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ARTICLE

Determination of Holo- and Apo-Riboflavin Binding Protein in Avian Egg Whites through Circular Dichroism and Fluorescence Spectroscopy

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The hen egg white contains proteins able to strongly bind, with a definite stoichiometry, small molecules such as biotin and riboflavin, or ions such as Cu^{2+} or Fe^{3+}. The complexation process modifies the spectral properties of these low-molecular-weight species. On the basis of these changes, it is possible, in principle, to measure the quantity of the binding protein and to evaluate the protein−substrate interactions. Here, we present a method to determine the concentration of both the apo and holo forms of the riboflavin-binding protein (RFBP) present in avian egg white, by measuring the circular dichroism (CD) related to the controlled addition of riboflavin (or vitamin B2) to the egg white. At the same time, front-face fluorescence is used to confirm the concentration of apo-RFBP obtained from CD data. The method is based on data only from spectroscopy, and no process involving either extraction, chromatography, electrophoresis, or mass spectrometry is involved. We study the egg whites from four different avian species, reporting and comparing the concentration of the apo- and holo-RFBP and the molar circular dichroism spectra ($\Delta\varepsilon$) of riboflavin in the RFBP binding site. Finally, egg whites from different hen individuals are analyzed, and a surprising variation of the RFBP concentration is found.

KEYWORDS: Riboflavin; vitamin B2; riboflavin-binding protein; thin egg white; circular dichroism; front-face fluorescence; pigeon; guinea hen; quail

INTRODUCTION

Proteins isolated from the whites of avian eggs, particularly of the hen type, have been the target of many studies for more than six decades (*1*). Some of these proteins show unique properties in the selective complexation of particular molecules because of the exceptionally high complexation constant (K_a) to the target species, combined with relatively low K_a to other molecules, even chemically quite similar to the main substrate. For example, very high K_a values characterize the complex of avidin with biotin ($K_a = 10^{13}\ M^{-1}$) (*2*,*3*), of ovotransferrin with ions such as Cu^{2+} and Fe^{3+} ($K_a = 10^{29}\ M^{-1}$) (*4*, *5*), and of riboflavin (RF) with riboflavin-binding protein ($K_a = 7.7\ 10^{8}\ M^{-1}$, with 1:1 stoichiometry). However, riboflavin-binding protein (RFBP) from hen egg white shows K_a for other important flavins such as flavin mononucleotide (FMN) and flavin adenin dinucleotide (FAD), respectively, 3 and 4 orders of magnitude lower with respect to the one for riboflavin (*6*, *7*).

We know that the egg white contains a large number of proteins, some of them in high concentrations (*8*−*10*). However, it is *possible* that in the egg white the described proteins bind their own substrate, independently of the presence of the many other proteins. Moreover, it should not be surprising that the spectral properties of both the substrates and the associated proteins are strongly affected by the binding process. We will show that this happens with RFBP and RF, and we will focus on the circular dichroism (CD) and fluorescence properties of the egg white in order to measure the concentration of apo- and holo-RFBP. We will measure CD and fluorescence spectra in the region 395−550 and 460−800 nm, respectively, where these quantities pertain to RF, the substrate of the transport RFBP. It is known that the complexation of RF to the RFBP in aqueous solution causes the complete quenching of the fluorescence of the RF isoalloxazine moiety (*11*). In fact, the RF fluorescence quenching due to RFBP has been used in the fluorometric determinations of the concentration of RF in urine, beer, and milk by titrating the substrate with pure apo-RFBP water solutions (*12*−*14*).

Several CD studies have been carried out to clarify the secondary and tertiary structure changes on flavoproteins upon flavins binding and to study the kinetics of denaturation−renaturation processes (*15*−*21*). In these investigations, CD spectra in the medium and far UV spectral region have revealed to be particularly useful. On the other side, concerning the near UV and visible region, a strong CD rising in the 350−550 nm spectral region has been observed during the complexation of flavins to a group of flavoprotein oxidases and dehydrogenases (*22*). Finally, Nishikimi and co-workers showed the rise of CD due to the binding of RF to pure hen RFBP (*23*, *24*). Edmonson and Tollin (*22*) offered a

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simple and reasonable qualitative interpretation of the effects on the near UV−vis CD of flavins due to their complexation to oxidases. In fact, the authors proposed that the geometry of the flavoprotein binding site forces the substrate flavin molecule into a *single* asymmetric conformation, which depends on the geometry of the protein binding site and is different from the average conformation in water. This explanation seems valid also for hen RFBP. We also observe that the conformation of RF in the binding site of hen RFBP in solution is likely to be close to that found in the crystalline state (*25*). Nishikimi suggested that the strong CD observed arises from the direct asymmetric perturbation of RF wave functions by the aromatic aminoacids surrounding RF in the protein binding site (*24*).

Here, we report the set up of a methodology to determine the concentration of native RF, apo-, and holo-RFBP in avian egg white by direct titration of egg white with an aqueous solution of RF. The titration is followed by two independent spectroscopic techniques, namely, CD and fluorescence. We also describe the experiments we performed to validate the method presented. Finally, we show some applications to determine the RFBP and riboflavin content in the egg whites of hen and other avian species.

