Molecular Weight of the Photoprotein Aequorin*

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ABSTRACT: Aequorin was studied by means of sedimentation analysis, gel filtration, gel electrophoresis, and functional group analysis. Except for gel filtration through Sephadex G-100 and sodium dodecyl sulfate gel electrophoresis, which indicated molecular weights between 15,000 and 23,000, the data from all other methods indicated a molecular weight of 31,000 ± 1000, and a one to one ratio of functional groups to aequorin molecules, with no evidence of subunits. Aggregation of the native protein, however, was shown to occur at rates which, though negligible at pH 9, become significant and faster as the pH is lowered, especially below pH 6.5. The rate was also faster at 20° than at 7°. Reversal of aggregation occurred at pH 9.0–9.5. After aequorin had been denatured by heat, sodium dodecyl sulfate, or urea, or after it had undergone the light-emitting reaction triggered by Ca^{2+} yielding the blue fluorescent substance "BFP," the protein product in each case showed a tendency to dimerize. The dimerization as well as aggregation could be inhibited or reversed by *p*-benzoquinone.

The photoprotein aequorin, isolated from the luminescent jellyfish *Aequorea* (Shimomura *et al.*, 1962, 1963), emits light by an intramolecular reaction when a trace amount of Ca^{2+} is added. Molecular weights of the protein have been previously reported as 18,300–23,000 by gel filtration (Hastings and Morin, 1968; Shimomura and Johnson, 1969; Blinks *et al.*, 1969; Hastings *et al.*, 1969), 32,000 from amino acid analysis, and 30,000 from sedimentation–diffusion (Shimomura and Johnson, 1969). Because of the obvious importance of establishing the correct molecular weight, as well as the ratio between functional groups and aequorin molecules, especially in reference to quantitative aspects of the as yet obscure mechanism of the light-emitting reaction, additional methods have been undertaken as described in this paper. These methods include gel filtration on Bio-Gel P-100 as well as Sephadex G-100, polyacrylamide gel electrophoresis, sedimentation equilibrium, and functional group analyses. Among them, it was soon found that sedimentation equilibrium analysis under various conditions often indicated a molecular weight of 38,000 or higher. This result suggested the possibility of a slow aggregation, which seemed all the more reasonable in view of the much longer period of time (*ca.* 24 hr) required by this method than by the previously employed sedimentation diffusion method (<2 hr). Moreover, the newly tried method of sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated a value of 20,000, approximately the same as had Sephadex G-100. However, the results of both the previous study and of the further experiments reported herewith, while not providing conclusive evidence from some points of view, are predominantly in favor of first, a molecular weight of 31,000±1000, second, the nonexistence of subunits, and third, a one to one ratio of functional groups to aequorin molecules.

Materials and Methods

Aequorin was extracted and purified as previously reported (Shimomura and Johnson, 1969, 1970). Chemicals used in this study were: cysteine, Na_2EDTA, glycylglycine, Tris (all from Sigma Chemical Co.), β-mercaptoethanol, *p*-benzoquinone (Matheson Coleman & Bell), sodium dodecyl sulfate (Mann Research Laboratories), Sephadex G-25 and G-100 (Pharmacia Fine Chemicals, Inc.), and Bio-Gel P-100 (Bio-Rad Laboratories). All other chemicals were ACS grade or the best grade commercially available. *p*-Benzoquinone was recrystallized from water, and ether and chloroform were always redistilled before use.

Sedimentation experiments were carried out with a Spinco Model E ultracentrifuge equipped with schlieren optics. The sample solutions of aequorin were prepared by dialysis or by gel filtration with Sephadex G-25 (fine). Sedimentation equilibrium analyses were performed by the method of Schachman (1957). The data apply to complete equilibrium which was usually attained after more than 20-hr centrifugation.

