Articles

Proton NMR of Aequorin. Structural Changes Concomitant with Calcium-Independent Light Emission†

Bruce D. Ray,‡ Steffan Ho,§ Marvin D. Kemple,*,‡ Franklyn G. Prendergast,§ and B. D. Nageswara Rao‡

Department of Physics, Indiana University–Purdue University at Indianapolis, Indianapolis, Indiana 46223, and Department of Pharmacology, Mayo Medical School, Rochester, Minnesota 55905

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ABSTRACT: Aequorin, a Ca(II)-sensitive bioluminescent protein from jellyfish, emits light at 469 nm from an excited state of a substituted pyrazine (oxyluciferin) which results from the oxidation of a chromophore molecule that is noncovalently bound to the protein. The chromophore is oxidized when Ca(II) or other activating metal ions are bound by aequorin. In the absence of Ca(II), spontaneous emission of light, referred to as Ca(II)-independent light emission, occurs at a rate $<10^{-6}$ of that for Ca(II)-induced emission. Proton nuclear magnetic resonance (NMR), circular dichroism (CD), and fluorescence were used to study structural changes of aequorin accompanying Ca(II)-independent light emission. Time course studies by ^{1}H NMR and CD demonstrate that as a result of Ca(II)-independent light emission, aequorin progressively changes from a rigid, fully active form showing little segmental mobility to a practically unfolded, discharged (i.e., inactive) form in which a number of amino acid residues are significantly mobile. This slow discharged protein (SDP) is distinct in nature and conformation from aequorin which has been discharged by Ca(II), i.e., the blue fluorescent protein. The rate of Ca(II)-independent discharge of aequorin is substantially reduced in the presence of excess Mg(II); the time constant for inactivation at 5 °C is 30 days with no Mg(II) present and 70 days with Mg(II) present. The NMR spectra are nearly identical at a given stage of inactivation whether or not Mg(II) is present. Oxyluciferin remains bound to SDP. If it is removed, however, by column chromatography, the resulting apo-SDP partially refolds, and the segmental mobility acquired in the formation of SDP is significantly attenuated particularly for some of the aromatic amino acid residues.

Aequorin is a bioluminescent protein of molecular weight 21 000 isolated from the jellyfish *Aequorea forskålea* (Shimomura et al., 1962, 1963; Blinks et al., 1976). The protein emits blue light with a spectral maximum at 469 nm upon binding Ca(II) or other activating metal ions. Light is emitted from the excited state of a 2,3,6-substituted pyrazine derived from oxidation of a noncovalently bound chromophore, an imidazolopyrazinone (Shimomura & Johnson, 1972; Cormier et al., 1973a,b). This oxidation proceeds in the absence of exogenous oxygen or cofactors and is presumed to be mediated by an intrinsic oxygen moiety the chemical identity of which is yet to be established (Shimomura et al., 1962; Prentice & Ward, 1983). In the absence of Ca(II) or other activating ions (Izutsu et al., 1972, 1974; Prendergast et al., 1977), there is spontaneous emission of light by the protein referred to as calcium-independent light emission (Allen et al., 1977) which occurs usually at a much lower rate ($<10^{-6}$) than that in the presence of Ca(II). At least one amino acid side chain—a thiol—has been identified as part of a putative "active site" in the protein; modification of this moiety results in complete loss of Ca(II)-triggered bioluminescence (Shimomura et al., 1974; Shimomura & Johnson, 1978; Prendergast & Mann, 1978). Thus, it appears that the binding of Ca(II) causes a dramatic increase in the rate of oxidation of the chromophore most likely by promoting free catalytic expression by the protein. However, the conversion of the chromophore into a light emitter by the protein in vitro is not reversible (what happens in vivo is not known), and an individual aequorin molecule emits light but once.

Very few structural details of aequorin are known thus far. The amino acid residues involved in the chromophore or metal ion binding sites have not yet been identified. Determination of the amino acid sequence of the protein is nearing completion (H. Charbonneau, personal communication), but efforts to determine structure by X-ray crystallography have not been successful. However, aequorin has a number of features, such as its interaction with a variety of metal ions and its moderate molecular weight, which make it amenable to investigation by magnetic resonance [nuclear magnetic resonance (NMR)[1] and EPR] techniques. An EPR study of aequorin spin-labeled

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‡ Indiana University–Purdue University at Indianapolis.

§ Mayo Medical School.

[1] Abbreviations: apo-SDP, slow discharged protein [Ca(II)-independent discharged aequorin] with oxyluciferin removed; BFP, blue fluorescent protein [Ca(II)-discharged aequorin]; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; SDP, slow discharged protein [Ca(II)-independent discharged aequorin]; Tris, tris(hydroxymethyl)aminomethane; TSP, (trimethylsilyl)propionic acid; UV, ultraviolet; vis, visible.

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at an essential sulfhydryl group inclusive of some characteristics of Mn(II) binding to the modified protein has been completed recently (Kemple et al., 1984). The present paper describes results of ^{1}H NMR investigations on aequorin. These data help to establish the general characteristics of the protein with particular focus on the changes in conformation concomitant with Ca(II)-independent light emission[2] as revealed by ^{1}H NMR spectra. A time course of the ^{1}H NMR spectrum was obtained along with a measurement of the bioluminescent activity of the protein over a period of several weeks during which the protein was completely discharged. Similar measurements were performed on an aequorin sample in the presence of a large excess concentration of the inhibitor cation Mg(II) in order to assess its role in retarding the discharge of the protein by Ca(II)-independent light emission. Corroborative evidence of the structural and conformational changes suggested by the ^{1}H NMR spectra as well as complementary data for the interpretation of the observed changes in the NMR spectra was obtained from CD and fluorescence measurements.

