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9. Supported by ONR (Nonr 2216-23), ACS (PRF 2068-A2), and NSF (GB 5262). I thank M. N. A. Peterson for inviting me to participate in the Spheres Expedition during which I made the experiments and for advice and discussion. F. B. Phleger criticized the manuscript, and W. R. Riedel provided the radiolarian sediment. Discussions with G. R. Heath and T. C. Moore were helpful.

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Chaetopterus Photoprotein: Crystallization and Cofactor Requirements for Bioluminescence

Abstract. *The* Chaetopterus *photoprotein has been isolated in an amorphous form (molecular weight, 120,000) which in* $(NH_4)_2SO_4$ *solution converts to a crystalline form (molecular weight, 184,000) having the same specific light-emitting activity; quantum yields are 0.0093 and 0.0143, respectively. Two new cofactor requirements have been separated from impure extracts: a macromolecule resembling a nucleoprotein, and a lipid-like substance.*

Bioluminescence of extracts of *Chaetopterus variopedatus* requires molecular oxygen, a hydroperoxide, ferrous iron, and a specific protein (*1*). Because the total light was proportional to the amount of the purified protein and because no diffusible substrate, in the usual sense, appeared to be involved, the general term photoprotein was suggested for this type of system, as distinct from the classical luciferin-luciferase type that characterizes most of the different bioluminescence systems yet successfully extracted (*2*). Two other photoprotein systems are now known: those of certain jellyfish (*3*) and of euphausid shrimps (*4*). Each of the three systems requires a specific protein to which the total light is related, but the specific proteins, as well as cofactors required for light emission, are different in all three.

Essentially final purification of the *Chaetopterus* photoprotein (CP) has shown that it can exist in at least two forms: one amorphous (CPA), which in solution of ammonium sulfate converts to crystalline (CPC). Two new cofactor requirements for luminescence have been separated from impure extracts: cofactors 1 and 2; the latter (or a substance with the same effect) occurs also in impure preparations of hyaluronidase. Our methods and results were as follows.

The 12th segment was cut from live specimens and immediately stored with dry ice. Batches of 100 g of the frozen material were homogenized for 4 minutes in a mason jar, the receptacle of a Teflon-bladed Omnimixer containing glass beads, 500 ml of saturated ammonium sulfate plus 40 g of undissolved ammonium sulfate, and a trace of $NaHCO_3$. The homogenate was squeezed through rayon gauze and centrifuged, and the supernatant was discarded.

The precipitate and floating material were stirred into a 500-ml mixture of 0.0002*M* oxine (8-hydroxyquinoline) and 0.01*M* tris, *p*H 7.2, giving an extremely viscous mixture that was allowed to stand 1 hour, during which time the viscosity decreased. The mixture was centrifuged, the precipitate was discarded, and 200 g of ammonium sulfate was added to the supernatant. The precipitate that formed was removed by centrifugation and combined with similarly prepared precipitates from two to three additional batches.

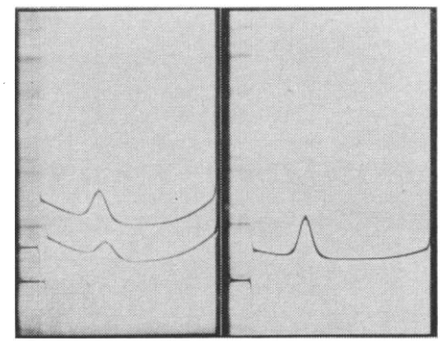

Fig. 1. Ultracentrifugal patterns of (left) the amorphous form (CPA) of *Chaetopterus* photoprotein at two different concentrations at 4.5°C and (right) the crystalline form (CPC) at 2.4°C. Speed, 59,780 rev/min; time, 64 minutes (CPC), 80 minutes (CPA).

The combined precipitates were dissolved in a 200-ml mixture of 0.0002*M* oxine and 0.01*M* sodium phosphate, *p*H 7.0, and dialyzed for 1.5 hours against the same mixture of oxine and buffer.

The dialyzed material was centrifuged and the precipitate was discarded. The supernatant was filtered through a column of DEAE-cellulose (3.5 by 15 cm), and the filtrate was subjected to fractional precipitation by ammonium sulfate; the fraction that came down at 0.3- to 0.5-saturation was saved. After this step, all buffer solutions were mixtures of 0.0001*M* oxine and 0.01*M* sodium phosphate, *p*H 6.0, which we shall call "buffer."

The fractionally precipitated material was dissolved in 50 ml of buffer, dialyzed against buffer, and then adsorbed on a column of DEAE-cellulose (3.5 by 15 cm). Stepwise elution by graded concentrations of NaCl gave a CP fraction at about 0.2*M* NaCl and a cloudy cofactor fraction at about 0.5*M* NaCl. The latter was further purified by DEAE-cellulose chromatography with gradient NaCl elution, the active material (cofactor 1) being eluted at 0.3*M* NaCl. It proved to be undialyzable and heat-resistant (about 50 percent loss of activity in 1 minute at 100°C); it was almost colorless, with an ultraviolet-absorption maximum at 278 mμ (optical density, 0.65), a minimum at 268 mμ (O.D., 0.63), and a shoulder at 255 mμ (O.D., 1.02), suggestive of a nucleoprotein. A slight blue fluorescence appeared under ultraviolet illumination. The active material is acidic, judged by its strong adsorption on DEAE-cellulose. Relatively low concentrations sufficed for maximum activation of luminescence.