The characterization and quantification of RF and RFBP can be of interest in both food and life sciences. In fact, on the one hand the content in RF is investigated in the food industry quality control procedures because of the nutritional significance of RF. On the other hand, RFBP is extensively studied since its biological function seems to be more complex than that of a simple storage protein, for example, RFBP being involved in RF uptake and trafficking (*26*). As far as we know, this is the first time that an analytical method based on the neat and strong rising of a CD signal of RF at 395−550 nm, due to its complexation to the avian RFBP present in such a complex protein mixture (*27*) as the egg white, has been described.

MATERIALS AND METHODS

Materials. Apo-RFBP was purchased from Sigma (apo-form from hen egg white, Sigma R8628; nominal RF binding capacity/mg of the protein, 10 μg) and was used as ca. 1 mg/mL water solutions. Aqueous solutions of RF (Sigma R9504, >99% pure) with a concentration of about 0.060 mg/mL were prepared, and the exact RF concentration was spectrophotometrically determined by using the value ε(445 nm) = 12500 M^{-1} cm^{-1} in water (*18*).

Grocery stores provided unfertile hen and quail (*Coturnix coturnix*) eggs which were generally used after a maximum of seven days from deposition. Pigeon (*Columba Livia*) and guinea fowl (*Numida Meleagris*) eggs were a gift from Professor R. Zamboni (University of Pisa). The study on single eggs was performed on unfertile *Isa Brown* chicken eggs, a gift from Avicola Deri (Nodica-Pisa, Italy).

Instruments. Absorbance spectra were measured with a Jasco V-550 spectrophotometer using quartz cells with 1−10 mm optical path length in order to measure absorbance values smaller than 1.5 au.

Circular dichroism spectra were taken with a Jasco J40AS spectropolarimeter with cylindrical cells, 1 cm path length. Spectra were scanned with 2 nm spectral bandwidth, 1 mdeg/cm sensibility, 1 s low response time, and 10 nm/min scanning speed, relatively high for a J40AS machine, in order to avoid too long of a light exposure of the sample (vide infra). With the same purpose, the scanning was usually limited to the 395−550 nm spectral interval, where electronic transitions pertain to the RF chromophore. In the wavelength range of the actual measurements, the sensitivity limit (rms noise) was 0.3 mdeg, i.e., more than 10-fold higher than the one of most modern spectropolarimeters.

Fluorescence spectra were recorded by using a ISA Fluoromax II photon-counting spectrofluorometer accessorized for front-face measurements with a cell holder designed to set the incidence angle of the excitation beam at 31°. As usual in front-face (FF) fluorescence spectra, the positioning of the cell is critical for the reproducibility of the spectra. We used a homemade cell-holder, able to guarantee that the position was exactly the same in each measurement. A 480 photographic filter, Φ = 40 mm (transmittance, T = 50% at 480 nm, abs > 2 for $\lambda \leq$ 450 nm) was located after the sample holder, near the cuvette and perpendicular to the local optical axis of the instrument, to strongly reduce the excitation light scattered in the emission beam. Spectra were measured with the sample in a fluorometric quartz cell (1×1 cm cross-section), stirred by a Teflon-coated magnetic bar. The excitation and the emission slits were 1.5 and 2.0 nm, respectively; the integration time constant was 0.4 s, and the wavelength increment during spectra scanning was 2 nm. Fluorescence emission, at λ_{exc} = 450 nm, was measured up to 800 nm. The spectral intensity was determined as the ratio S/R of the emission signal, S (counts, i.e. photons, per second, cps), to the light intensity from the excitation monochromator, R (μA), measured with a photomultiplier and a photodiode, respectively. No further correction of the spectra was performed. In order to reduce the noise/signal ratio to a negligible value, we used the fluorescence intensity integrated between 500 and 600 nm, i.e., in the fluorescence highest intensity spectral range of RF where the transmittance of the 480 filter was constant, about 92%. Analogously, we used the CD integrated in the 400−500 nm range.

A HPLC Jasco 880-Pu equipped with a Shimadzu SP-10 spectrophotometric detector was isocratically used to measure the concentration of RF in some of the native thin egg whites (TEWs) examined. A 4.6 mm × 250 mm S5 ODS2 Waters column equipped with a RPC18 precolumn, 10 mm, mobile phase CH_3CN/H_2O 1/4 v/v was used. The RF retention time was 9 min and 50 s at a flux of 0.5 mL/min and at a column temperature of 45 °C. The wavelength of the detector was set at 450 nm.