To avoid confusion between different forms of the protein and the molecules involved in light emission, the following nomenclature is used: Native aequorin refers to a protein molecule which has a bioluminescent response to the addition of Ca(II), and native chromophore to the molecule bound to native aequorin that is converted into a light emitter in the presence or absence of Ca(II). Aequorin which has bioluminesced in the absence of Ca(II) is referred to as slow discharged protein (SDP) whereas that which bioluminesced due to Ca(II) is called blue fluorescent protein (BFP) (Shimomura & Johnson, 1969). The light-emitting molecule which is responsible for the blue fluorescence of BFP (subsequent to bioluminescence) is termed oxyluciferin. Although it has not yet been unequivocally determined whether the light emitters in SDP and BFP are identical, since there is no evidence thus far to the contrary, we shall also refer to the light emitter in SDP as oxyluciferin. Apo-SDP is used to describe SDP from which oxyluciferin has been removed, usually by column chromatography. The chromatographic procedure yields a substantial amount of a blue fluorescent compound which has an absorption maximum at 350 nm as opposed to 330 nm for oxyluciferin bound to either BFP or SDP. This molecule is referred to as etioluciferin or AF-350 (Shimomura & Johnson, 1969, 1972) and appears to be a hydrolysis product of oxyluciferin.

^{1}H NMR, CD, and fluorescence measurements made on apo-SDP are also included in this paper. A comparison of these results with those on SDP provides information on the binding of oxyluciferin to SDP.

Materials and Methods

Aequorin, isolated from the jellyfish *Aequorea forskålea*, was purified and assayed by established procedures described elsewhere (Blinks et al., 1978). Protein concentrations were measured spectrophotometrically by using $\epsilon_{280nm}^{0.1\%} = 1.95$ cm^{-1} with the chromophore attached and 1.0 cm^{-1} for the apoprotein. The protein was lyophilized from a solution containing 10 mM MOPS, 150 mM KCl, and 0.5 mM EDTA and was stored under liquid nitrogen in sealed glass ampules each containing 10 mg of the protein. In view of the extreme sensitivity of aequorin to even low concentrations of activating metal ions, solvent H_2O and all buffered solutions used were decontaminated by passing them through a Chelex-100 column. Metal-free 99.96% D_2O purchased from Cambridge Isotope Laboratories was used as solvent for NMR samples. Tris-d_{11} used as a pH buffer for NMR samples was purchased from MSD Isotopes and was prepared for use by dissolving it in H_2O to 0.25 M, adjusting the pH to 7.8, passing it through a Chelex column, lyophilizing it, and redissolving it in D_2O to a final concentration of 0.5 M. A D_2O solution of TSP-d_4 (disodium salt), purchased from Cambridge Isotope Laboratories, was used as an internal chemical shift reference. Ultrapure grade $MgCl_2$ was obtained from Puratronic (Alfa). A solution of 0.5 M $MgCl_2$, 150 mM KCl, and 3 mM NaCN, which acts as a scavenger for Ag contamination from pH electrodes, was prepared at pH 8.0 in H_2O and was subsequently lyophilized and redissolved in D_2O. Aliquots of this buffer solution were added to protein samples to achieve a desired concentration of Mg(II). The pH values were measured with a Beckman Altex Model 3500 digital pH meter and were not corrected for deuterium isotope effects. UV–vis absorption spectra were obtained with a Perkin-Elmer λ_3 spectrophotometer.

The oxyluciferin was removed from SDP by either of two procedures: A 20-mL column of Sephadex G-25 was prepared by washing the Sephadex with 10 volumes each of type I water (electrical resistivity of 18 MΩ cm^{-1}), 20 mM KCl solution (pH 8.0) in type I water, and 50% acetone in type I water, 20 volumes of acetone, and 10 volumes of pH 8.0 20 mM KCl solution in type I water before packing the column. The inactivated aequorin was applied to this column and eluted with 20 mL of pH 8.0 20 mM KCl solution in type I water. The apo-SDP is in the eluate. The column was then washed with 20 mL of type I water, and the material containing the fluorescent moiety was eluted with 30 mL of 50% acetone in type I water. This eluate was evaporated to dryness, resuspended in 95% ethanol, and adjusted to pH 8. Its optical absorption spectrum from 500 to 200 nm was then recorded. The spectrum has strong absorption bands at 280 and 350 nm and is similar to the previously reported spectrum (Shimomura & Johnson, 1972) for etioluciferin (AF-350). Apo-SDP was lyophilized and used for preparing NMR samples as described below. Apo-SDP was also prepared by passage of SDP through a 1 cm × 7 cm column of hydrophobic XAD-2 resin (Rohm & Haas). The oxidized chromophore adsorbed avidly to the resin, and the apoprotein was eluted in the void volume. The adsorbed etioluciferin was eluted from the resin with ethanol.

NMR samples were prepared by first dissolving ~10 mg of aequorin in 1 mL of a pH 8.0 aqueous solution of 150 mM KCl and 3 mM NaCN. The solution was then dialyzed for ~16 h at 5 °C against two changes of a 50-mL aqueous solution of 150 mM KCl and 3 mM NaCN containing 0.1 g of Chelex for the dual purpose of removing the buffer used in the storage sample and eliminating any residual metal ion contamination in the sample. Thomas 3787-1445 dialysis tubing with a molecular weight cutoff of 3500 was used, although aequorin has a molecular weight of 21 000, since our experience showed significant protein loss from dialysis tubing with higher values of molecular weight cutoff. The dialysis tubing was soaked in a pH 8.0 solution of 0.1 M EDTA at 40 °C for about an hour before it was used. EDTA was

[2] The presence of a Ca(II)-independent dark pathway for the inactivation of aequorin cannot be completely excluded; however, there are plausible reasons for considering that the inactivation of the protein as described here is primarily due to Ca(II)-independent light emission. The rate of loss of activity of the protein is consistent with the measured rate of Ca(II)-independent light emission (Allen et al., 1977). In addition, chromatographic and fluorescence studies indicate that the light-emitting moieties of Ca(II)-discharged aequorin and Ca(II)-independent discharged aequorin are the same.

subsequently removed from the dialysis tubing by extensively rinsing the tubing with type I water. Dialyzed aequorin was lyophilized and redissolved in 760 μL of D_2O, 20 μL of 0.5 M Tris-d_{11} (pH 7.8), and 20 μL of 10 mM TSP-d_4 to give two identical NMR samples.