Gel filtrations were carried out with Bio-Gel P-100, 100–200 mesh, or Sephadex G-100 in a column 1.6 × 40–80 cm. Approximately 5 mg (5 ± 1 mg) of protein in 1 ml of buffer was added to the column in each case. The following buffered solutions were used: (1) 0.02 M sodium acetate containing 0.01 M EDTA and 0.2 M NaCl (pH 6.0), (2) 0.02 M glycylglycine–NaOH containing 0.01 M EDTA and 0.2 M NaCl (pH 9.0), (3) 8 M urea containing 0.02 M sodium acetate–0.01 M EDTA and 0.1% β-mercaptoethanol (pH 6.0), and (4) 0.1% sodium dodecyl sulfate containing 0.02 M Tris-HCl–0.01 M EDTA–0.2 M NaCl–0.01% β-mercaptoethanol (pH 7.5). The first two buffers were used at 3°, and the last two were used at 25°. Each column was calibrated with ribonuclease, chymotrypsinogen A, ovalbumin (all from Pharmacia Fine Chemicals, Inc.), and bovine serum albumin (Schwarz–Mann). When buffer system 3 or 4 was employed, aequorin and the standard proteins were kept in the same buffer overnight at 25° before application to the column. The elution curves were measured by OD at 280 nm, and, for native aequorin, the OD elution curve was supplemented by measurements of bioluminescence activity.

Gel electrophoresis with 7% polyacrylamide gel at pH 9.5 was carried out using apparatus and chemicals obtained from Canal Industrial Corp., Rockville, Md. EDTA (5 mM) was included in all solutions, persulfate was replaced by riboflavin, and electrophoresis was run in a cold room. Usually, 20 μg of protein was applied for each tube.

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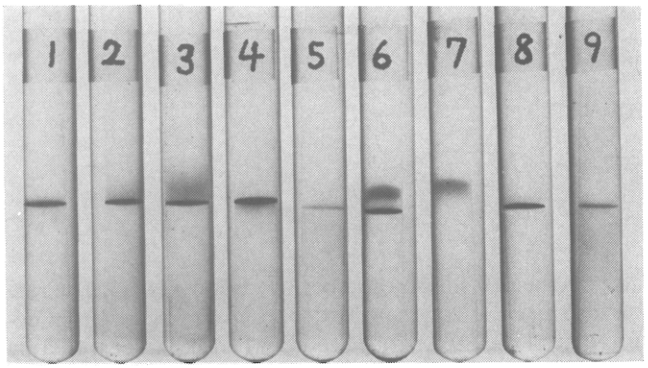

FIGURE 1: Polyacrylamide gel electrophoresis of aequorin under various conditions. The specimen of aequorin was from the same lot as used for Figures 2 and 3. Electrophoresis was at pH 9.5, at 3°. Top to bottom, cathode to anode. (1) Native aequorin without pretreatment; (2) 24 hr at 3° in pH 6.0 buffer; (3) 24 hr at 20° in pH 6.0 buffer; (4) 24 hr at 3° in pH 9.5 buffer; (5) BFP freshly prepared; (6) BFP, 24 hr at 20° in pH 6.7 buffer; (7) aequorin, 24 hr in 8 M urea containing 0.1% mercaptoethanol at 20°; (8) sample 6, 2 hr after addition of 1 mM *p*-benzoquinone; (9) sample 7, 2 hr after addition of 1 mM *p*-benzoquinone.

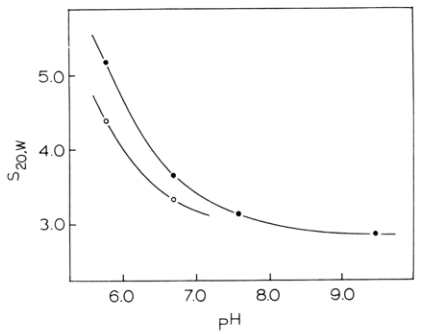

FIGURE 2: The influence of pH on the sedimentation constant of aequorin, 6 mg/ml, at 7° (open circles) and at 20° (solid circles). The measurements at 59,780 rpm, which started 3 hr after preparing the solutions, took approximately 2 hr. The buffers used were 0.01 M sodium acetate (pH 5.8), 0.01 M sodium phosphate (pH 6.7), 0.01 M Tris-HCl (pH 7.6), and 0.01 M glycylglycine–NaOH (pH 9.5), each containing 0.01 M EDTA and 0.1 M NaCl. Broadening of the peak was notable when s exceeded 4. At the highest pH, the symmetrical gaussian curve typical of homogeneous protein solutions was obtained.

The molecular weight determination by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was kindly performed by Dr. H. Ozaki, Department of Zoology, Michigan State University, in his laboratory. It was carried out by the procedure of Weber and Osborn (1969) on a 10% gel, using insulin, cytochrome *c*, trypsin, ovalbumin, and catalase as the standards.

