983) it was apparent that, in different sulfhydryl-containing proteins, groups labeled with Acrylodan allowed for

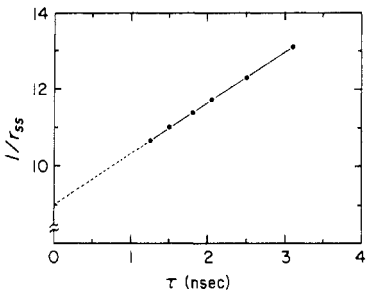

FIGURE 6: Plot of the reciprocal of fluorescence anisotropy vs. fluorescence lifetime at 25 °C for Acrylodan-labeled aequorin. Fluorescence lifetimes were varied by oxygen quenching and were calculated by assuming that the quenching is entirely collisional from the relation $\tau = (\tau_0 F/F_0)$ where τ_0 and F_0 are the fluorescence lifetime and intensity measured in the absence of oxygen and τ and F are the fluorescence lifetime and intensity at a chosen partial pressure of oxygen (Lakowicz & Weber, 1973). τ_0 was measured as described in the text.

varying degrees of access of solvent to the fluorophore. Of the proteins studied by these authors, the most apolar environment was found to be for Acrylodan-labeled carbonic anhydrase; the emission maximum for this derivative was approximately 494 nm. The fluorescence emission spectrum of Acrylodan-labeled aequorin (Figure 5) has the emission maximum at 495 nm, very similar to that observed for Acrylodan-labeled carbonic anhydrase which suggests that the fluorophore is moderately inaccessible to solvent molecules.

The data for the lifetime-resolved anisotropy studies are shown in Figure 6. Rotational correlation times were calculated by use of equations given under Materials and Methods. The results show that the fluorophore has an apparent correlation time of 8.5 ns which is considerably less than the rotational correlation time (28 ns) calculated from fluorescence depolarization studies on the calcium-discharged blue fluorescent protein (F. G. Prendergast, unpublished results). Evidence of rotational freedom of the fluorophore also comes from the low zero time anisotropy value, $r_0' = 0.11$, observed for Acrylodan-labeled aequorin by extrapolation of the linear plot to zero time (see Materials and Methods). The real limiting anisotropy r_0 was determined to be 0.335 in this case by measurements of Acrylodan in a glass of 75% glycerol/water at −70 °C. As pointed out earlier, a low value for the zero time anisotropy compared with the real limiting anisotropy is indicative of marked motional freedom of the probe. Thus, while the fluorophore may be ensconced in a region that is moderately inaccessible to solvents, nonetheless it must have considerable freedom of reorientation.

Neither the fluorescence emission spectrum nor the apparent mobility of the probe determined from lifetime-resolved anisotropy measurements was significantly altered by the addition of calcium to the medium. This result corroborates the data found for spin-labeled aequorin.

Discussion

The fact that chemical modification of a thiol residue in aequorin leads to loss of Ca-activated luminescence implies strongly the involvement of this group in the catalytic events leading to bioluminescence. By inference the thiol moiety must be in the proximity of the active site of the protein. The EPR spectrum of the protein labeled with the thiol-specific maleimide spin-label yields a rotational correlation time of 3.6×10^{-10} s at 20 °C which is about 2 orders of magnitude shorter than the value noted earlier for Ca^{2+}-discharged aequorin (28 ns). The hyperfine interaction constants measured from the spectra indicate appreciable accessibility of the spin-label to the solvent as does the ease with which the attached label can be reduced by ascorbate. Furthermore, fluorescence measurements show that the rotational correlation time of a thiol-attached fluorophore (8.5 ns) is shorter than that of discharged aequorin but longer than that of the free fluorophore (5.0×10^{-10} s). Thus, the overall rotational motion of the protein does not contribute greatly to the measured correlation times of either label.

Although the EPR and fluorescence results demonstrate that the respective labels attached to the protein have considerable freedom of reorientation, the correlation time for the motion of the spin-label is a factor of approximately 20 shorter than that for the fluorophore. Further, the attached spin-label is readily accessible to the solvent while the fluorophore itself appears to be relatively inaccessible to the solvent. These differences raise the question as to whether the spin-label and the fluorophore modify the same sulfhydryl group. However, it should be noted that both labels inactivate the protein equally well and that the stoichiometry measurements with each label yield a value of one label per protein molecule. These results are in agreement with those of Shimomura & Johnson (1978) which showed that NEM labeled and inactivated native aequorin with a 1:1 stoichiometry. In fact all sulfhydryl-labeling experiments of native aequorin thus far report a 1:1 stoichiometry. [A stoichiometry of 2:1 (label:protein) was noted for NEM labeling of *discharged* aequorin (Shimomura & Johnson, 1978)]. It is therefore highly unlikely that the spin-label and the fluorophore are attached to two distinct sulfhydryl groups. If there is heterogeneity of labeling in either case, it must be below the experimental limits of detection.