For CD measurements, only phosphate was used to buffer solutions since it has minimal absorbance in the far-UV region (180–240 nm). The various protein samples were dissolved in a solution of 0.1 M sodium phosphate, 1.0 mM KCN, and a 0.1 mM EDTA buffered at pH 8.0. The EDTA concentration was maintained low because EDTA absorbs significantly at wavelengths <210 nm. All solutions were treated with Chelex so that 0.1 mM EDTA was sufficient to prevent discharge of aequorin by contaminant Ca(II). All fluorescence measurements were made on the same solutions used for the CD spectrophotometry.

NMR Measurements. ^1H NMR spectra were obtained at 5 °C by using a Nicolet NT-300 NMR spectrometer equipped with a Nicolet 1280 computer, a 293C pulse programmer, and a variable temperature controller. The spectra were obtained in the Fourier-transform mode of operation with quadrature detection. The deuterium signal from solvent D_2O was used to provide a field-frequency lock. Residual proton signals from HDO in the D_2O solutions were suppressed by continuous monochromatic radio-frequency irradiation during the delay period following data acquisition. Good spectra were obtained with typical accumulations of 1000 scans for over an hour. An FC-20-84 cascade Flexi-cool system equipped with a P11S nonmagnetic copper tube in tube heat exchanger (FTS Systems Inc.) was employed to cool the nitrogen gas used for sample temperature control.

CD Measurements. CD spectra were measured with a Jasco Model J500A spectropolarimeter interfaced to a Jasco Model DP500N data processor (Japan Spectroscopic Co., Inc., Tokyo, Japan). The instrument was calibrated with an aqueous solution of d_{10}-camphorsulfonic acid (Aldrich Chemical Co., Inc.) at 290.5 nm (λ_{max}), where $\Delta\epsilon = 2.37\ M^{-1}\ cm^{-1}$ (Vallee & Holmquist, 1980). Unless otherwise noted, spectra were measured with a 2-s time constant and a scan rate of 50 nm/min. These conditions were determined not to distort the CD spectra. A slit width of 1 nm was used while the spectra were recorded. The accuracy of the spectra was improved by signal averaging with a minimum of 16 scans per spectrum. CD spectra of protein-free solutions were measured similarly to provide base-line values which were then subtracted from the spectra of the protein solutions. Far-UV spectra were measured with a 2-mm path-length cell and an approximate protein concentration of 0.1 mg/mL. Near-UV spectra were measured through use of a 5-mm path-length cell and an approximate protein concentration of 2 mg/mL.

The CD spectra were measured in degrees of ellipticity (θ), and these were converted to mean residue ellipticity by use of the equation:

$$[\theta]\ (\text{or}\ [\theta']) = \frac{\theta_{obsd} M_r\ (\text{or MRW})}{10cd}$$

where $[\theta]$ is the molar ellipticity and $[\theta']$ is the mean residue ellipticity (degrees centimeter squared per decimole), θ_{obsd} is the observed ellipticity, M_r = molecular weight, MRW = mean residue weight, d = path length in centimeters, and c = concentration in grams per milliliter.

The secondary structures of the forms of aequorin studied were estimated from far-UV CD spectra with the assumption that each spectrum comprises a linear combination of the CD signals from α-helix, β-sheet, and random structure as described by the equation:

$$[\theta'](\lambda) = f_\alpha[\theta']_\alpha(\lambda) + f_\beta[\theta']_\beta(\lambda) + f_R[\theta']_R(\lambda)$$

where $[\theta'](\lambda)$ is the spectrum of unknown composition, $[\theta']_\alpha(\lambda)$, $[\theta']_\beta(\lambda)$, and $[\theta']_R(\lambda)$ are reference spectra for α-helix, β-sheet, and random structures, respectively, and f_α, f_β, and f_R are the fractions of the three structural forms in the protein molecule ($f_\alpha + f_\beta + f_R = 1$) as described by Chen et al (1972). The values for the reference spectra over a range from 245 to 187 nm in 1-nm intervals were tabulated by Yang (1976) from the data of Chen et al. (1972, 1974). These values were calculated from the CD spectra of five globular proteins whose conformational compositions are known from X-ray data. Conformational compositions in our measurements were determined with an HP 9836 computer by a least-squares minimization procedure based upon the method of determinants.

Fluorescence Measurements. Fluorescence spectra and lifetimes (τ) were measured with an SLM 4800 fluorometer. For studies of intrinsic tryptophan fluorescence, an excitation wavelength of 295 nm was used. The intrinsic blue fluorescence of SDP was excited with light at 330 nm, i.e., approximately at the absorption maximum of oxyluciferin bound to BFP (or SDP) (Shimomura & Johnson, 1969). For all fluorescence lifetime measurements, unpolarized light was used for excitation. The fluorescence emission of interest was then selected by the use of either cut-on or interference filters; a polarizer was placed in the emission path and oriented at a magic angle (35°) to minimize the effects of Brownian motion on the measurement of τ. Lifetimes were measured by use of the phase-modulation method of Spencer & Weber (1969). POPOP (τ = 1.35 ns) and *p*-terphenyl (τ = 0.95 ns) were used as reference compounds to minimize the effects of targeting errors and the wavelength dependence of phototube response on the measurement of τ (Lakowicz et al., 1981). Modulation frequencies of either 18 or 30 MHz were usually employed. Measurements of steady-state fluorescence anisotropy (r_{ss}) were made by use of the SLM 4800 fluorometer operated in the "L" format (Lakowicz, 1984). For measurement of r_{ss} of tryptophan fluorescence, the excitation wavlength was 300 nm with a 1-nm band-pass. Tryptophan emission was selected by the use of a Schott WG 345 (cutoff) filter for studies with native aequorin and apo-SDP and a 5-nm band-pass interference filter centered on 345 nm when there was also blue fluorescence (i.e., with BFP and SDP). For r_{ss} determination of the blue fluorescence, excitation at 350 nm was used and the blue fluorescence selected with a Schott KV 418 filter. Anisotropies were calculated in the standard fashion (Lakowicz, 1984); the use of filters in the emission path rather than a monochromator makes it unnecessary to calculate the so-called "G" factor which is a correction term for differential transmission of the two components of polarized light by a monochromator.

