iological roles played by these collagens will be the subject of future investigations.

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Chemical and Physical Properties of Aequorin and the Green Fluorescent Protein Isolated from *Aequorea forskålea*†

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ABSTRACT: The calcium-sensitive photoprotein aequorin and the green fluorescent protein were isolated and purified from *Aequorea forskålea*. Purified aequorin shows electrophoretic microheterogeneity but appears as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Amino-terminal sequence determination by the automated Edman technique revealed a single NH_2-terminal sequence: Val-Lys-Leu-(Thr)-Pro-Asp-Phe-Asn-Asn-Pro-(−)-Trp-Ile-Gly-Arg-His-aequorin exists as a single polypeptide chain. Apparent molecular weights were determined by sedimentation equilibrium, urea–sodium dodecyl sulfate/polyacrylamide gel electrophoresis, gel filtration of native protein, and gel filtration of denatured protein in 6 M guanidine hydrochloride; all methods suggest an apparent molecular weight of 19 500 ± 2000 for aequorin. Under some conditions the protein dimerizes by disulfide bond formation. Aequorin has a sedimentation coefficient of 2.31 S and a Stokes radius of ~19 Å; the extinction coefficient ($E_{1cm}^{1\%}$) was calculated to be 27.1. Amino acid analysis revealed a slight preponderance of acidic residues; no carbohydrate moieties were found. Aequorin contains at least one free sulfhydryl group, chemical modification of which results in irreversible loss of luminescent activity. The apparent molecular weight of the green fluorescent protein is 30 000 when determined by urea–sodium dodecyl sulfate/polyacrylamide gel electrophoresis and gel filtration of denatured protein in 6 M guanidine hydrochloride. Amino acid analysis revealed an absence of tryptophan.

The jellyfish *Aequorea forskålea* exhibits a bright green bioluminescence when stimulated chemically or physically. The biochemical system responsible for the bioluminescence comprises two proteins which occur in close association in vivo, aequorin and a green fluorescent protein. Aequorin has the property of emitting light when exposed to calcium ions (Shimomura et al., 1962, 1963a,b; Shimomura & Johnson, 1969) and requires other exogenous cofactors. The light emitted by aequorin is distinctly blue, however, and the green light seen in vivo is probably a consequence of transfer of energy from aequorin to the green fluorescent protein.

The intriguing properties of this system have attracted the interest of two types of investigators, those interested in the phenomenon of bioluminescence per se and those wishing to utilize the protein as a biological calcium indicator (Blinks et al., 1976). Irrespective of specific interests, however, the physicochemical properties of the protein must clearly be known. Of these probably the most fundamental is molecular weight. Yet, despite a number of studies from several laboratories (Shimomura et al., 1962; Shimomura & Johnson, 1969; Blinks et al., 1969; Hastings & Morin, 1969; Kohama et al., 1971), there has been no agreement on the molecular weight of aequorin.

Published estimates of the molecular weight of aequorin fall into two groups: those near 20 000 and those near 30 000. Thus

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Hastings & Morin (1969) and Blinks et al. (1969) using gel filtration and $NaDodSO_4$[1]-polyacrylamide gel electrophoresis reported a value of 20 000. Shimomura & Johnson (1969) and Kohama et al. (1971), however, concluded that the molecular weight of aequorin was ~31 000 largely on the basis of data obtained from sedimentation studies, although they too had found that $NaDodSO_4$-polyacrylamide gel electrophoresis and gel filtration of native protein on Sephadex G-100 yielded an apparent molecular weight for aequorin of 20 000. Review of all the available data does not provide reasons for these disparities.

Uncertainty also exists with respect to the molecular weight of the green fluorescent protein (GFP). Morise et al. (1974) reported that $NaDodSO_4$-polyacrylamide gel electrophoresis of green fluorescent protein purified by preparative isoelectric focusing showed three main bands with apparent molecular weights of 65 000, 54 000, and 31 000. No other studies were reported and in an effort to explain these results the authors postulated that the monomer molecular weight of green fluorescent protein was ~15 000, and that the bands seen on $NaDodSO_4$-polyacrylamide gels represented polymeric forms.