Preparation of the Thin and Thick Egg White. In the whole hen egg albumen, some gelatinous material (thick albumen) or fibrils deriving from inner and outer shell membranes or from chalazae is present. This makes it difficult to measure the volume of this nonhomogeneous material, to mix it with the titrating RF solution and, finally, to perform the CD experiments. Thus, we separated the TEW, i.e., the watery part of the egg white located farthest from the yolk, from the whole albumen (*28*). After the yolk and chalazae were removed, the whole egg white was transferred to a mesh screen with rhombic openings, ca. 1.3 × 0.4 mm. The filtrate, i.e., TEW, was stored at 4 °C until use, never longer than 96 h. Typically, from a large sized egg (82 g) ca. 18 mL of TEW was obtained in 1 h of filtration. TEW was generally clear, homogeneous, and fluid. In some cases, it was useful to eliminate some dishomogeneity by centrifugation at 5500 rpm for 15 min. Measurements on thick egg white were performed on samples of thick egg white mixed with 20% TEW. This material was then fluid enough to be treated similarly to TEW.

Titrations. Typically, when TEW was abundant, for example due to the use of three hen eggs, we decided to add RF in the appropriate quantity to 7−12 samples with ≥2 mL measured volume of conveniently homogenized TEW. We did not repeat the measurement procedure in triplicate, as usual, as we considered our samples a kind of *average* and thus suitable to reveal possible mistakes and flaws in some of the operations performed before the spectroscopic measurements. After a minimum of 15 min of slow magnetic agitation of the samples, we first measured the fluorescence spectra, 10 times more rapid than CD, and then the CD spectra. The temperature of the titrated samples (aqueous apo-RFBP or TEW) was maintained in the range 15−20 °C during titrations and spectroscopic measurements. When only a small amount of TEW was available, we used a sequence of 8−10 RF addition steps to a single 2.5 mL native TEW sample. Finally, in the case of single-egg studies, with ca. 13 mL of TEW, we used five samples of 2.5 mL each with two-step additions of RF. For example, first we measured the fluorescence and CD spectra of the samples where 0, 50, 150, 250, and 350 μL of RF solution were added. After that, the last four samples were added with 50 μL of RF and resubjected to fluorescence and CD measurements.

Irradiations. We irradiated samples at λ_{exc} = 450 nm after enlarging the excitation monochromator slits of the Fluoromax II fluorometer up to 28 nm spectral bandwidth. Thus, we obtain a 350-times increase of the irradiation intensity with respect to the 1.5 nm-wide slits used in the fluorescence scanning. This means that in a 15 min irradiation, we delivered to the sample 5000 times the energy delivered during the scanning of a single fluorescence spectrum.

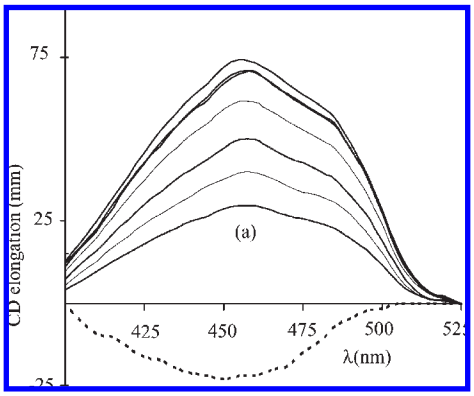

Figure 1. CD spectra of 2.2 mL of TEW added with 0, 50, 100, 150, 200, 250, 300, and 350 μL of RF (with a concentration of 166 μM) uncorrected for dilution. Curve a corresponds to the sample where RF had not been added. The dashed curve represents the CD elongation of the RF water solution used in the titrations. In the spectrum, 1 mm of elongation corresponds to 0.1 mdeg.

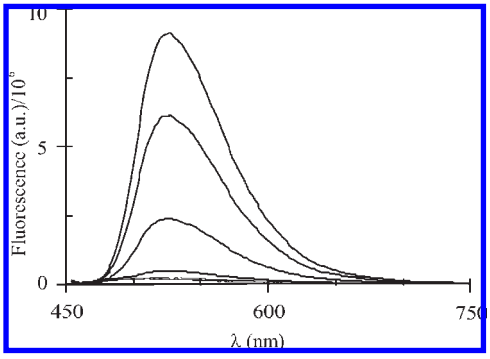

Figure 2. Emission spectra of 2.2 mL of TEW added with 0, 50, 100, 150, 200, 250, 300, and 350 μL of RF (with a concentration of 166 μM), uncorrected for dilution. The fluorescence curves corresponding to the 250, 300, and 350 μL additions can be clearly identified.

RESULTS

CD and Fluorescence Measurements of TEW and TEW Added with RF. Figure 1 reports the CD spectra of eight samples of the watery fraction of hen egg white, identical in origin and added with increasing volumes of a RF water solution (0.0625 mg/mL). The signal intensity gradually increases up to a defined limit after which it decreases very slowly. Obviously, this point corresponds to the maximum binding capacity of the protein. The only measurable contribution coming from aqueous RF added in excess seems to be that caused by the dilution of the sample. **Figure 2** shows the fluorescence spectra of the same samples of **Figure 1**. FF fluorescence technique was used because the sample absorbance, up to 0.4 abs units at the excitation λ, was too high for linearity of fluorescence with RF concentration, when measured in the traditional right-angle arrangement. A comparison of the spectra of **Figures 1** and **2** demonstrates that, upon addition of RF, when the CD growth stops the fluorescence starts to increase substantially. These aspects are represented in **Figure 3**, where we report the area of CD spectra between 400 and 500 nm and the area of fluorescence spectra, between 500 and 600 nm, both corrected for dilution, as a function of added RF. The cross-points in the CD and fluorescence titrations correspond to similar addition volumes, 226.0 μL (from CD data) and 220.4 μL (from fluorescence). The systematic coincidence in all our experiments of the CD and fluorescence titration end-points as well as the