Results

The aromatic and aliphatic regions of the ^1H NMR spectra of native aequorin in the presence of EDTA, obtained at different stages after the preparation of the sample, are shown in panels A and B, respectively, of Figure 1. The sample was stored throughout at 5 °C. The number of days elapsed from the preparation of the sample and the corresponding activity of the protein expressed relative to that of the protein initially are indicated for each of the spectra in the figures. The spectra of the fully active protein show rather broad resonances. However, when the activity decreases to approximately half the initial value, some sharp resonances begin to appear in both the aromatic and aliphatic regions. The appearance of the sharp resonances increases progressively with time and loss

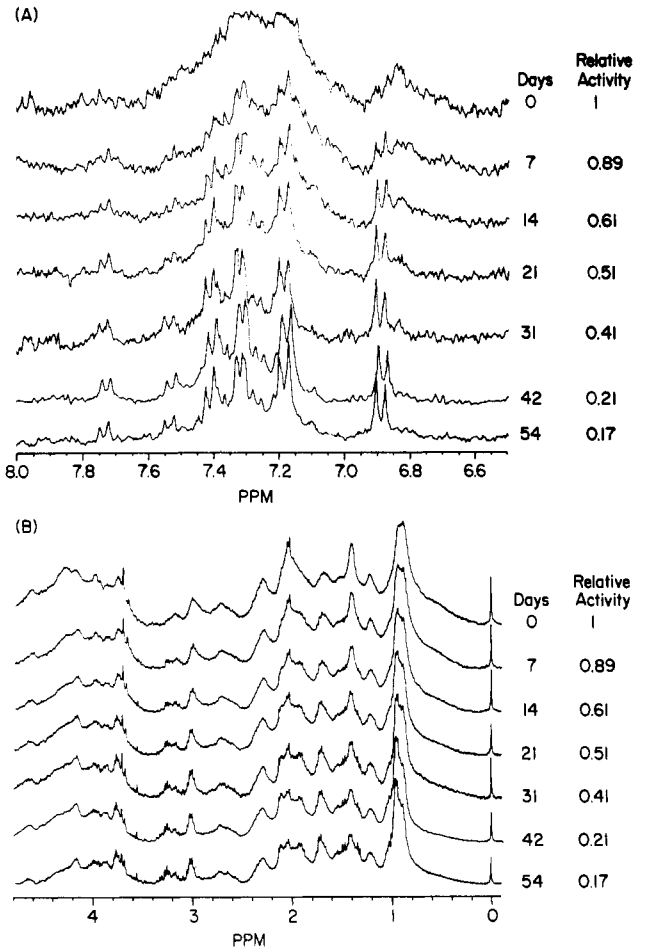

FIGURE 1: Aromatic region (A) and aliphatic region (B) of the 300-MHz ^{1}H NMR spectrum of native aequorin in the course of loss of bioluminescent activity due to Ca(II)-independent light emission at 5 °C. The time elapsed from initial preparation of the sample and the fraction of remnant bioluminescent activity are indicated. Other NMR parameters were the following: pulse length, ~9 μs; spectral width, ±2 kHz; data size, 8K; line broadening, 1 Hz; number of scans, 1124 for the initial sample and 1000 for all others. Solvent ^{1}H signals were suppressed by irradiation during a 3-s recycle delay. The sample consisted of a pH 7.8 solution of 9.8 mg/mL aequorin, 150 mM KCl, 12.5 mM Tris-d_{11}, 0.1 mM EDTA, 3 mM NaCN, and 0.2 mM TSP-d_4 in 99.96% D_2O.

of activity until spectra with a significant number of sharp features are obtained for the inactive protein in dramatic contrast with that of the initial protein. Thereafter, the spectra show no further changes.

Two questions need examination before interpretation of the data given in Figure 1A,B. One is whether the fully active protein is aggregated and that therefore the spectral changes described above arise from a loss of such aggregation with inactivation. Gel filtration data (on Sephadex G-50 in 10 mM EDTA and 25 mM HEPES at pH 7.5) of purified aequorin at a concentration of 20 mg/mL show a biphasic elution profile, one peak corresponding to monomer and the other, smaller peak to a molecular volume suggesting an aequorin dimer. No other aggregated form of the protein was evident. The presence of a small amount of dimer cannot account for the broad resonances in the NMR spectrum of the active protein. The second question is whether there was slow proteolysis of the protein over the period of time in which the spectra were recorded. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the slowly discharged aequorin showed the protein to be structurally intact. Thus, the changes in the NMR signals are not due to either initial aggregation or

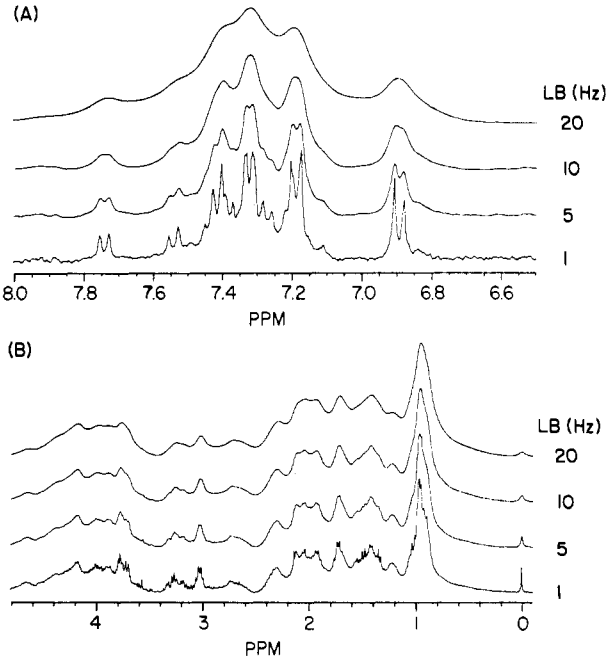

FIGURE 2: Aromatic region (A) and aliphatic region (B) of the 300-MHz ^{1}H NMR spectrum of SDP subjected to 1-, 5-, 10-, and 20-Hz line broadening (LB), respectively. The experimental conditions are the same as those given in Figure 1.

subsequent degradation of the protein but are indicative of a progressive alteration in the structure of the protein.