In order to determine the content of the functional group "AF-350,"[1] the splitting from the protein was done by an improved method. Aequorin[2] (83 mg) in 12.5 ml of 10 mM Tris buffer containing 10 mM EDTA and 5 mM cysteine (pH 7.0) was treated with 12.5 g of urea (Ultra Pure from Mann Research Laboratories) at room temperature for 2 hr with occasional stirring. After dilution of the mixture with 12.5 ml of water, AF-350 was separated out by extracting four times with 1-butanol. The combined butanol extracts were mixed with an equal volume of ether, and the butanol–ether layer was washed with water three times. The combined water layers were reextracted with butanol three times, and the combined butanol extract, after mixing with an equal volume of ether, was washed with water three times. The water layer was discarded. The first and second extracts in butanol–ether were combined and evaporated to dryness under vacuum at 40°. AF-350 in the residue was purified by two successive chromatographies on silicic acid (1 × 17 cm column, Silic AR CC-7, 200–325 mesh, Mallinckrodt Chemical Works), with water-saturated ether as the solvent. The AF-350 band was identified by its strong, blue fluorescence under uv light. The final eluate containing AF-350 was mixed with two volumes of chloroform, then dried with anhydrous Na_2SO_4, and finally evaporated to dryness under vacuum at 40°, leaving purified AF-350 as the residue.

Results and Discussion

In the interests of critically interpreting the data, it is perhaps useful to emphasize at the start that the aequorin used in all experiments was very highly purified and that all the solutions of this protein were initially homogeneous according to polyacrylamide gel electrophoresis as illustrated in Figure 1 (tube 1). In one phenomenon, *viz.*, that of aggregation, however, some quantitative variations occurred, depending on the long term as well as immediate history of the preparation.

Aggregation Phenomena. In our earlier study there was evidence of aggregation after keeping aequorin in solution for as much as 20 hr after dissolving the protein in buffer, from the stockpile routinely preserved in saturated ammonium sulfate plus EDTA at −30°. At the time of the earlier study, aggregation was not a significant factor in the experiments involved. The nature and longer duration of certain experiments in the present study as well as a faster rate of aggregation found in some preparations, made it necessary to take the possibility of aggregation into account, and to investigate some of the factors which by influencing the rate influenced the amount formed in a given period of time under specified conditions.

Repeated experiments showed that aggregation is sensitive to pH. Typical data are illustrated in Figure 2, showing that the rate, which is practically negligible at pH 9 or 9.5 (*cf.* Figure 1, tube 4), becomes appreciable as the pH is lowered and increases rapidly in the region below about pH 6.5. The rate of aggregation is evidently faster also at 20° than at 7°. While some variations in rates occurred depending on the history of the preparations used in repeated experiments, the important fact was found that, in every instance, the sedimentation constant approached a value of 2.9 as a minimum at pH 9.5 which is the same value reported previously for pH 5.8 at 3 and 20°, based on measurements made *immediately* after preparing the protein solution from the stored material (Shimomura and Johnson, 1969).

[1] Abbreviations used are: BFP, blue fluorescent protein, the product of the bioluminescence reaction of aequorin; AF-350, the functional chromophore group split off from aequorin by urea treatment, having an uv absorption maximum at 350 nm and a blue fluorescence (Shimomura and Johnson, 1969).

[2] The bioluminescence of aequorin is believed to be an intramolecular reaction in which at least two groups on the same protein molecule are involved (Shimomura *et al.*, 1962). One of the reactive groups incorporates a chromophore which, after the bioluminescence reaction, becomes highly blue fluorescent, and a group containing this chromophore can be isolated; we have designated it "AF-350" (Shimomura and Johnson, 1969, 1970). No information is available at present for the counterpart group which supposedly reacts with the chromophore group.

TABLE I: Molecular Weight of Aequorin by Various Methods.

Amino acid analysis		32,000[a]
Sedimentation–diffusion		30,000[a]
Sedimentation equilibrium		30,300
Gel filtration		
With Bio-Gel P-100		31,000
With Sephadex G-100		23,000
With Sephadex[b]		18,300[c]
		21,000[d]
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis		
	95%	20,000
	5%	15,000
Functional group (AF-350) analysis		30,000

[a] Shimomura and Johnson (1969). [b] The type of Sephadex used by these authors for molecular weight determinations was not stated. [c] Hastings and Morin (1968). [d] Blinks *et al.* (1969); Hastings *et al.* (1969).