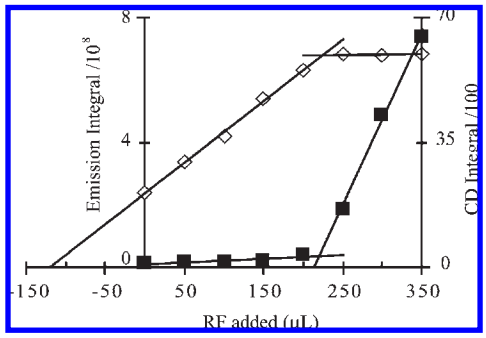

Figure 3. The emission at λ_{exc}= 450 nm of TEW integrated in the 500−600 nm range (■) and the TEW ellipticity integrated in the 400−500 nm range (□) as functions of the volume of the added RF aqueous solution.

similarity of the shape of all CD curves (**Figure 1**) seem to exclude any association of RF with other proteins having effects on the CD spectra.

From the experimental data shown above, we can calculate the molar concentrations of the apo- and holo-RFBP in the hen TEW as 17.0 μM (16.6 μM, from fluorescence) and 9.06 μM, respectively. In fact, the concentration of apo-RFBP (17.0 μM) in TEW can be estimated directly from the volume of RF added at the end-point of the titration in **Figure 3**, taking into account the 1:1 stoichiometry of the complex formed. The concentration of holo-RFBP can be determined from the CD titration curve of TEW (open squares in **Figure 3**), as the quantity of RF which would be necessary to generate the CD signal present in the original TEW sample, before any RF addition. We obtain a value of 120.1 μL representing the quantity of RF present in the holo-RFBP in 2.2 mL of TEW, thus corresponding to the above 9.06 μM of holo-RFBP.

Here, we assume that the minor flavins (FAD, FMN, 4′-5′-FMN, and 10-FMF), eventually present in our egg white (*29*), give a negligible contribution to the CD in this spectral region. This was verified with the flavins FAD and FMN, whose concentration with respect to the overall flavin content (327 μg/100 g of hen egg white) had been measured as 3.5% and 2.0% (*29*), respectively. We added commercial FAD or FMN, dissolved in water to hen TEW, and then we measured the corresponding fluorescence and CD spectra. We found out that the addition of both FAD and FMN induces flavin fluorescence, but the quantum yield is significantly smaller than the one of RF *after saturation of TEW*. In fact, the quantum yield of FMN fluorescence in TEW is ca. 3-fold higher with respect to FAD and ca. half with respect to RF. We also found out that FAD has no effect on the CD at $\lambda > 400$ nm, while the addition of FMN increases the positive CD signal of TEW at $\lambda > 400$ nm. The CD signal due to pure FMN is ca. 1/3 of that of pure RF (before site saturation) at the same molar concentration.

Hence, the dichroism of FMN causes an error of the order of only 2.0 × 1/3% in the concentration of holo-RFBP if we assume that the measured CD is due only to RF. Thus, the contribution of FAD and FMN is negligible.

We also calculated the molar circular dichroism $\Delta\varepsilon(\lambda)$ of RF complexed to the RFBP in TEW on the basis of the measured CD spectra in the spectral region of interest. RF is already present in the holo-RFBP of native egg white, and further RF has been added at each step before saturation of the binding site. Both contributions to the RF concentration are known. Thus, at each additional step we can evaluate the $\Delta\varepsilon(\lambda)$ of RF with increasing precision as CD elongations increase up to site saturation. After this point, the dichroic signal may change only because of dilution and eventual complexation to different proteins. For this reason, we took into account only the $\Delta\varepsilon(\lambda)$ corresponding to 50, 100,

150, and 200 μL RF additions to calculate an averaged $\Delta\varepsilon$. The four spectra relative to a mixture of TEWs from two hen eggs have been used together with 29 additional $\Delta\varepsilon$ values deriving from eight individual hen TEWs, to calculate the average $\Delta\varepsilon(\lambda)$ spectrum shown as a black curve in **Figure 4** and the corresponding standard deviation shown as a green curve in **Figure 4**. The shape of $\Delta\varepsilon(\lambda)$ in **Figure 4** is identical to that published by Nishikimi (*24*) where the maximum at 458 nm is 8.5 M^{-1} cm^{-1}, different from our 9.8 M^{-1} cm^{-1} essentially because of a different molar CD attributed by the authors to the camphorsulfonic acid used as a CD standard (*30*).