Mg(II) is known to inhibit Ca(II)-independent light emission by aequorin (Allen et al., 1977). The ^{1}H NMR spectra of an aequorin sample similar to that used in Figure 1 except for the presence of 2.5 mM $MgCl_2$ were recorded as a function of time (data not shown). The spectra of Mg(II)–aequorin display a close similarity to those of EDTA–aequorin as a function of protein activity although the time taken for a given loss of activity is much longer in the presence of Mg(II). The fractional activity as a function of time is exponential for both with a time constant of 30 days for EDTA–aequorin and 70 days for Mg(II)–aequorin.

The extent of line narrowing of some of the resonances in Figure 1 can be estimated by comparison with Figure 2. In Figure 2, the resonances in the proton NMR spectrum of SDP are progressively artificially broadened by the standard noise-reduction technique of multiplication of the free induction decay by an exponential function before Fourier transformation. For resonance lines of Lorentzian shape, this procedure simply increases the width of the lines by the amounts listed in Figure 2. A broadening of the SDP resonances by 20 Hz produces a spectral profile similar to that of native aequorin. The aromatic resonances of some of the tyrosine residues of SDP which occur at ~6.8 and 7.2 ppm have line widths of ~4 Hz. Assuming this width to be entirely caused by dipolar interactions between neighboring protons of the tyrosine ring (proton separation ~2.4 Å) yields an effective rotational correlation time (τ_R) of ~10^{-8} s on the basis of standard relaxation theory (Abragam, 1961). At a proton resonance frequency of 300 MHz ($\omega = 1.9 \times 10^9$ rad/s), $\omega\tau_R >> 1$, and therefore, the line width is directly proportional to τ_R. As the native protein slowly discharges, line widths of those amino acid residues that acquire segmental mobility typically decrease by a factor of ~5, indicating that the effective rotational correlation time of these tyrosyl moieties in the undischarged native protein is ~5×10^{-8} s, which is in the range of rotational correlation times expected for a protein of aequorin's size. This suggests that most of the residues have negligible

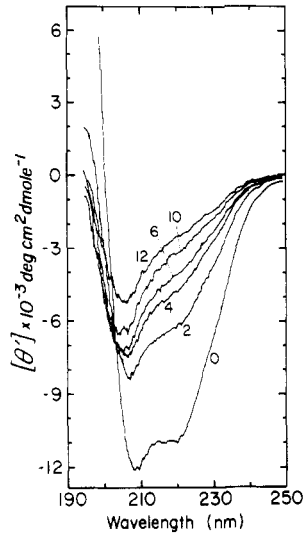

FIGURE 3: Far-UV CD spectra of aequorin during time course of inactivation by Ca(II)-independent emission at 20 °C. Each spectrum presented is the average of 16 original scans of the protein sample from which background signals due to solvent alone have been subtracted. The protein concentration was 0.15 mg/mL, and the solution contained 0.1 M sodium phosphate, 1.0 mM KCN, and 0.1 mM EDTA at pH 8.0. All experiments were conducted at 20 °C. The spectra, labeled according to the number of days from the initial preparation, show a progressive decrease in negative ellipticity at 222 nm and a concomitant increase in negative ellipticity at 206 nm as the protein discharges. The far-UV CD spectrum of the completely inactivated protein (SDP) corresponds to the spectrum labeled 12, and the secondary structure calculated for that spectrum is given in Table I.

Table I: Percentages of Apparent α-Helix, β-Sheet, and Random Structure of Various Forms of Aequorin Calculated on the Basis of Far-UV CD Spectra at 20 °C

form of aequorin	α-helix	β-sheet	random structure
native aequorin	31	20	49
BFP	28	26	46
SDP	11	22	67
apo-SDP	11	22	67

freedom of internal motion in the undischarged protein. The reduction of the effective rotational correlation time concomitant with the loss of bioluminescent activity is primarily caused by the acquisition of segmental mobility by these residues. Line-width changes in the proton NMR spectra arising from changes in secondary structure were observed in earlier studies on histones (Bradbury et al., 1975).

It should be noted that the proton NMR spectrum of the native protein (Figure 1) is not identical with that obtained by artificially broadening the SDP spectrum (Figure 2), indicating that the formation of SDP produces shifts of some of the resonances in addition to the line narrowing discussed above.

In order to examine the putative structural changes suggested by these NMR data, we measured the near- and far-UV CD spectra of aequorin under conditions similar to those employed for the NMR experiment. The far-UV CD spectrum of active aequorin given in Figure 3 shows that there is a strong negative Cotton effect which is maximal at 206 nm. An analysis of the ellipticity values in terms of α-helix, β-sheet, and random structure, given in Table I, suggests that although active aequorin has some organized secondary structure, there is substantial random-coil. Discharging the protein with Ca(II) causes only a small change in secondary structure (Table I). On the other hand, progressive inactivation of the protein by Ca(II)-independent light emission is accompanied by a marked

Table II: Peak Position, Lifetime, and Steady-State Anisotropy of Tryptophan Fluorescence and Blue Fluorescence of Various Forms of Aequorin at 25 °C[a]

form of aequorin	peak position, λ_{max} (nm)	lifetime, τ (ns)	steady-state anisotropy, r_{ss}
tryptophan fluorescence			
native aequorin	337	3.6	0.100
BFP	332	3.5	0.136
SDP	352	3.4	0.056
apo-SDP	337	3.7	0.100
blue fluorescence			
BFP	469	3.6	0.280
SDP	435	2.1	0.215

[a] The uncertainty in the r_{ss} values is ±0.001.

increase in the random form (Figure 3). The near-UV CD signal, which presumably arises from tryptophanyl moieties in the protein, is also progressively lost with time (S. Ho et al., unpublished results) which suggests either an increase in mobility of the ring(s) or a loss of μ–μ coupling (Strickland, 1974). The loss of a CD signal at 222 nm and the gain in signal at 206 nm parallel the loss of protein activity. This pattern in CD signals suggests a progressive change toward a final, largely random form of the completely inactive protein.