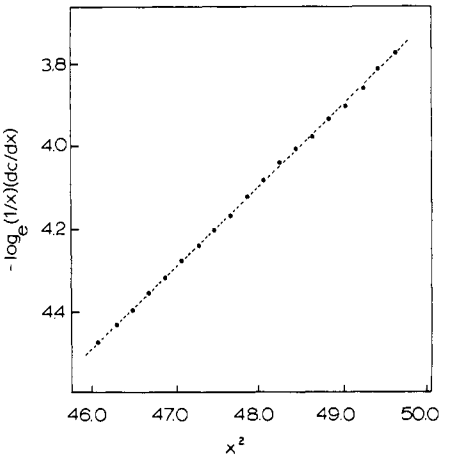

FIGURE 3: Sedimentation equilibrium data of aequorin, 6 mg/ml, in 0.01 M glycylglycine containing 0.01 M EDTA and 0.1 M NaCl, pH 9.5, at 7°, after 20.5 hr at 10,485 rpm. Bar angle: 75°.

Aggregation did not affect the light-emitting capacity of aequorin. Moreover, aggregation could be largely reversed by adjusting the pH of the solution to between 9.0 and 9.5. Tubes 2 and 3, of Figure 1 show that the protein after having aggregated at pH 6 dissociates, *i.e.*, disaggregates (except for a small part of the aggregated protein which had formed at 20°), at the high pH involved in electrophoresis.

Sedimentation Equilibrium. In view of the foregoing results, the experiments directed at determination of the molecular weight by the sedimentation equilibrium method were carried out with solutions at pH 9.5. Under these conditions, homogeneity of the protein was attested not only by polyacrylamide gel electrophoresis at this pH, but also by the pattern of sedimentation at 59,780 rpm. The results, illustrated in Figure 3, indicate a molecular weight of 30,300; the straight line gives proof again of homogeneity of the material. A partial specific volume of 0.729 was used in the calculation as before (Shimomura and Johnson, 1969).

Gel Filtration of Aequorin. The results with Bio-Gel P-100 indicated a molecular weight of 31,000 for native aequorin in acetate buffer at pH 6.0 and in glycylglycine buffer at pH 9.0, both at 3°. The results obtained with Sephadex (18,300–23,000) by several investigators are in pronounced contrast to this figure (Table I). At the lower pH but not at pH 9.0, spontaneous aggregation occurred, causing a spreading out, but *no change in position* of the main peak. The amount of aggregation in this instance was reduced apparently because of a slower rate of the process in the gel as compared to the rate in a liquid solution.

The discrepancy in the results obtained with Bio-Gel P-100 and Sephadex G-100 is difficult to explain. A satisfactory explanation would apparently involve not only more detailed information concerning the structure and properties of aequorin, but also a fuller understanding than is presently available concerning the theory of gel filtrations. Even so, some experiments were conducted in an effort to find a clue as to the cause of the discrepancy. Thus, suspecting an unusual interaction between aequorin and Sephadex, a polysaccharide, but not between aequorin and Bio-Gel, an acrylamide, the disaccharide sucrose was included in the elution buffers on the premise that it might influence the behavior of the protein in the process of filtration through Sephadex. Experiments showed, however, that the presence of sucrose had no influence on the behavior of aequorin on columns of either Sephadex or Bio-Gel, nor was there any evidence of adsorption of aequorin by itself on Sephadex G-25 (fine or superfine). These results suggest that the difference in behavior on the two types of gels resides in spatial rather than in the chemically interacting properties of the molecules involved.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The results of this method indicated the presence of two species of molecules, one in a major and the other in a relatively minor amount. The molecular weight of the former seemed 20,000 and the latter 15,000, in amounts (as computed from densitometric data) of 95 and 5%, respectively. These molecular weights, for reasons that are not clear, are much closer to the results obtained by filtration through Sephadex G-100 than by filtration through Bio-Gel P-100 and by the other methods described.