In an effort to resolve the apparent uncertainties we restudied the problem with a variety of methods. Results from all our experiments show that aequorin and the green fluorescent protein are single polypeptide chains with molecular weights ~20 000 and 30 000, respectively.

Materials and Methods

Acrylamide was recrystallized from ethanol and *N,N'*-methylenebis(acrylamide) was recrystallized from *n*-hexane prior to use. Iodoacetamide, Chloramine-T, and sodium dodecyl sulfate were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiobis(nitrobenzoic acid) was purchased from Pierce Chemical Co., Rockford, Ill. $Na^{125}I$ was obtained from Amersham/Searle Radiochemicals. Urea was deionized with Bio-Rad AG-501XD. Sequencer grade 1 *N,N'*-dimethylallylamine (DMAA), phenyl isothiocyanate, heptane, 1-chlorobutane, ethyl acetate, heptafluorobutyric acid, benzene, and 4% SP400 on Chromosorb-W were purchased from Beckman Instruments, Inc., Palo Alto, Calif. *N,O*-Bis(trimethylsilyl)-acetamide and standard Pth-amino acids were obtained from Pierce Chemical Co., Rockford, Ill. Dithiothreitol was obtained from Sigma Chemical Co., St. Louis, Mo. Acetonitrile, UV, was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Mich., and was used without further purification. Water used in the preparation of sodium acetate buffers for use in the high pressure liquid chromatograph was purified on a Mili-Q four place Millipore system purchased from Millipore Corp., Bedford, Mass.

The extreme sensitivity of aequorin to even traces of Ca^{2+} requires careful preparation of all solvents to prevent Ca^{2+} contamination. Water used for experiments with aequorin in the absence of Ca^{2+} chelators was deionized, glass-distilled, and then doubly quartz-distilled and stored in a plastic carboy. In addition, solutions were passed through a column of Chelex 100 resin (200–400 mesh) prior to use. Guanidinium chloride (Ultra-Pure) was obtained from the Heico Chemical Co., Delaware Water Gap, Pa. Guanidinium chloride solutions were prepared by dissolving the solid salt in distilled water by constant stirring overnight at room temperature. The solution was then filtered through a Millipore filter and the concentration adjusted to 6 M (with water) using a refractometer.

Purification of Aequorin and Green Fluorescent Protein. Methods used for the extraction and purification of aequorin and for the assay of luminescent activity have been described briefly by Blinks et al. (1976) and will shortly be published in detail (Blinks et al., 1978). A brief account follows.

Aequorin, extracted from specimens of *Aequorea* (collected at Friday Harbor, Wash.), was purified in 10 mM EDTA (ethylenediaminetetraacetic acid) by a five-step sequence:

1. Differential ammonium sulfate precipitation (25–75% of saturation cut).
2. Gel filtration on Sephadex G-50 (fine).
3. Ion-exchange chromatography on QAE-Sephadex (A-50) with pH step and [NaCl] gradient elution.
4. Gel filtration on Sephadex G-50 (fine).
5. Ion-exchange chromatography on DEAE-Sephadex (A-50) with [NaCl] gradient elution.

As far as step 3, the green fluorescent protein copurifies with aequorin but at this point is fairly selectively eluted by the pH step. For further purification, fractions of green fluorescent protein from the DEAE-Sephadex column were pooled and then were concentrated in an Amicon ultrafiltration cell fitted with a PM 10 membrane. The concentrated sample of green fluorescent protein was then dialyzed at 4 °C against 10 mM EDTA (two changes, 3 L each). The sample was loaded onto DEAE- or QAE-Sephadex equilibrated in 10 mM EDTA, pH 5.5. A pH step elution identical with that described above was made, and the fractions with green fluorescence were pooled, concentrated to about 10 mL, and dialyzed against (four changes, ~1 L each) 10 mM ammonium acetate, pH 6.0, with washed Chelex-100 beads placed outside the dialysis tubing. The solution of protein was then lyophilized. The lyophilized protein appeared as a highly fluorescent green solid, which when redissolved still showed significant residual bioluminescent activity. Final purification of the green protein was achieved by preparative polyacrylamide gel electrophoresis in a home-made apparatus.