Among the sharp features that appear in the NMR spectra of the inactive protein are a number of doublets in the aromatic region with a separation of about 9 Hz. Such doublets occur in the spectra of aromatic protons of tyrosine and tryptophan. In addition, the suggested chemical structure of oxyluciferin contains both a *p*-hydroxyphenyl and a *p*-hydroxybenzyl moiety which must give rise to similar doublets. The appearance of these doublets with the progressive inactivation of the protein along with the fact that oxyluciferin is readily dissociable from SDP (or BFP) whereas the native chromophore is not suggests that some of the sharp resonances in the aromatic region may be due to oxyluciferin. Two other observations pertinent to the sharp features in the aromatic region are (i) these resonances represent a significantly larger number of protons than those in the aromatic moieties of the oxyluciferin alone, indicating that SDP has a number of aromatic residues which possess appreciable mobility, and (ii) some of the residues in SDP are still sufficiently immobile that discernible resonances from them are not apparent in the spectrum; e.g., no clear signals are observed for the C(2) protons of histidines in the region 7.0–8.5 ppm, implying that some structure persists in this form of the protein.[3]

The indication of significant mobility for a number of aromatic moieties in SDP raises the question whether oxyluciferin is bound to SDP. The likely answer to this question comes from examining the optical absorbance and fluorescence of aequorin in its various forms. The UV–vis absorption spectrum of SDP (data not shown) has a peak at 330 nm similar to the peak attributed to oxyluciferin bound to BFP (Shimomura & Johnson, 1969). The fluorescence results are summarized in Table II, and the spectra are given in Figure 4. In spite of differences in detail, the overall similarity of the optical absorption and fluorescence spectra of SDP and BFP strongly suggests that oxyluciferin is bound to SDP (as it is to BFP). However, the r_{ss} values for BFP are larger for both tryptophan and blue fluorescence (Table II) than they are for SDP. The red shift of the SDP tryptophan fluorescence emission and the decrease in the value of r_{ss} are clear indications of increased mobility of the tryptophan residues in SDP

[3] These resonances are observed in some denatured forms of the protein (unpublished results).

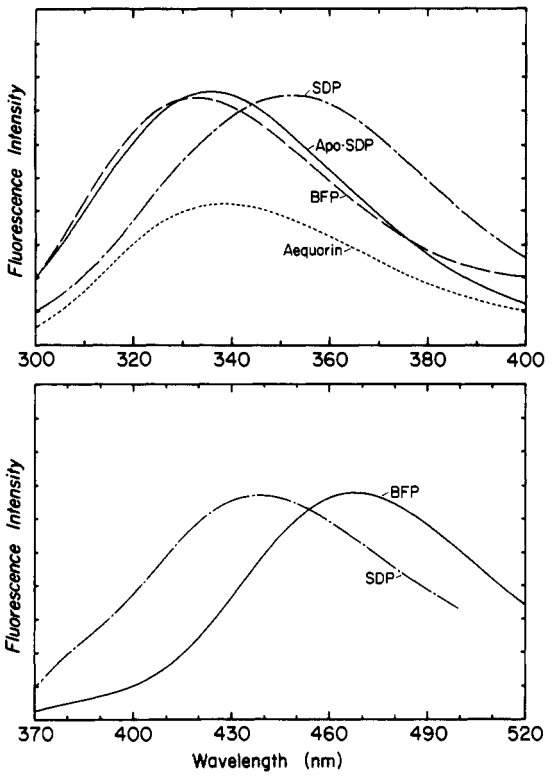

FIGURE 4: Corrected emission spectra. (Upper panel) Tryptophan fluorescence of native aequorin (---), BFP (--), SDP (-·-), and apo-SDP (—). (Lower panel) Blue fluorescence of BFP (—) and SDP (-·-). Fluorescence intensities are not shown to the same scale. Experiments were conducted at 25 °C on protein samples prepared as described in the text.

as compared with BFP. Likewise, the smaller r_{ss} value for blue fluorescence of SDP indicates that the oxyluciferin is more mobile in SDP. Nevertheless, this moderately high anisotropy of blue fluorescence of SDP shows that the oxyluciferin remains bound in SDP, albeit less tightly than it is bound to BFP. It is unclear whether the differences in the absorption and fluorescence spectral parameters between SDP and BFP arise only from conformational and motional factors or at least in part from different chromophore oxidation products.

Apo-SDP was prepared by column chromatography of SDP as described earlier. All blue fluorescence is lost from the protein since the chromophore adsorbs to the resin. The aromatic and aliphatic regions of the ^{1}H NMR spectrum of apo-SDP are shown along with those for the fully active and fully discharged protein in Figure 5A,B. The spectra of apo-SDP show a broadening of some resonances compared with those of SDP. This is a somewhat unexpected reversal of the line narrowing which accompanies the formation of SDP. The intrinsic tryptophan fluorescence of the apo-SDP shown in Figure 4 is maximal at 337 nm, the same wavelength as that of native aequorin but much lower than that of SDP (see Table II). The average anisotropy of tryptophan fluorescence in apo-SDP given in Table II was found to be 0.100, considerably higher than in SDP (0.056), indicating a significant reduction in mobility of the tryptophan residues in apo-SDP. The far-UV CD data (Table I) give essentially the same result for apo-SDP and for SDP. By comparison, BFP and native aequorin have a more ordered structure than both SDP and apo-SDP.

