

The Riboflavin Flavoprotein from Egg Yolk

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A flavoprotein with riboflavin as the prosthetic group has been isolated from the soluble fraction of egg yolk. The complex is a glycoprotein with a molecular weight of 37 000. It proved to contain one molecule of riboflavin per one molecule of protein. Absorption maxima of the flavoprotein were found to lie at 276, 375 and 458 m μ . The protein-bound riboflavin is incapable of reducing on the dropping electrode. The dissociation constant of the complex as established on the basis of fluorometric measurements equals 2.65×10^{-9} M.

Recently, flavoproteins with riboflavin as the prosthetic group, have been reported to occur in avian egg white¹ and yolk². The riboflavin proteins are incapable of oxidizing reduced pyridine nucleotides¹. They resemble the avidin-biotin system and probably constitute a storage form of vitamin B₂ for developing embryo. In this paper some properties of a flavoprotein as isolated from egg yolk have been described. A preliminary account on the subject was published earlier².

EXPERIMENTAL

Preparation procedures and properties

Partially saturated flavoprotein was prepared from fresh hen eggs according to the method described previously². To completely saturate apoprotein with riboflavin, the original preparation was dialyzed in 0.05 M Na-phosphate buffer, pH 7.0, containing 39.5 μ g riboflavin per ml, followed by dialysis against several changes of distilled water. The dialysis residue was then lyophilized and the resulting product was used for analysis.

The flavoprotein proved to comprise about 10 μ g riboflavin per mg protein as determined both microbiologically³ and spectrophotometrically⁴. It gave a single peak in the ultracentrifuge (Fig. 1.). The molecular weight calculated by Ehrenberg's modification of the Archibald approach to sedimentation equilibrium method⁵ amounted to 37 400. The minimum molecular weight calculated on the basis of the riboflavin content gave approximately the same result. The preparation proved to be homogeneous also when checked with starch gel electrophoresis in the range of pH from 4.5 to 8.5 and density gradient electrophoresis

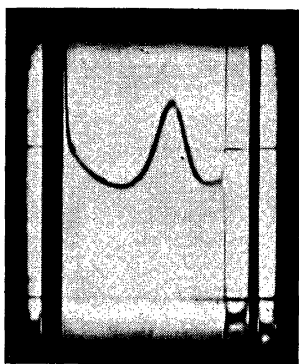


Fig. 1. Ultracentrifuge pattern of egg yolk flavoprotein. The sedimentation measurements were made with a Spinco model E ultracentrifuge at a rotor speed of 59 780 rpm at 20°C. 1% flavoprotein in Na-phosphate buffer, pH 7.0, $\mu = 0.2$ was used. The exposure was made after 92 min of sedimentation with a phase plate angle of 30°.

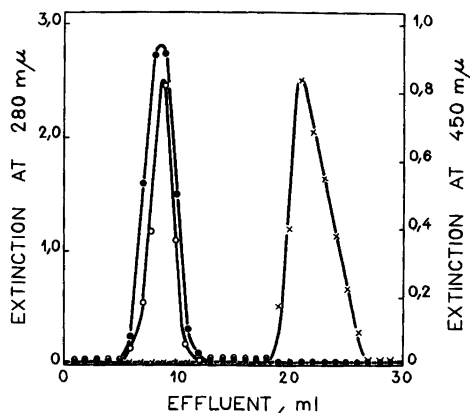


Fig. 2. Gel filtration of the flavoprotein on Sephadex G-25. 10 mg in one ml was applied to the column (1.4 × 18 cm) and eluted with 0.1 M citrate buffer, pH 3.0 at a rate of 5 ml/hr. O—O absorbancy at 280 mμ, X—X at 450 mμ, ●—● absorbancy at 450 mμ when flavoprotein was filtrated at pH 7.0 (0.05 M phosphate buffer); 3°C.

at pH 5.0. The pure substance is readily soluble in water and in salt solutions in a wide range of pH. It contains 13% N, 0.2% P and about 8% hexoses. Neutral flavoprotein solution is very resistant to heat as it neither precipitates nor dissociates flavin on boiling for 30 minutes.

The riboflavin-free protein was prepared by passing the flavoprotein through a Sephadex G-25 column (Pharmacia, Uppsala). The column was equilibrated and eluted with 0.1 M citrate buffer of pH 3.0. At this pH riboflavin undergoes dissociation and entirely separates from the protein component while in the range of pH 3.5 to 8.0 the complex appears in one peak as the excluded solute (Fig. 2.). Fractions containing riboflavin were pooled together, extracted with butanol and dried in vacuo. Further purification of riboflavin involved paper chromatography with the use of saturated water isoamyl alcohol⁶, as a solvent. The yellow spot which migrated in parallel to the reference spot of authentic riboflavin was then eluted from the chromatograms with water and resulting solution evaporated in vacuo.