Electrophoretic Studies. $NaDodSO_4$-polyacrylamide gel electrophoresis was carried out with the technique of Weber & Osborn (1969); urea-$NaDodSO_4$ gel electrophoresis was performed with the technique of Swank & Munkres (1971) as modified by Downing et al. (1975). Coomassie blue stain was used for the $NaDodSO_4$ or urea-$NaDodSO_4$ gels; gels were destained electrophoretically. For molecular weight determinations all gels were scanned for light absorption by the stain at 560 nm with a Gilford Model 24105 gel scanner. For the detection of ^{125}I in urea-$NaDodSO_4$ gels, each gel was cut transversely into approximately 100 slices and a Beckman LS-100C γ counter was used for determination of radioactivity.

Sedimentation Equilibrium. Studies were performed as described by Yphantis (1964) with a Beckman Spinco Model E ultracentrifuge equipped with RITC and Rayleigh interference optics. An AN-D rotor was used for all experiments. Rayleigh interference patterns were photographed on Kodak type IIG spectroscopic plates. For all sedimentation equilibrium studies, a double sector cell fitted with sapphire windows was used; experiments were done at 22 °C.

Sedimentation equilibrium determinations were performed on native aequorin and on aequorin in denaturing solvent. For experiments with native protein, samples were prepared as follows: purified, lyophilized aequorin was dissolved in 0.1 M NaCl, 0.01 M sodium EDTA, pH 6.0, to a final concentration of 0.10 to 0.20 mg/mL by weight and the sample dialyzed vs. solvent in the cold (4 °C) for at least 24 h. The denaturing solvent used for sedimentation equilibrium studies was 6 M guanidinium chloride made 0.1 M in 2-ME; the 6 M guani-

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; GFP, green fluorescent protein; DMAA, *N,N'*-dimethylallylamine; DNP, dinitrophenyl; Nbs_2, 5,5'-dinitrobis(2-nitrobenzoic acid).

dinium chloride solution was prepared in the manner described above. Samples of lyophilized protein were dissolved in appropriate volumes of 6 M guanidinium chloride, 0.1 M in 2-ME, and then dialyzed against solvent at room temperature in a flask sealed with Parafilm for 24 h. In all experiments the dialyzing solution was used as reference solvent. The experiments were performed with 3-mm columns of solution and samples were analyzed at a minimum of two rotor speeds. The plot of the natural logarithm of the fringe displacement (expressed as ln c where c refers to concentration) vs. the square of the radial position (r^2) was linear for all runs. Molecular weights were calculated as described by Yphantis (1964). When sedimentation studies were performed with native, undischarged aequorin, the sample taken from the cell at the end of the experiment was assayed for luminescent activity.

Partial specific volumes of the proteins were calculated from the amino acid composition (see below) by the method of Cohn & Edsall (1941). However, it is apparent that a chromophore is presumably present in aequorin and must contribute to the partial specific volume. The structure of the chromophore in aequorin has been reported (Shimomura & Johnson, 1972; Hori et al., 1975, 1977; Ward & Cormier, 1975). We assumed that only 1 mol of chromophore is bound per mol of aequorin and the approximate contribution of the chromophore to specific volume of the protein was then calculated by the method of Cohn et al. (1934) and Cohn & Edsall (1941).