The line broadening observed in the NMR spectrum of apo-SDP could obtain in principle if apo-SDP were to aggregate more readily than SDP. That was, however, not the case at lower concentrations of the protein; the migration of

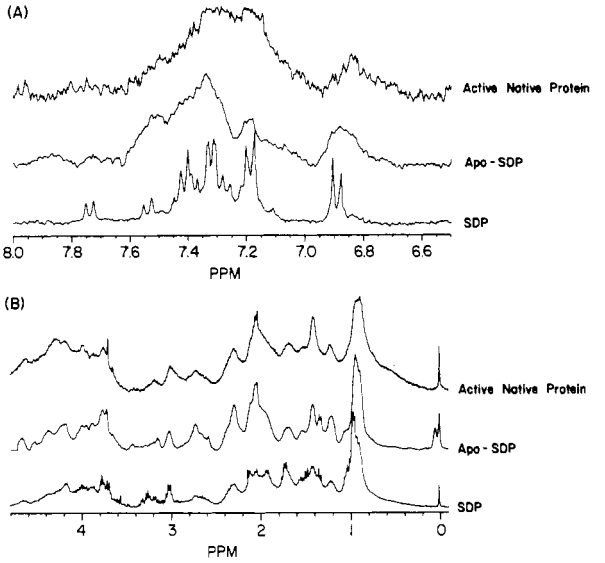

FIGURE 5: Comparison of the aromatic regions (A) and aliphatic regions (B) of the 300-MHz ^{1}H NMR spectra of native aequorin (top), apo-SDP (middle), and SDP (bottom). The NMR parameters were the same as Figure 1 but with 1124, 16808, and 808 scans (top to bottom). Samples compositions were as given in Figure 1 except that the apo-SDP sample contained 6 mg/mL protein and no EDTA.

a sample of apo-SDP (initial concentration 2 mg/mL) on a column of Sephadex G-50 suggests a Stokes radius consonant with that of a monomeric protein. The line widths of a number of resonances of apo-SDP are intermediate with respect to those of native aequorin and SDP, which means that the corresponding effective rotational correlation times have intermediate values. Thus, although the far-UV CD data indicate no detectable change in the overall percentage of random structure of the protein when apo-SDP is prepared from SDP, the NMR spectra show that the motional freedom acquired by some of the amino acid residues in SDP is partially lost upon the removal of the oxyluciferin. As discussed earlier, some of the sharp resonances in the aromatic region of the NMR spectrum of SDP (bottom of Figure 5A) arise from tyrosine and tryptophan residues. The spectrum of apo-SDP shows that these residues have lost their mobility. The shift of the tryptophan fluorescence maximum of apo-SDP back to the value for native aequorin and near to that of BFP along with the increase in the anisotropy suggests that the partial refolding of the protein that occurs with the removal of oxyluciferin sequesters the tryptophan residues from the solvent as well as diminishes the rotational mobility of these residues.

Discussion

In all physicochemical studies on aequorin in which protein solutions are likely to be stored for several days at 5 °C, Ca(II)-independent light emission is an unavoidable factor since it takes place inexorably albeit at a low level. A characterization of the protein at different stages of inactivation as a function of time is, therefore, of clear value for any investigation of the structure of aequorin and of the mechanisms mediating its bioluminescent action. The ^{1}H NMR results presented in this paper provide clear evidence that (i) as a result of Ca(II)-independent light emission, aequorin progressively changes from a conformationally rigid, fully active form to a discharged form in which a number of amino acid residues are significantly mobile, (ii) binding of Mg(II) protects aequorin from Ca(II)-independent discharge, (iii) oxyluciferin remains bound to the protein after slow discharge, and (iv) removal of oxyluciferin from SDP reduces the mobility of some of the amino acid residues. The far-UV CD data

support the inference from NMR data that the slowly discharged protein is appreciably unfolded relative to undischarged aequorin or BFP.[4] The near-UV CD spectra of SDP suggest either a complete loss of the μ–μ coupling observed in native aequorin and BFP or a marked increase in the mobility of the tryptophan residues, or both (S. Ho et al., unpublished results). The shift in the tryptophan fluorescence emission spectrum to 352 nm (from 337 nm in the native protein) suggests that the emitting tryptophan residues have become exposed to solvent which in turn would indicate a likely increase in the mobility of these residues. The fact that the anisotropy in the blue fluorescence of SDP remains high corroborates the notion that oxyluciferin is protein bound in both discharged forms although there are significant differences between SDP and BFP in terms of their fluorescence and absorption properties, their CD spectra, and their NMR spectra which merit further examination. SDP, however, is clearly a well-defined state of the protein as evidenced by the fact that EDTA–aequorin and Mg(II)–aequorin show very similar NMR spectra at similar levels of residual activity and that while sharp resonances appear with progressive inactivation, the spectrum shows no further sharpening once the discharge is complete.

The appearance of sharp resonances in the ^{1}H NMR spectrum of SDP arising from a number of amino acid residues is useful for the NMR studies of aequorin. As is clear from Figures 1 and 5, the spectrum of the native protein has broad features indicating a rather rigid conformation with little segmental mobility of individual residues. It is, therefore, not feasible to identify specific resonances with individual residues or types of residues. On the other hand, the spectrum of SDP shows a considerable number of sharp resonances that are amenable to assignment (B. D. Ray et al., unpublished results). Since SDP is a well-defined form of the protein and the Ca(II)-independent discharge has a half-life of at least 20 days at 5 °C and pH 7.8, it offers the strategy of preparing the protein at any desired level of activity, and a corresponding level of unfolding in the native structure, for the purpose of investigating other properties such as the interaction of the protein with cations, provided the results of such experiments are obtained in about 2 days (or less) during which the structure of the protein is not significantly altered.

The reversal in the appearance of sharp resonances in the ^{1}H NMR spectrum of SDP upon the removal of oxyluciferin is somewhat unexpected and is particularly striking in the aromatic region of the NMR spectrum, where the sharp resonances first begin to appear as the protein is progressively inactivated. These features are clearly relevant to the binding of oxyluciferin to SDP and possibly to the binding of the chromophore to the native protein.