Finally, during the CD and fluorescence measurements on RF-enriched hen TEW, we observed that the fluorescence of the samples with free RF, i.e., ultra saturated in RF, after the CD scan was lower than the fluorescence measured before this experiment. Thus, we applied an intense irradiation on samples 6 and 11, **Table 1**, containing RF in excess. After 15 min of irradiation at 450 nm, we found up to 80% and 75%, respectively, reduction of the fluorescence, while their CD spectra remained unchanged. The same irradiation experiment was performed on 2.6 mL of TEW and 2.6 mL of water, both samples added with only 100 μL of RF (0.0630 mg/mL). The fluorescence of water−RF solution was reduced by some 30%, while the very low fluorescence and the intense CD of the TEW remained unaltered. Clearly, only the excess RF is photolabile, and the bound fraction continues to show null fluorescence and strong CD signals. This important fact is directly connected to the complete quenching ability of the binding site. In fact, after photon absorption the excitation energy of the complexed RF is very efficiently taken away so that use of this energy is possible neither for RF emission nor for RF photoreactions (*11*). In order to minimize photolysis, (a) fluorescence spectra were measured before the CD ones, and (b) the CD scanning time was reduced as much as possible.

Titration of Pure RFBP Dissolved in Water. In order to verify that the CD spectra in **Figure 1** depend *only* on the RF complexed to the RFBP present in the TEW, we also measured the CD titration curves of pure commercial hen apo-RFBP dissolved in water (**Figure 5**). We proved that curve a of **Figure 1** is almost coincident with the CD curve a in **Figure 5**, corresponding to a 9.3 μM concentration of RF complexed to RFBP. This evidence supports our interpretation that the CD signal present in TEW, curve a in **Figure 1**, is due to native RF in the RFBP site. The fluorescence spectra of pure apo-RFBP in water at $\lambda_{exc} = 450$ nm, measured at the same steps of the CD titration, are reported in **Figure 6**. Finally, **Figure 7** describes the overall results of the CD and fluorescence measurements, showing the same linear increase

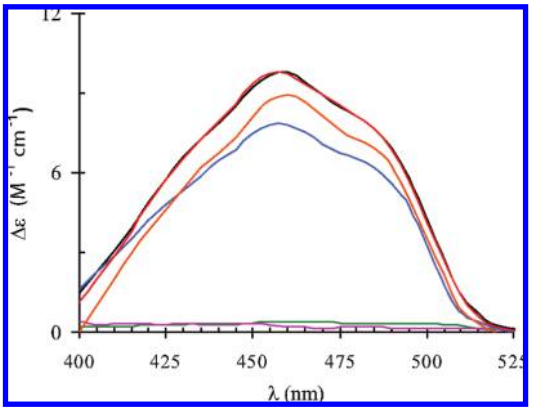

Figure 4. Molar CD spectra of RF in the RFBP binding site in TEWs from eggs of different species: quail (red), pigeon (orange), guinea hen (blue), and hen (black). The molar CD of RF from the titration of a water solution of pure apo-RFBP is also reported (yellow), but it perfectly overlaps the black curve relative to the hen TEW and is not visible. The green and the magenta curves represent the standard deviation for the measurements in hen TEW and in the water solution of pure apo-RFBP, respectively.

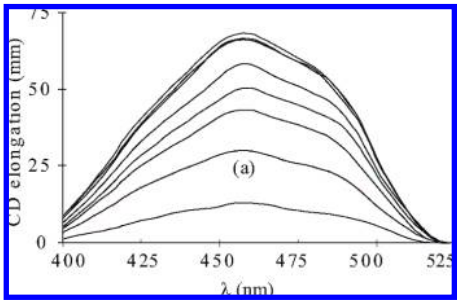

Figure 5. CD elongations measured on addition of 0 (zero at any of the wavelengths reported), 50, 125 (curve a), 200, 230, 260, 310, 360, and 410 μL of a water solution of RF, 0.0630 mg/mL, to a 2.2 mL solution, ca. 24 μM, of pure hen apo-RFBP. The corresponding CD curves increase progressively on addition, up to the maximal curve at 310 μL of RF addition, after which they decrease because of dilution. The curves reported are not corrected for dilution. In the spectrum, 1 mm of elongation corresponds to 0.1 mdeg.

Table 1. Concentration of Apo- and Holo-RFBP in the TEW of 12 Different Hens, 6 of Them Classified As Young and 6 As Old (See Text)[a]