Determination of Sedimentation Coefficient. The sedimentation coefficient of aequorin was measured in a Beckman Spinco Model E ultracentrifuge equipped with RITC and UV absorption optics. Since aequorin has a high extinction coefficient ($E_{1cm}^{1\%} = 27.1$), sedimentation velocities for very low aequorin concentrations could easily be determined with absorption optics. Thus, a solution of aequorin (100 μg/mL) was dialyzed vs. 0.1 M NaCl, 0.1 M NaCl, 0.020 M EDTA at 4 °C for 24 h; its sedimentation velocity was determined by ultracentrifugation at 60 000 rpm in a double sector cell equipped with sapphire windows. The sedimentation coefficient (s_{obsd}) was then determined as described by Chervenka (1970). An extrapolated sedimentation coefficient (s^0) value was not obtained by determination of the concentration dependence of the sedimentation velocity. However, at a concentration of 100 μg/mL of protein, it is unlikely that significant protein–protein interaction would occur during transport. Consequently, s_{obsd} was used to calculate a corrected sedimentation coefficient ($s_{20,w}^0$).

Molecular Weight by Gel Filtration of Denatured Protein. The apparent molecular weights of the reduced and carboxamidomethylated proteins were also determined by gel filtration in 6 M guanidinium chloride. Samples of protein were either radioiodinated or labeled with a fluorescein derivative. Radioiodination was effected by the method of Greenwood et al. (1963); the protein samples were then dialyzed against 10 mM ammonium acetate, 0.1 mM EDTA (eight changes, 1 L each) for 24 h. Finally, the solutions were lyophilized and the dry protein subsequently dissolved in 1 mL of 6 M guanidinium chloride and reduced and carboxamidomethylated by the method of Gurd (1967). After the addition of blue dextran (void volume marker), dinitrophenylalanine (DNP-alanine, internal volume marker), and sucrose (to increase the density of the solution), 100 μL of the radioiodinated solution was chromatographed on a column (1.5 × 87 cm) of Sepharose 6 B (lot 120C-0030) equilibrated with 6 M guanidinium chloride (Mann & Fish, 1972). Alternatively, protein samples were labeled with fluorescein (Mann & Fish, 1972). The apparent molecular weight was obtained in the manner suggested by Ackers (1967) and Mann & Fish (1972).

Fractions of the protein eluted from the gel filtration column were pooled and dialyzed extensively against 0.2 M acetic acid (six changes, 2 L each, in 24 h) to remove all traces of guanidinium chloride. Aliquots were then tested for homogeneity and apparent molecular size by urea–$NaDodSO_4$/polyacrylamide gel electrophoresis.

Gel filtration of native aequorin was performed on Sephadex G-100 and Bio-Gel P-100. A 1 × 100 cm column of Sephadex G-100 was equilibrated with 0.1 M NaCl, 0.01 M EDTA, pH 5.5, and this solution was used as eluent. Protein samples were dissolved in eluent (0.2 mL) and 60 mg of sucrose added for increased density; 1.2-mL fractions were collected. The elution of aequorin was followed by measurements of absorbance at 280 nm and by assay of luminescent activity. Similar experiments were performed on a column of Bio-Gel P-100 equilibrated in 0.10 M NaCl, 0.20 M EDTA, pH 6.8. The positions of all peaks were determined with respect to the cumulative weight of the fractions collected. Glycine was used as a marker for the determination of the internal volume (V_i) of the column and its elution position was determined by the fluorescence of a fluorescamine derivative.

Determination of Extinction Coefficient. Protein concentration was determined by drying a weighed solution of aequorin (of known absorbance at 280 nm) to constant weight and also by the synthetic boundary method of Babul & Stellwagen (1969). The data from the latter method were not corrected for the contribution of the chromophore of aequorin to the measured molar refractivity.

Calculation of the Diffusion Coefficient. The diffusion coefficient was calculated in two ways, by use of the Svedberg and Stokes–Einstein equations, respectively. The Stokes radius, $a°$, used in the latter calculation was estimated by gel filtration on a Sephadex G-100 column calibrated with proteins of known Stokes radius.