The assignment of a number of resonances in the NMR spectrum of SDP will be communicated separately. The well-defined NMR spectra of SDP and apo-SDP offer the possibility of comparing these proteins with other discharged forms of the protein such as BFP and apo-BFP [with and without Ca(II)]. Furthermore, we have recently reported (Kemple et al., 1984) that modification of the essential sulfhydryl group in aequorin also results in a form of the protein in which the chromophore readily dissociates. It will be of interest to determine whether the final form of this modified protein is similar to that of apo-SDP. Metal ions or other moieties can be attached to that thiol and used to perturb the NMR spectra of nearby residues, thereby yielding assignment and geometrical information.

Although the NMR and optical spectral properties of the SDP are likely to be useful for the reasons stated above, the marked structural perturbation attending slow discharge will make certain other experiments difficult if not impossible. Ca(II)-independent light emission occurs even in lyophilized powders. Although the process is retarded by the presence of Mg(II) ions, it is incessant even with such ions present. Thus, while the X-ray crystal structure of aequorin would undoubtedly be of great value, it may well be difficult to obtain stable crystals in view of the substantial changes in the secondary and tertiary structure of aequorin attending Ca(II)-independent light emission. On the other hand, since SDP appears to partially refold upon removal of oxyluciferin, it may be that incubation with native chromophore might promote complete refolding to yield a fully "recharged" aequorin.

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Registry No. Mg, 7439-95-4; oxyluciferin (*Aequorea forskalea*), 96758-72-4.

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Adrenal Microsomal Hydroxylating System: Purification and Substrate Binding Properties of Cytochrome P-450$_{C\text{-}21}$[†]

S. Narasimhulu* and C. R. Eddy
University of Pennsylvania, Philadelphia, Pennsylvania 19104
M. Dibartolomeis, R. Kowluru, and C. R. Jefcoate
University of Wisconsin—Madison, Madison, Wisconsin 53706
Received October 30, 1984; Revised Manuscript Received March 5, 1985

ABSTRACT: The substrate–cytochrome P-450$_{C\text{-}21}$ binding reaction has been investigated in detail by using the purified cytochrome. The apparent substrate dissociation constant (K_D^{app}) depended on the enzyme concentration, indicating that the binding reaction does not follow simple two-component mass action equilibrium. However, the binding data fit reasonably well to a model in which the P-450$_{C\text{-}21}$ exists in a monomer–dimer equilibrium and the substrate does not bind to the dimer. The intrinsic dissociation constant (K_1) and the dissociation constant for the dimerization reaction (K_2) were calculated from the titration data by a pattern search procedure. K_1 and K_2 were found to be essentially independent of the enzyme concentration, indicating the appropriateness of the assumed model. In the present study, all factors that increased the dissociation of the dimer, as indicated by an increase in K_2, decreased K_D^{app} so that it approached the intrinsic constant K_1. These results suggest that there is mutual interaction of the substrate binding and self-association reactions of cytochrome P-450$_{C\text{-}21}$ in the purified preparation.

Several hydroxylation reactions are involved in the transformation of cholesterol to steroids with hormonal activity. These reactions are catalyzed by highly specific cytochrome P-450 enzymes present in the adrenal cortex. The enzymes which catalyze 11β-hydroxylation [P-450$_{11_\beta}$ (Wilson et al., 1965)] and side-chain cleavage [P-450$_{SCC}$ (Simpson & Boyd, 1966)] reactions are localized in the mitochondria. The enzymes which catalyze C-21 and C-17α hydroxylations (P-450$_{C\text{-}21}$ and P-450$_{C\text{-}17\alpha}$) and side-chain cleavage (P-450$_{lyase}$) are localized in the endoplasmic reticulum (Inano et al., 1969).

All of the adrenal cytochrome P-450 enzymes have been purified from one source or the other, and various aspects of ligand–P-450 interactions have been studied (Whysner & Harding, 1968; Cheung & Harding, 1973; Lambeth et al., 1979; Jefcoate, 1982). The cytochrome P-450$_{C\text{-}21}$ has been purified from bovine adrenocortical microsomes (Kominami et al., 1980; Bumpus & Dus, 1982) as well as pig adrenocortical microsomes (Yuan et al., 1983). Using the purified P-450$_{C\text{-}21}$ and the reductase, Kominami et al. (1984) have investigated one of the important aspects of electron transport, that is, interaction of reductase and P-450. However, interaction of substrates with purified P-450$_{C\text{-}21}$ has not yet been investigated. This is important for reasons indicated below.

Cytochrome P-450$_{C\text{-}21}$ as isolated is in the low-spin state (Kominami et al., 1980). It is generally agreed that binding of substrates to low-spin cytochrome P-450 enzymes transforming these to the high-spin state is obligatory for electron transfer (White & Coon, 1980). The low- to high-spin-state transition is reflected as the characteristic substrate-induced blue shift of the Soret absorption band (Whysner et al., 1970) designated as type I spectral change (Remmer et al., 1966). Studies (Narasimhulu, 1971a,b; Narasimhulu et al., 1966) using bovine adrenocortical microsomes had indicated that binding of type I substrates to the cytochrome P-450$_{C\text{-}21}$ is essential for electron transfer. As a result, the addition of type I substrates to bovine adrenocortical microsomes strikingly increases the extent of P-450 reduced (Narasimhulu & Eddy, 1984). Therefore, especially at concentrations below the saturation level, any changes in the substrate dissociation constant would be expected to alter the parameters of the reduction. Therefore, knowledge of factors which can alter the substrate–P-450 binding reaction is important.

Factors such as temperature and detergents were found to have a striking effect on the apparent substrate dissociation constant for P-450$_{C\text{-}21}$ in the adrenocortical microsomes

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* Address correspondence to this author at the Harrison Department of Surgical Research, University of Pennsylvania.

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