hen	age	apo-RFBP (CD)	holo-RFBP (CD)	apo-RFBP (F)	total RFBP (CD)	holo-RBFB/ apo-RFBP (CD)	% holo-RBFB
1	young	15.95	10.02	15.70	25.97	0.628	38.59
2	young	16.09	11.02	15.95	27.12	0.685	40.66
3	young	13.36	13.46	14.10	26.82	1.008	50.19
4	young	16.85	12.05	17.11	28.91	0.715	41.69
5	young	12.31	5.48	12.47	17.79	0.445	30.79
6	young	12.05	10.39	11.87	22.45	0.862	46.30
7	old	15.75	9.84	15.69	25.59	0.624	38.44
8	old	12.23	8.29	12.61	20.52	0.678	40.39
9	old	14.72	9.80	14.28	24.52	0.666	39.97
10	old	12.16	7.15	12.51	19.31	0.588	37.04
11	old	13.29	12.11	13.19	25.40	0.912	47.69
12	old	13.00	9.83		22.83	0.756	43.07
	average young	**14.4** (**2.1**)	**10.4** (**2.7**)	**14.5** (**2.1**)	**24.8** (**4.1**)	**0.72** (**0.19**)	**41.4** (**6.7**)
	average old	**13.5** (**1.4**)	**9.5** (**1.7**)	**13.6** (**1.3**)	**23.0** (**2.6**)	**0.70** (**0.12**)	**41.1** (**3.8**)
	average young + old	**14.0** (**1.8**)	**9.9** (**2.2**)	**14.1** (**1.7**)	**23.9** (**3.4**)	**0.71** (**0.15**)	**41.2** (**5.2**)

[a] The concentration is given as (μM), and the standard deviation is reported in parentheses.

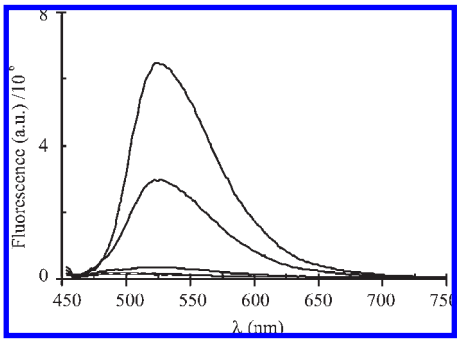

Figure 6. Emission spectra of hen apo-RFB samples added with 0, 50, 125, 200, 230, 260, 310, 360, and 410 μL of RF, uncorrected for dilution. The fluorescence curves corresponding to the 310, 360, and 410 μL additions can be clearly identified.

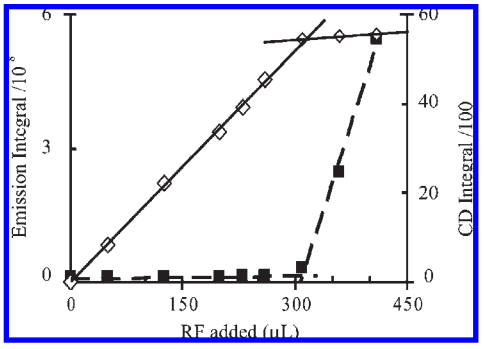

Figure 7. Emission at λ_{exc}= 450 nm of a pure apo-RFBP water solution integrated in the 500−600 nm range (■) and the TEW ellipticity integrated in the 400−500 nm range (□) as functions of the added RF aqueous solution volumes.

Table 2. Concentration of Apo- and Holo-RFBP in the TEW of Different Avian Species[a]

	hen	quail	guinea hen	pigeon
apo-RFBP	14.0 (1.8)	13.2	13.81	8.46
holo-RFBP	10.0 (2.2)	6.58 (1.10)	5.41	0
total RFBP	23.9 (3.4)	19.81	19.22	8.46
holo-RFBP/(total RFBP)	0.412 (0.052)	0.332	0.281	0
holo-RFBP/apo-RFBP	0.71 (0.015)	0.497	0.392	0

[a] The concentration is given as (μM). The standard deviation, where determined, is reported in parentheses.

of the CD signal upon addition of RF as in the experiments with egg whites (**Figure 3**). We note that in **Figure 7** the linearity starts from zero concentration of RF, as expected. Thus, the procedure we employ to calculate the holo-RFBP present in TEW (**Figure 3**) is correct. From the CD measurements recorded at each titration step reported in **Figure 5**, we calculated the molar $\Delta\varepsilon(\lambda)$ of RF in the binding site of pure hen RFBP dissolved in water, and the average curve is reported as a yellow curve in **Figure 4**. The coincidence of yellow curve with the black one (i.e., the $\Delta\varepsilon(\lambda)$ calculated from titrations of hen TEWs) is so good that actually the yellow curve cannot be distinguished. This fact confirms that CD spectra measured during the titration of TEW are due to RF in the binding site of RFBP.

HPLC Determination of RF Content in TEW. Finally, we verified the content of RF in TEW by using nonspectroscopic techniques. We applied methods reported in literature for the extraction of RF from food and the chromatographic analysis of this analyte in the extracts (*29*, *31*−*33*). However, when we extracted RF from TEW by solvent mixtures containing CH_3CN, we observed the coagulation of proteins, of which TEW is particularly rich, ca. 100 mg/mL (*27*). The extraction of RF from this material gave incomplete results even after two extraction cycles. Thus, we choose to inject diluted TEW directly in the RP-HPLC column. As the direct injection of TEW, even in such small volumes as 3 μL, was immediately followed by the onset of extremely high backpressure, peak enlargement, and irreproducibility of measurements, we found it expedient to dilute TEW 3-fold with water and sonicate the resulting solution for 30 s in order to have the possibility of repeating injections, each of 3 μL. The drawback of our HPLC method is that after 8−10 injections the above problems begin to manifest, and it becomes necessary to change the precolumn filter and to perform a regeneration cycle for the column. Clearly, with the much higher sensibility offered by fluorometric detection it is possible to use much more diluted TEW samples, to have the possibility of many more HPLC reproducible injections. This procedure was revealed to be appropriate. In fact, in a set of eight HPLC injections of a diluted hen TEW sample, we obtained an average value of 11.58 μM for the holo-RFBP to be compared to the value of 11.46 μM from the CD measurements, i.e., a 1.03% difference. However, it should be noted that the standard deviation in the HPLC determinations was neatly higher than the above difference: 5.1%.