Amino Acid Analysis and NH_2-Terminal Amino Acid Sequence Determination of Aequorin. Protein samples were hydrolyzed in evacuated tubes with 6 M HCl for 6, 24, 48, and 72 h. Cysteine was determined as cysteic acid after performic acid oxidation (Moore, 1963). The number of free sulfhydryl groups was determined in the native and Ca^{2+}-discharged photoprotein by titration with Nbs_2 (according to Habeeb, 1972); a value of 13 000 was used for the molar extinction coefficient of Nbs_2. The tryptophan content of apoaequorin was determined by the method of Edelhoch (1967). Tryptophan values were also determined according to the method of Liu & Chang (1971). Glucose was assayed enzymatically by the method of Bergmeyer et al. (1974) (Table I).

Approximately 8 mg of purified lyophilized aequorin (a mixed sample from *Aequorea* purified in each of 3 years) was dissolved in 1.0 mL of Ca^{2+}-free 6 M guanidinium chloride. The protein in guanidinium chloride was reduced and carboxamidomethylated. Apoaequorin was then prepared by dialysis vs. 50 volumes of 6 M guanidinium chloride (two changes, 8 h) which removes the chromophore and finally against 10 mM ammonium acetate (eight changes, 1000 volumes, over 24 h). The reduced and carboxamidomethylated apoaequorin precipitated out of solution essentially quantitatively and was collected by centrifugation, lyophilized, and stored as a fine white powder. Lyophilization of the supernatant produced negligible residue. Two hundred nanomoles (approximately 6 mg) of apoprotein so prepared was dissolved in 0.6 mL of 50% acetic acid. NH_2-terminal amino acid analysis was then performed by the automated method of Edman & Begg (1967) using a Beckman Model 890C automated protein sequencer. Sequencing methods used in this laboratory are discussed in detail by Downing et al. (1975).

TABLE I: Amino Acid Composition of Aequorin and Green Fluorescent Protein (GFP) per 20 000 Molecular Weight.

amino acid[a]	amino acid/aequorin (mol/mol) this paper[b]	Shimomura & Johnson[c]	amino acid/GFP (mol/mol) this paper[d]	Morise et al.[e]
Lys	14	13	22	23
His	4	4	11	11
Arg	8	6	7	8
Asp	24	24	32	37
Thr	8	8	19	19
Ser	10	8	10	11
Glu	35	24	28	30
Pro	6	8	14	14
Gly	16	15	27	27
Ala	10	12	14	14
Val	7	8	20	19
Met	4	4	4	6
Ile	8	9	18	15
Leu	8	12	24	21
Tyr	6	7	12	13
Phe	7	8	14	14
Trp[f]	5	5		
Trp[g]	(4.8)			
½-cystines[h]	3	3		3.22
cysteines[h]	1	1		
glucose		3		
amino sugar				

[a] The composition was determined from 6, 24, 48, and 72 h hydrolysates. [b] Calculated on the basis of molecular weight 20 000. [c] Calculated from the data of Shimomura & Johnson (1969) on the basis of molecular weight of 20 000. [d] Calculated on the basis of molecular weight of 30 000. [e] Calculated from the data of Morise et al. (1974) on the basis of molecular weight of 30 000. [f] Determined by the method of Liu & Chang (1971). [g] Determined by the method of Edelhoch (1967). [h] Determined as cysteic acid and by titration with Nbs_2 (Habeeb, 1972).

Residues were identified as phenylthiohydantoins by high pressure liquid chromatography (Downing & Mann, 1976) and by gas liquid chromatographic analysis.