The so obtained flavin was further identified by paper electrophoresis at pH 5.1 and 8.0⁷ and microbiologically³. Moreover, the purified material was analyzed in the Unicam SP-100 IR-spectrophotometer (Cambridge, England) using a KBr pellet. The spectrum was found essentially identical with synthetic riboflavin, showing characteristic absorption bands at frequencies 1248, 1181, 1080 and 851 cm^{-1} ⁸. All these results have demonstrated that free riboflavin is the prosthetic group.

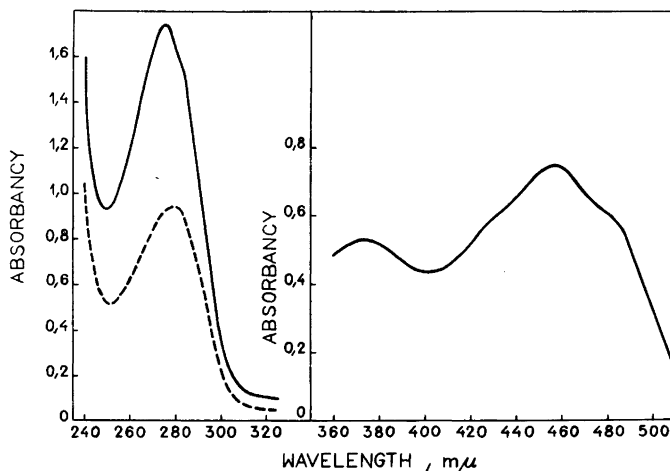


Fig. 3. Absorption spectra of the pure flavoprotein in 0.05 M Na-phosphate buffer at pH 7.0. Protein concentrations were 3.0 and 1.0 mg per ml, respectively, for obtaining visible (360 to 510 $m\mu$) and ultraviolet (240 to 320 $m\mu$) spectra. Dotted line depicts the UV spectrum of apoprotein prepared by gel filtration (one mg per ml, pH 7.0).

The absorption spectrum of the flavoprotein at pH 7.0 is shown in Fig. 3. There occur three maxima: at 276, 375, and 457–459 $m\mu$ with the shoulder at about 485 $m\mu$. The ratio of extinction at 276 $m\mu$ to that at 458 is 6.5. The molar extinction coefficient as determined from the absorption at 276 $m\mu$ and the molecular weight is 6×10^7 cm^2/M . The apoprotein reveals only one peak at 280 $m\mu$ with an extinction coefficient by about 45 % lower than that of the flavoprotein.

Polarographic titrations

Titration were performed in the polarograph type PO3 (Radiometer, Copenhagen) in a 2 ml cell adapted for deaeration with N_2 . The potential of the dropping electrode was referred to a saturated calomel reference electrode. The riboflavin solution in 0.05 M Na-phosphate buffer, pH 7.0 was polarographed in the range from -0.2 to -0.8 V and the diffusion current was measured by the height of polarographic waves⁹.

Lingane and Davis¹⁰ have shown that riboflavin being reduced at neutral pH gives a well defined polarographic wave with $\pi/2$ -potential of -0.47 V. In our studies we have observed that protein-bound riboflavin is no longer able to reduce on the dropping electrode. Since the cathodic diffusion current was proportional to concentrations of the free riboflavin undergoing reduction, the stoichiometry of the reaction: riboflavin–protein could readily be followed in the polarograph. Quantitative studies were made by titrating the apoprotein solution with riboflavin under standard conditions. A typical experiment is shown in Fig. 4. Curve A represents a blank titration in which increasing amounts of riboflavin were added in a final volume of 2 ml to the polarographic cell, Curve B illustrates the course of titration of 0.016 μM apoprotein under the same

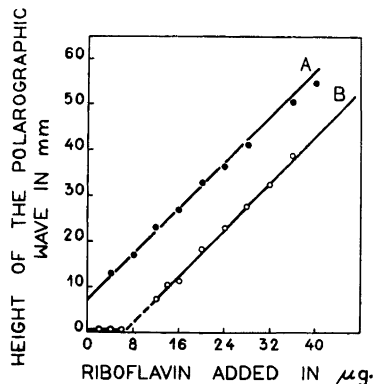


Fig. 4. Polarographic titrations of apoprotein with riboflavin solution. A — blank titration, B — titration of $0.016 \mu\text{M}$ of apoprotein. The solution of the riboflavin was added to the polarographic cell by means of an "Agla" microburette. Trace of silicone (Dow Corning Co., USA) was added to the solution as the anti-foam agent.