Spectra of Other Avian Species and Individual Variability of Hen Eggs. The spectroscopic methodology we developed has been applied to investigate the individual variability and effect of age on the amount of apo- and holo-RFBP in hen TEWs (**Table 1**) and to determine RFBP in the TEW from different avian species (**Table 2**). We present data on thin egg whites, but we have tested that the concentration of both apo- and holo-RFBP in thin and thick egg whites are equal, at least in the cases of hen and pigeon eggs (data not shown). Differences in the composition of individual hen eggs from birds of the same origin, age, feeding, and living environment could be believed to be minor if not negligible, while the age of these birds could be considered an important parameter on this respect. Therefore, we examined the concentration of holo- and apo-RFBP in 12 hen egg whites, including six young and six old hens. The individual values and the average content of holo- and apo-RFBP for the different ages are reported in **Table 1**. We also investigated egg whites from different avian species. **Figure 4** reports the $\Delta\varepsilon(\lambda)$ of quail, guinea hen, and pigeon together with the data for hen. These spectra have been obtained from CD titration data of the respective thin egg whites. The average concentrations of the corresponding apo- and holo-RFBP are shown in **Table 2**. The hen values are from **Table 1**; the values from different chicken TEWs conform to these averages. It should be noticed that holo-RFBP is basically absent in the TEW of pigeon and that its quantity in the TEW of guinea hen was significantly lower than that in the hen case.

CD Simplified Procedures. The previously described process to determine the concentration of holo- and apo-RFBP requires about 10 fluorescence and CD measurements on RF-enriched samples. It is possible to reduce this process to only two CD measurements in a narrow wavelength range around 460 nm, the first on native TEW and the second on a TEW oversaturated with RF. In fact, if the CD of native TEW derives exclusively from holo-RFBP, the concentration of the holo-RFBP can be immediately calculated by taking into account the value of molar $\Delta\varepsilon$ of RF in the hen binding site (black curve in **Figure 4**). Thus, we calculate the concentration of RF in native TEW, i.e., the concentration of holo-RFBP, by the following

equation:

$$[\mathrm{RF}] = [\text{holo-RFBP}] = CD_{\max}/(32980 \times 9.8) \quad (1)$$

where $CD_{\max}$ represents the elongation in cm (1-cm cell path length, with sensibility of 1 mdeg/cm) measured at the maximum near 460 nm. The divisor 32980 derives from 32.98, the conversion factor between molar dichroism (M^{-1} cm^{-1}) and molar ellipticity (deg M^{-1} cm^{-1}), multiplied by 1000, because of the use of millidegrees (mdeg). Finally, 9.8 is the maximum molar $\Delta\varepsilon(460)$ of RF in hen RFBP. We verified that the values calculated with such a simplified technique for the samples of **Table 1** are on the average 3.32% different in absolute value from the values determined with the complete titration and reported in **Table 1**. The concentration of apo-RFBP can be determined in a similar way from the measure of the CD elongation at the maximum, after saturation of the RFBP site. Thus, we can add an excess of RF solution to TEW and measure the CD of the resulting solution. If the CD signal after saturation of the RFBP binding site changes only because of the dilution accompanying the addition of aqueous RF, we can correct the measured CD (CD_{meas}) for the dilution of TEW, knowing the volume of the sample before RF addition (V_{unadd}) and the volume after addition (V_{add}). Thus, we obtain the CD signal that we would measure in the case of totally saturated RF binding site in original TEW (CD_{corr}):

$$CD_{\text{corr}} = CD_{\text{meas}} \cdot V_{\text{add}}/V_{\text{unadded}} \quad (2)$$

The value of CD_{corr} at the maximum near 460 nm can be used to calculate the overall concentration of RF complexed to the RFBP, i.e., the total concentration of RFBP in TEW by the following equation:

$$[\mathrm{RF}]_{\text{total}} = CD_{\text{corr}}/(3298 \times 9.8) \quad (3)$$

It must be noticed that this value does not change for excess addition of aqueous RF. Then, the concentration of apo-RFBP is simply the difference between the values from eqs 3 and 1. We reconsidered the experimental data in **Table 1**. In all of the cases, the addition of 400 μL of RF 0.0630 mg/mL to TEW corresponded to ca. 50% excess relative to the effective content of apo-RFBP. Thus, we used the elongation at maximum CD near 460 nm of each native TEW and that correspondent to the addition of 400 μL to obtain the concentration of apo-RFBP. The average absolute value of the difference of the values from this simplified procedure and the values of **Table 1** was 4.18%. In conclusion, we could determine the concentration of apo- and holo-RFBP in TEW with a measuring time of half an hour within some 5% precision.