Results

Electrophoretic Studies. The results of urea–$NaDodSO_4$ electrophoretic studies on aequorin and the green fluorescent protein are shown in Figure 1. Figure 1 is a photograph of a urea–$NaDodSO_4$/polyacrylamide gel of reduced and carboxamidomethylated (standard) proteins of known molecular weight and of an overloaded urea–$NaDodSO_4$ gel of an aequorin preparation. First, the gels of the aequorin preparation show that the protein samples applied were relatively free of impurities; densitometric analysis indicated that less than 2% of the protein was outside the main band. Second, the gels show that, when aequorin is run under nonreducing conditions, two bands are obtained, one with an apparent molecular weight of 20 000 and the other an apparent molecular weight of 37 000 (Figures 1b,c). When the protein, prepared in the same way, is run in the presence of 2-ME (Figure 1e) or is run as a reduced and carboxamidomethylated sample (Figure 1f), then only a band corresponding to an apparent molecular weight of ~20 000 is observed. From this one may conclude that intermolecular disulfide bridging may occur during sample preparation and may result in appearance of a higher molecular weight species of aequorin. (It is noteworthy that the data from sedimentation analysis gave no indication of dimerization either when native or when denatured protein was examined.) Additionally, the sample of aequorin used for the gels shown in Figure 1e had been shown to comprise at least six electrophoretically heterogeneous species, yet appeared as a single band on urea–$NaDodSO_4$ gels (Figure 1e). This indicates that all the microheterogeneous species of aequorin have very similar apparent molecular weights.

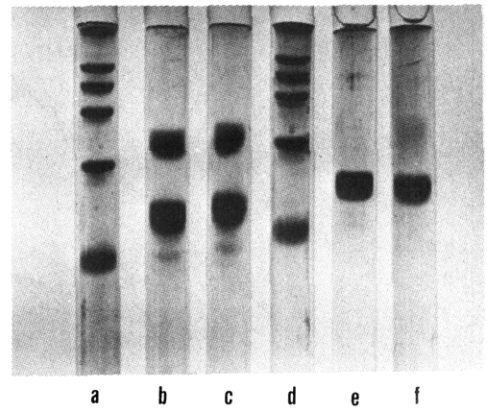

FIGURE 1: Urea–$NaDodSO_4$ polyacrylamide gel electrophoresis of aequorin. (a) Standard proteins: the proteins used were bovine serum albumin, catalase, ovalbumin, carbonic anhydrase, and cytochrome *d*. All had been reduced and carboxamidomethylated: a relates to gels b and c. (b) Aequorin treated with 2-ME and heated to 90 °C in urea–$NaDodSO_4$ sample preparation buffer, but not run in the presence of 2-ME. (c) Aequorin prepared by incubation at 37 °C overnight in urea–$NaDodSO_4$ sample preparation buffer but without addition of reducing agents. (d) Standard proteins: relate to gels e and f. (e and f) Aequorin prepared for electrophoresis by incubation in urea–$NaDodSO_4$ sample preparation buffer at 37 °C in the presence of 2-ME (e) or after reduction and carboxamidomethylation (f). The samples were also run in the presence of 2-ME.

Urea–$NaDodSO_4$ gels of the green fluorescent protein showed only a single band of protein of apparent molecular weight 30 000. If the protein was not heated to 90 °C during sample preparation, the electrophoretic pattern often showed two or three bands with apparent molecular weights greater than 30 000. Although the green fluorescent protein is known to exhibit electrophoretic microheterogeneity, urea–$NaDodSO_4$ gel electrophoresis of a mixture or of isolated isospecies of green fluorescent protein species gave a single band.

Sedimentation Equilibrium and Velocity Studies. Sedimentation equilibrium studies performed on aequorin in denaturing solvents and on native aequorin dissolved in aqueous salt solutions yielded values for the molecular weight which were essentially identical—19 500 and 19 000, respectively. Figure 2 illustrates the results obtained in one such experiment. The data indicate the homogeneity of the preparation and indicate, moreover, that aequorin is a single polypeptide chain.

The very small differences in the value of $\bar{\nu}$ caused by the contribution of the chromophore (0.729 without and 0.731 with the chromophore) clearly do not significantly affect the apparent molecular weight of aequorin derived from sedimentation equilibrium studies in nondenaturing solvent systems.

The sedimentation coefficient of aequorin was determined at a single concentration (100 μg/mL) and found to be 2.31, a value significantly less than that of Shimomura & Johnson (1969). The data did not suggest the existence of dimers at this concentration.