Dimerization by Urea, Sodium Dodecyl Sulfate, and Heat. Gel filtration of solutions of aequorin in 8 M urea through Bio-Gel P-100 revealed three peaks, namely, mol wt 63,000 (88% of protein), mol wt 30,000 (12%), and a low molecular weight, blue fluorescent compound. This third compound was identified as AF-350 (mol wt 277; Shimomura and Johnson, 1969). Gel electrophoresis of urea-treated aequorin revealed a strong but slightly broadened band on the cathode side of the native aequorin band (Figure 1, tube 7). This strong band must correspond to the protein of mol wt 63,000, so we will call it a "dimer band." Gel filtration through Bio-Gel P-100 in 0.1% sodium dodecyl sulfate buffer gave two protein peaks without splitting off AF-350, namely, mol wt 60,000 (75%) and mol wt 32,000 (25%).

The product of the bioluminescence reaction, *i.e.*, the blue fluorescent protein BFP, without added denaturant behaved almost exactly the same as native aequorin in gel filtration and in gel electrophoresis; however, after leaving the solution some time, the BFP dimerized (Figure 1, tubes 5 and 6), whereas aequorin under the same conditions showed merely a reversible aggregation.

When gel electrophoresis of native aequorin was carried out at an ambient temperature of 25° instead of 3°, a distinct dimer band was observed. This result is most likely due to heat denaturation caused by the ohmic heat which raises the temperature of the gel probably to 40° or higher.

When aequorin is denatured, a blue fluorescence appears due either to the denatured protein itself (as in the case of sodium dodecyl sulfate), or to a moiety split from the native protein (as in the case of urea), indicating that a reaction chemically similar to the bioluminescence reaction has taken place in both instances. Considering that the native aequorin did not form a dimer, and that the urea-denatured aequorin gave rise to a dimer accompanied by the splitting off of AF-350, it follows that the group involved in fluorescence is not involved in dimerization. Moreover, a counterpart group which persists after light emission or after denaturation seems to be essential for dimerization. Further, it was found that the aggregation and the dimerization described so far were all inhibited and even reversed by the addition of 1 mM *p*-benzoquinone (Figure 1, tubes 8 and 9), thereby resulting, in the subsequent gel electrophoresis, in a sharp single band corresponding to the band of native aequorin (Figure 1, tube 1). This is an interesting result because benzoquinone was already known to be a very powerful inhibitor of the bioluminescence of this photoprotein (Shimomura *et al.*, 1962). In addition, we have recently found that AF-350 contains a *p*-hydroxyphenyl group and in the native aequorin this group is probably in the quinoid form (unpublished data). Neither β-mercaptoethanol, nor *n*-heptyl alcohol which increases the total photon emission of purified aequorin, had any influence on aggregation or dimerization.

Protein Functional Group Ratio. The splitting off of the functional chromophore from 83 mg of aequorin, after purification as described, yielded 0.68 mg of AF-350, mol wt 277. The loss in weight as impurities during fractionation of AF-350 was 11% of the total, as judged by uv absorbancy at 350 nm. Assuming that the splitting was complete and that no decomposition occurred in the course of purification, the molecular weight of the protein computed on the basis of a single functional chromophore group amounts to 30,000. This result is consistent with the molecular weight of aequorin according to the bulk of the evidence from the various methods discussed above. It would then follow that one molecule of aequorin contains only one group of functional chromophore.

Absence of Subunits. Finally, throughout the experiments described in this paper, no evidence was encountered for the existence of subunits in the aequorin molecule of molecular weight close to 31,000, except possibly for the seemingly equivocal results obtained with gel filtration on Sephadex G-100 and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which have yielded values for molecular weight ranging between 15,000 and 23,000. These values however, are not strongly suggestive of the possibility of two equal subunits combining to give a molecule of mol wt 31,000. Moreover, account should be taken of the fact that if the sedimentation constant of 2.9 S is correct (Shimomura and Johnson, 1969, and present data), the 15,000–23,000 molecular weight range is impossible. Thus if any choice as to the most likely correct molecular weight is to be made on the basis of the total evidence available, a range of 15,000–23,000, from Sephadex gel filtration and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, seems outweighed by the value 31,000 ± 1000, from Bio-Gel P-100 filtration, amino acid composition, analyses of data pertaining to sedimentation and diffusion, sedimentation equilibrium, and the ratio of quantity of functional group to quantity of protein.

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