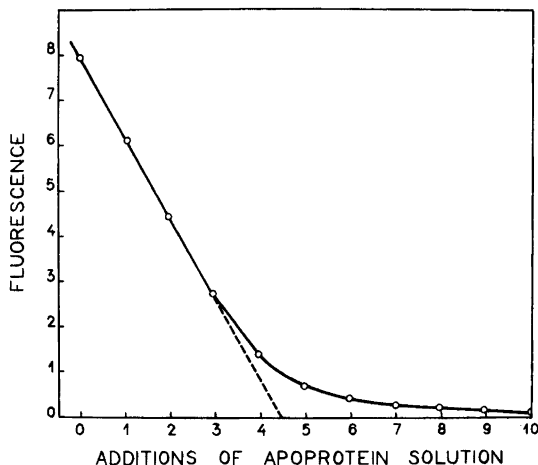


Fig. 5. Fluorometric titrations of riboflavin with partially saturated flavoprotein. To the 2 ml volume of 0.05 M Na-phosphate buffer, pH 7.0, containing $4.06 \times 10^{-4} \mu\text{M}$ of riboflavin, $2 \mu\text{l}$ portions of flavoprotein solution were added. Each addition represents $3.46 \mu\text{g}$ of free apoprotein. Excited at $373 \text{ m}\mu$, fluorescence measured at $530 \text{ m}\mu$.

conditions. From the breaking point of latter curve the amount of riboflavin binding sites could be derived. It was found, that $0.016 \mu\text{M}$ apoprotein combined with $6.3 \mu\text{g}$ riboflavin which gives the molar ratio of the two components as 1 : 1.

Fluorometric titrations

In this set of experiments the riboflavin was titrated with a solution of partially saturated flavoprotein at neutral pH by following the disappearance of fluorescence. Fluorometric titrations were carried out with a spectrofluorometer constructed by Theorell¹¹. All determinations were done at 23.5°C in 0.05 M Na-phosphate buffer of pH 7.0. Fig. 5 shows the corresponding titration curve. Since the apoprotein solution was saturated with riboflavin in 69% of its maximal binding capacity, 4.4 additions were necessary to bind $4.06 \times 10^{-4} \mu\text{M}$ riboflavin present in the spectrophotometric cell. Admitting the molecular weight to be 37 000 one gets one molecule riboflavin per one molecule flavoprotein. Identical results were obtained by reversed titration, *e. g.* when a pure apoprotein solution was titrated with riboflavin.

From the fluorometric titration the dissociation constant of the complex can be calculated according to the formula:

$$K_{AR} = \frac{[A_F][R_F]}{[AR]}$$

where AR indicates flavoprotein, A_F and R_F indicate free apoprotein and free

riboflavin, respectively. Provided the complex did not fluoresce and the solution of riboflavin was devoid of any fluorescing impurities, the values of dissociation constant as established on the basis of fluorometrical measurements for several points of a curve varied between 2.06 and 2.94×10^{-9} M with a mean value of 2.65×10^{-9} M, at pH 7.0 and 23.5°C. This figure is of the order of magnitude reported for dissociation constants of numerous flavin enzymes and suggests that riboflavin is very strongly bound to the protein moiety.

DISCUSSION

Following the publication of Rhodes *et al.*¹, the present paper provides further evidence for the occurrence of natural flavoproteins in which riboflavin rather than a riboflavin nucleotide is the prosthetic group. In fact, attempts to conjugate the isolated apoprotein with either FMN or FAD were negative in as much as the resulting complex could readily be displaced by free riboflavin¹.

Like other flavoproteins, that from egg yolk completely quenches the fluorescence of riboflavin, shifting its absorption bands at 373 and 445 m μ toward greater wave length. As anticipated, the protein-bound riboflavin was unable to reduce on the dropping electrode. This property may be taken advantage of for further polarographic studies. The behaviour of the yolk flavoprotein on the dropping electrode points to the concomitant reduction of riboflavin at both N (1) and N (10) of the isoalloxazine ring. The quenching effect is probably due to the formation of a linkage between N (3) and tyrosine residue of the protein^{12,13}. According to Rhodes *et al.*¹ the ribitol group at N (9) also is essential for the formation of a flavoprotein complex. It appears then, that the conjugation of a flavin with protein involves several groups of the isoalloxazine ring, the structure of which specifically conforms to the binding sites of the protein moiety.

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