Gel filtration studies were performed on reduced and carboxamidomethylated aequorin and green fluorescent protein under denaturing conditions and the data yielded apparent molecular weights of 20 000 and 30 000, respectively, for these two proteins (in 6 M guanidinium hydrochloride). These values were obtained whatever method was used for labeling and detection of protein.

Gel filtration of active aequorin on columns of Sephadex

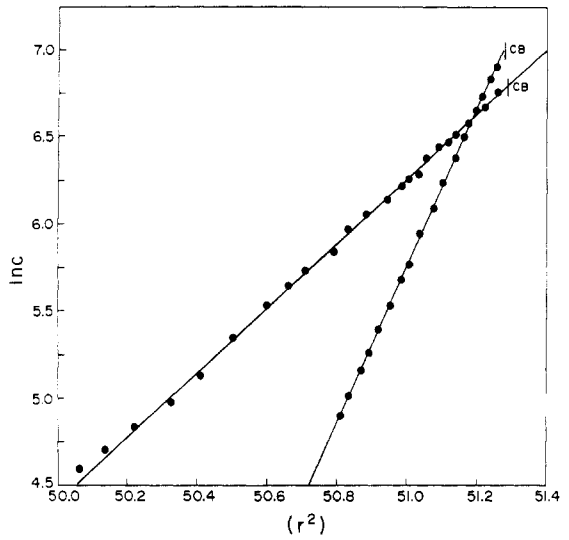

FIGURE 2: Molecular weight determination by high speed equilibrium sedimentation centrifugation in 6 M guanidinium chloride. The natural logarithm of the concentration (ln c) is plotted on a linear scale against the square of the distance from the center of rotation (r^2).

TABLE II: Summary of Physical Properties of Aequorin.

Molecular weight		
1. Sedimentation equilibrium		
Native	19 000	
Denatured	19 500	
2. $NaDodSO_4$ gel electrophoresis	20 000	
3. Gel filtration (native)		
Bio-Gel P-100	19 000	
Sephadex G-100	18 800	
4. Gel filtration denatured	20 800	
Sedimentation coefficient (s)	2.31×10^{13} (2.95)[a]	
Extinction coefficient ($E_{1cm}^{1\%}$) at 280 nm	27.1	
Molar extinction coefficient (ϵ, L mol^{-1} cm^{-1})	54 000 (83 000)[b]	
Frictional ratio (f/f_{min})	1.21	
Diffusion coefficient	9.4×10^{-7}	cm^2/s[c]
	8.8×10^{-7}	cm^2/s[d]

[a] From Shimomura & Johnson (1969). [b] Calculated from the data of Shimomura & Johnson (1969) based on a molecular weight of 20 000. [c] Calculated from the Svedberg equation (see text). [d] Calculated by the Einstein equation (see text).

G-100 and Bio-Gel P-100 yielded very similar values for molecular weight, 18 800 and 19 000. In order to rule out the possibility of adsorption of aequorin to Sephadex beads through hydrogen bonding to the hydroxyl backbone of the gel, experiments were also performed in the presence of (100 mM) glucose; glucose did not alter the elution volumes of the proteins. Data from these studies were used to estimate the Stokes radius of aequorin; this was found to be about 19 Å.

Other Physical Constants. The calculated values of diffusion coefficient ($D°$) for aequorin were 9.4 and 8.8×10^{-7} cm^2 s^{-1}, respectively (Table II). The frictional ratio (f/f_{min}) was calculated (Tanford, 1961) to be 1.21 which suggests that aequorin behaves hydrodynamically like a globular protein. The extinction coefficient ($E_{1cm}^{1\%}$) at 280 nm derived by dry weights was 27.1 while that derived by the synthetic boundary method was 25.6. The difference may, in part, reflect the influence of the chromophore on the refractive index increment measured in the synthetic boundary method of Babul & Stellwagen (1969).