The difference in the rotational correlation times and apparent solvent accessibility of the attached spin-label and fluorophore is not altogether unexpected. There is no reason to assume that the two structurally very different probes will locate in the same microenvironment on the protein even though they are attached to the same thiol. In the first place, they are attached differently to the thiol, the nitroxyl via a maleimide and the Acrylodan via the α,β-unsaturated bond. Second, the nitroxyl moiety is more polar than the 2-(dimethylamino)-6-acylnaphthalene group, and third, it is considerably less bulky. The nitroxyl will thus seek an aqueous environment and by virtue of its size have a significantly faster rotational rate. Because of its steric bulk, the motions of the fluorophore are likely to be more restricted than those of the EPR probe. Furthermore, the correlation times characterizing EPR and fluorescence data arise from correlation functions of different physical quantities and therefore need not be identical even on theoretical grounds.

A variety of sulfhydryl-modifying reagents readily inactivate aequorin which clearly suggests that a thiol group is reasonably accessible even though the integrity of this group is essential for the bioluminescence activity. While aequorin with the SH group chemically modified is unarguably different from the native protein, fluorescence and CD data show that there are no apparent major changes induced in the protein by this modification (S. Ho and F. G. Prendergast, unpublished results). There is, therefore, no reason to believe that metal ion binding to the spin-labeled protein is compromised relative to its binding to the "native" protein. The EPR data on the binding of Mn(II) to aequorin bolster this conclusion. Clearly Mn(II) binds to spin-labeled aequorin with a $K_D \lesssim 10^{-6}$ M. Pr(III) at concentrations equivalent to that of Mn(II) displaces Mn from aequorin. Ca(II) can also displace bound Mn(II) but only at concentrations very much in excess of that of Mn(II). Mg(II) even at concentrations 10^5 times greater than

Mn(II) cannot displace the latter from aequorin. No direct evidence is available that Mg(II) binds to aequorin; however, it is known that the Ca-activated as well as Ca-independent bioluminescence of aequorin is markedly reduced in a concentration-dependent manner in the presence of Mg(II) (Allen et al., 1977; Prendergast et al., 1977). Further, although ionic strength also reduces the sensitivity of aequorin to Ca(II), the effect of Mg(II) is not due to changes in ionic strength. Thus, it may be reasonably concluded that Mg(II) competes with Ca(II) for the same binding sites on aequorin but has a lower affinity. From the displacement data we can set up a rank order of affinities for metal ion binding to aequorin as Pr(III) > Mn(II) > Ca(II) > Mg(II). It is likely that all the lanthanides will have similar affinities inasmuch as they are similarly effective in eliciting bioluminescence (Prendergast et al., 1977). Other metal ions also bind to and (apparently) trigger bioluminescence from aequorin (Izutsu et al., 1972, 1974; Prendergast et al., 1977), but we do not yet have any data on their binding affinities.

The apparent 1:1 stoichiometry for Mn(II) binding to aequorin is important. This ion is not an effective activator of aequorin bioluminescence. On the basis of the Mn(II) displacement data, it cannot be determined whether other metal ions studied that *do* activate aequorin bioluminescence also have only one binding site. The Ca(II) concentration effect curves for aequorin bioluminescence strongly support the notion of at least three Ca-binding sites on aequorin (Allen et al., 1977; Blinks et al., 1976); a linear relation between light output and [Ca(II)] has never been observed. By the same token, the apparent weak ability of Mn(II) to promote bioluminescence could be simply related to the fact that it can bind to only one of three sites.

Finally, if Leigh's formula regarding the effect of Mn(II) relaxation on the spin-label EPR signal is applied, it can be inferred that the Mn(II) binding site must be ≥20 Å from the average location of the spin-label. Even allowing for the extra distance incurred by the "arm" of the spin-label, the data suggest that at least one of the metal binding domains of aequorin is likely to be ≥15 Å from the putative active site of aequorin. In other words, information regarding metal ion binding must be transmitted to the reactants at the active site via conformational changes in the whole protein molecule. The data presented here do not, however, allow any conclusions to be drawn regarding the nature or extent of these conformational changes. They are, however, sufficiently small that neither the spin nor the fluorescent labels are affected upon metal binding.