DISCUSSION

We have shown that the addition of RF to TEW is accompanied by CD and fluorescence phenomena which are entirely due to the complexation of RF to the RFBP present in the TEW, any interference from the many other molecular species present not being measurable. An adequate addition of RF to the complex mixture of the molecular species (particularly of proteins in high concentration) present in egg white leads to the saturation of the CD signal and to the *simultaneous* starting of RF fluorescence development. This process makes possible the precise determination of the RF quantity necessary to convert the apo-RFBP into holo-RFBP and thus the amount of apo-RFBP via two independent spectroscopic techniques, namely, CD and fluorescence. We performed TEW titrations on >30 different samples from hens and on >6 from other avian species, and we found only one case in which the CD titration end-point was more than 3% different from the fluorescence end-point, i.e., 5.2%. In addition, the CD of native egg white and its linear increase during the RF addition up to the saturation let us quickly and precisely determine the RF concentration in the samples and hence the native concentration of holo-RFBP.

It is now useful to briefly discuss the results regarding single hen TEWs presented in **Table 1**. The concentration of holo- and apo-RFBP in single chicken egg shows a high variability between individuals. Moreover, the ratio of the concentrations of holo-RFBP and apo-RFBP is far from being constant. The standard deviation gives an indication of the average dispersion of individual egg content versus the average one. We notice that the error in each determination, about 3%, is very low compared with this dispersion. Relatively high inter-hen variability was observed also for the egg white protein lysozyme (*34*).

The other interesting issue regards the differences between eggs of young chickens, 8 months old, and old chickens, 14 months old (**Table 1**). We observe a marginal even if systematic enrichment bias of RF and of the relative transport proteins, in younger chicken averaged values (rows 13 vs 14 in **Table 1**). However, these differences are amply exceeded by the values of the standard deviation. The fraction of holo-RFBP in total RFBP, averaged over the 12 eggs, 41.2% (row 15, **Table 1**), is in complete agreement with 42.2% results of White (*35*). Also, our averaged holo-RFBP concentration, 9.95 μM, is in agreement with the average value reported by White, 10.9 μM (*35*), and with the value 8.7 μM from more recent literature (*29*). The differences among these values can be considered acceptable when we take into account both the above-shown variability of eggs and the fact that the reported data refer to completely different groups of birds. Moreover, different analytical methods were used. In particular, White and co-workers (*35*) used a very complex fluorometric/photochemical method involving the determination of scattering. As the scattering and absorbance can heavily affect the usual right angle fluorescence measurements, we preferred to use a front-face fluorometric technique, as discussed in the Instruments section. In addition to the analytical data, the shape and the intensity of $\Delta\varepsilon$ spectra should reflect the conformation of RF in the RFBP binding site, which could be different in the egg whites of different avian species. Clearly, relevant differences in intensity and shape of the molar circular dichroism spectra have a structural meaning also for the involved protein binding sites. As we see in **Figure 4**, the differences of both intensity and shape of the spectra with respect to those of chickens are appreciable only for pigeons and, perhaps, for guinea hens. In these cases, the hypothesis of significant differences between the respective RFBP sites seems justified.

In conclusion, the method we propose can provide quantitative information on the egg alimentary value or on the effects of treatments done in a species-improvement perspective or, simply, the effects of bird feeding (*35*) and aging on the characteristics of the egg whites. In general, since the expression of proteins changes constantly, responding also to external stimuli, our analysis method seems particularly suitable to measure the amplitude of these responses directly on the protein expressed and could be adopted as a specialized part of proteome technologies (*36*). Obviously, the methodology here reported can be useful to characterize, quantitatively and qualitatively, eggs of nonavian origin (reptiles and fishes) as well. We also observe that small amounts of sample (< 1 mL) are required and that nondestructive analytical techniques are employed, thus being a convenient approach also when small sample amounts are available.

More in general, flavoproteins are known to be involved in most metabolic pathways in both prokaryotic and eukaryotic cells and in many other cellular processes, and often, their flavin-binding properties have been related to the ones of hen RFBP. For example, it was speculated that the recently isolated membrane RF carrier protein, RibU, has an RF binding site similar to that of hen RFBP. In fact, RibU and RFBP are characterized by very close RF complexation constants and comparable spectroscopic properties, such as light absorption and RF and tryptophan fluorescence quenching. Notice that this happens without any particular sequence similarity between RFBP and RibU (*37*, *38*). From the above spectroscopic and binding similarities, it is probable that the CD method we are presenting could be useful also in quali-quantitative determination of RibU as well as the mentioned enzymatic flavoproteins (*22*).

ABBREVIATIONS USED

TEW, thin egg white; RF, riboflavin; apo-RFBP, apo-riboflavin-binding protein; holo-RFBP, holo-riboflavin-binding protein; FF, front-face; CD, circular dichroism.

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