Amino Acid Composition and NH_2-Terminal Amino Acid Sequence. For the most part our estimates of the amino acid composition of aequorin and the green fluorescent protein given in Table I agree well with those of Shimomura & Johnson (also given in Table I, but calculated for molecular weights of 20 000 and 30 000 for aequorin and green fluorescent protein, respectively). In contrast to the report of Shimomura & Johnson (1969), we found no evidence that aequorin was a glycoprotein. Analysis for free sulfhydryl groups using Nbs_2 suggested at least one free sulfhydryl group in native aequorin. The most noticeable feature of the amino acid composition of green fluorescent protein was the lack of tryptophan and cysteinyl residues.

The NH_2-terminal amino acid sequence for apoaequorin was found to be Val-Lys-Leu-(Thr)-Pro-Asp-Phe-Asn-Asn-Pro-(−)-Trp-Ile-Gly-Arg-His. (If the holoprotein was used instead of apoaequorin there was considerable difficulty in distinguishing between the chromophore, and presumably derivatives of the chromophore, and the phenylthiohydantoin derivatives of the NH_2-terminal amino acids.) The sequence was derived from three separate runs on samples of aequorin purified from jellyfish caught in 1972, 1974, and 1975; identical results were obtained in each case.

Discussion

Studies on native and denatured samples of aequorin and the green fluorescent protein have consistently yielded the same estimates for the apparent molecular weights of these proteins. We therefore conclude that the molecular weight of aequorin is approximately 20 000 and of the green protein approximately 30 000. (A summary of the physical properties of the proteins is given in Table II.) Both proteins appear to be single polypeptide chains. The results obtained from sedimentation studies, urea-$NaDodSO_4$ gel electrophoresis and NH_2-terminal amino acid sequence determination indicate that the proteins employed in these studies were homogenous. Further, it is clear that the microheterogeneity of aequorin and green fluorescent protein does not reflect differences in apparent molecular weight. The reasons for the disparities between our results and those of Shimomura & Johnson (1969) and Kohama et al. (1971) are not apparent. Possible explanations may derive from the tendency of aequorin to aggregate or the tendency to form dimers through disulfide bond formation.

Both of these explanations seem unlikely for the following reasons: first, the concentrations of proteins used for sedimentation analysis by Shimomura & Johnson (1969), Kohama et al. (1971), and by us in this study were sufficiently low that aggregation should not have occurred; second, dimer formation seems to occur only in the presence of sodium dodecyl sulfate. In view of the differences between our estimate of the molecular weight of aequorin and the estimates of Shimomura & Johnson (1969) and Kohama et al. (1971), the other physical properties of aequorin that we report (whose values are dependent on the molecular weights of the protein) are inevitably different from those reported by Shimomura & Johnson (1969) and Kohama et al. (1971) (Table II).

At least one free sulfhydryl group is a major feature of the amino acid composition; chemical modification of this sulfhydryl group results in total loss of bioluminescent activity (Shimomura & Johnson, 1969; Prendergast, unpublished data). The NH_2-terminal sequence does not reveal any special features but does show that the electrophoretic microheterogeneity of aequorin is probably not due to structurally different species of protein.

The only data hitherto published regarding the physical

characteristics of the green fluorescent protein of aequorin are those of Morise et al. (1974). Our value of 30 000 for the molecular weight of the green fluorescent protein corresponds with one of the estimates quoted by Morise et al. (1974) who found apparent molecular weight values of 31 000, 54 000, and 65 000. Morise et al. have suggested the possibility that the green fluorescent protein might have a monomer molecular weight of ~16 000 in order to explain the values they obtained. We have not been able to find any evidence of a subunit of size *less than* 30 000, but on several occasions we have seen bands on $NaDodSO_4$ gel electrophoresis corresponding to species of greater molecular weight. In general, it would appear that unless the sample of green fluorescent protein is heated, preferably in a sample preparation solution containing urea in addition to $NaDodSO_4$, polymeric forms of the green protein which are relatively resistant to the effects of $NaDodSO_4$ may occur. It seems probable that in our preparations these problems were circumvented by use of 6 M urea in the sample preparation buffer which effectively prevented any association reactions.

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