Clearly these are but the initial studies, and much more can be learned about aequorin from further EPR studies of Mn(II) with native and discharged aequorin, through use of other spin-labels and other metal ions. Any structural information obtained therefrom will be complemented by NMR and fluorescence studies on the same systems. Such studies are in progress.

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Registry No. Mn, 7439-96-5; 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy, 15178-63-9; Acrylodan, 86636-92-2.

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^{13}C NMR Studies of the Molecular Dynamics of Selectively ^{13}C-Enriched Ribonuclease Complexes†

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ABSTRACT: ^{13}C spin–lattice (T_1) relaxation times determined at four frequencies (25, 68, 100, and 125 MHz) have been used to probe the molecular dynamics of ribonuclease S′ complexes prepared from synthetic amino-terminal peptides containing ^{13}C enrichment (ca. 90%) at selected sites [Niu, C., Matsuura, S., Shindo, H., & Cohen, J. S. (1979) *J. Biol. Chem. 254*, 3788]. It was found that the motion of the C^{α}–H bond of Ala-5 could not be determined by isotropic reorientation alone. The time scale and spatial restriction on the internal motion of this residue were determined by the model-free approach of Lipari and Sazbo [Lipari, G., & Szabo, A. (1982) *J. Am. Chem. Soc. 104*, 4546–4559]. It was found that the C^{α}–H bond, in addition to an overall correlation time of 20 ns, underwent internal motion with a correlation time of 0.5 ns and a generalized order parameter $\mathcal{S}$ corresponding to a cone semiangle of 23 °C. The C^{β}–H bond had a correlation time of 37 ps, reflecting the fast rotation of the methyl group, and had an $\mathcal{S}$ value close to that expected if the C^{α}–C^{β} and C^{α}–H bonds have the same degree of spatial restriction.

NMR spectroscopy is now widely used in studies of the conformation and mobility of proteins in solution (Cohen et al., 1983). But there have in general been many fewer applications of ^{13}C NMR than of ^{1}H NMR to proteins because of the lower sensitivity of ^{13}C NMR (1.6%) and its lower natural abundance (1.1%) compared to ^{1}H. However, it is particularly useful to observe ^{13}C relaxation in order to determine mobility in proteins, because of the averaging of proton relaxation rates due to spin diffusion in proteins (Kalk & Berendsen, 1976).

Several years ago, Niu et al. (1979) synthesized selectively ^{13}C-labeled amino-terminal peptides of ribonuclease (RNase) in order to study their fully enzymatically active RNase S′ complexes. The greater sensitivity resulting from use of the high ^{13}C enrichment (ca. 90%), and the absence of ambiguity in the assignments of the signals observed well above the background of natural abundance resonances, allowed single carbon atom sites in this enzyme to be studied in detail. Several examples of selectively ^{13}C-labeled proteins have been published (Eakin et al., 1975; Jones et al., 1976; Blakley et al., 1978; Deber et al., 1978; Schejter et al., 1978; Cohen et al., 1979; Harina et al., 1980; Matta et al., 1980; Wooten et al., 1981). These have frequently been prepared biosynthetically, resulting in the presence of multiple peaks in the spectrum and the need for subsequent assignment.

We now wish to report ^{13}C relaxation time measurements on the ^{13}C-labeled RNase complexes and their analysis in terms of protein mobility. We have carried out these relaxation time determinations at several observing frequencies in order to satisfy the needs for rigorous analysis, particularly to distinguish between different mechanisms of relaxation that may be operative (Doddrell et al., 1972; Lyerla & Levy, 1974; Norton et al., 1977). A few examples of ^{13}C relaxation time measurements of individually resolved resonances in proteins at several frequencies have been reported previously (Jones et al., 1976; Richarz et al., 1980; Ribeiro et al., 1980; Norton et al., 1977). Earlier work tended to be at a single lower field strength [for review, see Egan et al. (1977) and Howarth & Lilley (1978)].

Recently, a new model-free approach to the analysis of NMR relaxation data of macromolecules in solution has been developed (Lipari & Szabo, 1982). We have applied this theory to the ^{13}C relaxation data for the Ala-5 residue of RNase S′ with data obtained at four frequencies. Our conclusions are that, in addition to the overall motion, a component of local mobility is needed to satisfactorily explain the relaxation time data. Using the Lipari–Szabo theory, we are able to estimate the time scale and the amplitude of this local motion.

Experimental Procedures

Materials. The three ^{13}C-enriched (1–15) RNase amino-terminal peptides were synthesized and purified as previously

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