The eluate containing the photoprotein (CP) was further purified by Sephadex G-200 chromatography in buffer containing 0.5*M* NaCl, followed by saturation with ammonium sulfate and by centrifugation. The precipitate (CPA) was dissolved in a small amount of buffer, and the solution revealed a single peak in the analytical ultracentrifuge (Fig. 1). Ammonium sulfate was then added to produce slight cloudiness. In the course of several hours the crystalline photoprotein (CPC) formed (Fig. 2). After recrystallization this material also gave a single peak in the ultracentrifuge (Fig. 1). The s_{20} for CPA was calculated at 4.16×10^{-13}; for CPC, 5.73×10^{-13}. From the same centrifugal data, D_{20} was calculated, by

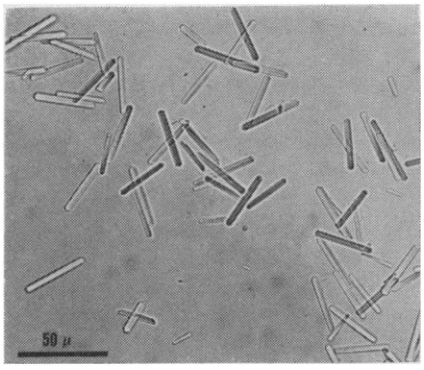

Fig. 2. Thrice recrystallized photoprotein (CPC) of *Chaetopterus*.

use of area-height and extrapolated zero time, as 3.04×10^{-7} for CPA and 2.80×10^{-7} for CPC.

From these data molecular weights of 128,000 for CPA and 184,000 for CPC were calculated. In all calculations the partial specific volume was assumed to be 0.73. Determination of molecular weight by Sephadex G-200 gave 110,000 for CPA. Both proteins were colorless and heat-labile, giving no evidence of peroxidase activity. One milliliter of solution having an optical density of 1.0 per centimeter at 280 mμ contained 0.48 mg of protein, dry weight.

In regard to specific requirements for bright luminescence, before separation of CP and cofactor 1 the only diffusible factors appeared to be Fe^{++}, O_2, and a hydroperoxide such as those that form spontaneously during storage of dioxane or tetrahydrofuran (*1*). We have found that tetrahydropyrane hydroperoxide, ethyl hydroperoxide, and dicumyl hydroperoxide have almost no activity in this system. With increasing purification of CP, luminescence activity progressively decreased in the presence of O_2 and optimum concentrations of cofactor 1, Fe^{++}, and dioxane. The activity could be restored, however, by addition of either a small amount of impure extract or (better) 0.1 to 0.2 mg/ml of an impure hyaluronidase preparation. With the purest photoprotein material the activation of luminescence in this way was more than 50-fold. The fact that more-highly purified hyaluronidase preparations gave less activation of luminescence indicated that the activating factor (cofactor 2) occurs as an impurity.

While its chemical nature remains unknown, cofactor 2 is heat-stable, undialyzable, resistant to acid and alkali, and soluble in 2:1 mixture of acetone and water. Perhaps it is a lipid. The activation mechanism appears to involve protection of photoprotein and cofactor 1 from nonluminescent destruction by Fe^{++} and peroxide, judged by progressive loss in activity during incubation in the absence of sufficient cofactor 2, and also by data on total light. The previously reported (*1*) catalytic activity of spent photoprotein solutions on the luminescence reaction of unspent solutions is most likely attributable to the presence in the former of cofactor 1 or 2, or both.

The kinetics of light-emission is subject to complex variation with varying proportions of components in the reaction mixture. The maximum total light is practically the same, however, for a given amount of CPA and CPC, with optimum amounts of the other components in each instance. Moreover the fact that under given conditions the maximum total light is proportional to the amount of photoprotein over a wide range of concentrations justifies the term photoprotein (*1*, *4*), although this relation does not hold with suboptimum concentrations of cofactor 2.

The maximum total light elicited from 0.043 mg of CPA or CPC at 25°C was 2×10^{12} photons. If one assumes the molecular weights of CPA and CPC to be 120,000 and 184,000, respectively, the quantum yields, or (number of photons emitted) : (number of photoprotein molecules reacted), amount to 0.0093 for the amorphous form and 0.0143 for the crystalline form. Since it is clear that the amorphous form (CPA) converts to the crystalline form in solution of ammonium sulfate, the ratio of molecular weights suggests that CPA is a dimer and CPC is a trimer of a monomeric form that is probably the most reactive form, inasmuch as we found by reaction kinetics that CPC is less reactive than CPA.

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Ribosome Biogenesis: Nonrandom Addition of Structural Proteins to 50S Subunits

Abstract. *Assemblage of structural proteins into 50S subunits was examined in* Escherichia coli *recovering from chloramphenicol treatment. Cells previously labeled with H^3-leucine for three generations were incubated for 30 minutes with chloramphenicol. Proteins synthesized during the initial 5 minutes of recovery from chloramphenicol treatment were labeled with C^{14}-leucine. Marked variation in the ratios of C^{14}- to H^3-leucine in ribosomal protein occurred in cells that had been treated with chloramphenicol; untreated cells displayed little variation. The results suggest that ribosomal proteins are assembled into 50S subunits in a nonrandom manner.*

The studies of Waller (*1*), Leboy *et al.* (*2*), and Traub *et al.* (*3*) suggested that 70*S* ribosomes contain 30 structural proteins. Proteins extracted from 50*S* particles can be separated by polyacrylamide-gel electrophoresis into 20 different bands, while from the 30*S* particles 10 or 11 distinct bands can be distinguished. Little is known concerning the assemblage of these various proteins into ribosome particles. A number of cases exist in which bacterial cells previously incubated under conditions of inhibited protein synthesis preferentially synthesize ribosomal protein when protein formation is allowed to resume. *Escherichia coli* synthesized these structural proteins at a greater rate than soluble protein during recovery from chloramphenicol treatment