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Fluorometric and Spectrophotometric Study of Heme Binding on the Apoprotein from a Cytochrome b_2 Derivative*

Françoise Labeyrie, Ann di Franco, Motohiro Iwatsubo, and Alain Baudras

ABSTRACT: A derivative of cytochrome b_2 (L-lactate: cytochrome *c* oxidoreductase, EC 1.1.2.3) has previously been obtained by trypsic digestion of the enzyme; a similar product is found in the supernatant upon recrystallization of the same enzyme. Both are called noyau cytochromique b_2. It has been possible to separate the heme constituent from this protein and to describe some of the properties of the apoprotein. When added, heme is bound quantitatively to the apoprotein at neutrality; the reconstituted hemoprotein seems quite similar to the original one; tyrosine and tryptophan fluorescence are then 95% quenched. The ultraviolet spectrum allows the determination of tyrosine and tryptophan content. Quantum yields for tyrosine and tryptophan, calculated from the fluorescence emission spectrum and aromatic acid content, gave values of 0.030 and 0.025, respectively, for the apoprotein, the latter increasing to 0.21 when in the presence of 7 M urea.

These data, along with previous results, permit the discussion of the relative position of the heme and flavin prosthetic groups.

While the first spectroscopically observed reconstitutions of hemoglobin from hematin and globin date from the end of the last century, only recently similar data have been obtained for *b*-type cytochromes. These experiments were performed by Strittmatter (1960) on cytochrome b_5 from liver microsomes (mol wt 12,700), then by Shichi and Hackett (1962) for cytochrome b_{555} and b_{561} from mung bean seedlings (mol wt 12,000).

It is known that cytochrome b_2 from bakers' yeast (L-lactate:cytochrome *c*-oxidoreductase EC 1.1.2.3.) is a molecule having a molecular weight of order of 200,000 and containing one heme and one FMN[1] prosthetic group per 77,000*g* protein (Appleby and Morton, 1954; Appleby *et al.*, 1960). One of these prosthetic groups, the flavin, is easily removable as was shown independently by Morton (1961) and Baudras (1962); the resulting apoenzyme can be reactivated (up to 80%) by saturation with FMN and the reconstituted flavoenzyme was even crystallized (Baudras, 1965). As far as the heme prosthetic group of cytochrome b_2 is concerned, it has been impossible in spite of many efforts in Morton's (1961) laboratory and ours (M. Rippa, A. Baudras, and M. Iwatsubo, unpublished results) to obtain a separation without extensive denaturation of the protein moiety.

Recently, a hemoprotein derivative of low molecular weight, called noyau cytochromique b_2 "t," was prepared from cytochrome b_2 by trypsic hydrolysis. Oxidation–reduction and spectral investigations have shown that the heme–protein linkage is essentially the same in cytochrome b_2 and in this derivative obtained by elimination of 85% of the protein moiety (Labeyrie *et al.*, 1966). A hemoprotein very similar if not identical which is called noyau cytochromique b_2 "s," was found in the supernatant upon recrystallization of cytochrome b_2 (M. Iwatsubo and A. di Franco, unpublished results).

The experiments presented in this paper show that it is possible to remove the heme from both the noyau

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[1] Abbreviation used: FMN, flavin mononucleotide.

cytochromique b_2 s and t and to reconstitute, by heme fixation, hemoproteins which appear to be similar to the original ones; this reconstitution was followed spectrophotometrically and fluorometrically. Some preliminary findings have already been given in a review article on yeast lactate dehydrogenases (Labeyrie and Slonimski, 1964).

Materials and Methods

Noyau Cytochromique b_2 s. The supernatant, after recrystallization of type I ferrocytochrome b_2 to the form ferricytochrome b_2 following the method of Morton and Shepley (1963), was found to contain a spontaneously formed noyau cytochromique b_2 which, under these conditions, does not crystallize. The supernatant of several preparations is collected over a period of several months at $-20°$ or until a sufficient volume is obtained to make a preparation worthwhile. The stocked supernatant is then defrosted, precipitated with ammonium sulfate (85% saturation), centrifuged, dissolved in a minimum volume of 20 mM Tris-acetate (pH 8.0), and passed on a Sephadex G-100 column which has been equilibrated with 20 mM Tris-acetate (pH 8.0), in order to separate any traces of type I ferricytochrome b_2 from noyau cytochromique b_2. This sort of molecular sieving (Sephadex G-100 columning) followed by precipitation with ammonium sulfate is repeated, in order to eliminate the remaining nucleotides and contaminating proteins, three times or until a ratio of $A_{413}:A_{280}$ of 5.6 is obtained. The determination of the millimolar absorbancy coefficient by the formation of a pyridine hemochromogen gives a value of $\epsilon_{413\,m\mu}$ 125 mM^{-1} cm^{-1} at the maximum of the γ band in the oxidized form. By an approach to equilibrium method, carried out at the Station Centrale d'Ultracentrifugation du CNRS under the direction of Mme. S. Filitti-Wurmser, a molecular weight of 10,800 $\pm$ 180 was found.

Noyau Cytochromique b_2 t. A solution ($\sim$30 mg/ml) of cytochrome b_2 (crystallized sample type I; *cf.* Appleby and Morton, 1959a) in 0.1 M phosphate buffer is hydrolyzed several hours at 30° by trypsin (0.8 mg/ml). The hydrolysate, after centrifugation, is eluted on a Sephadex G-100 column (3 $\times$ 40 cm). The elution diagram shows three peaks amongst which only the second one is red. It was shown previously that the heme component of cytochrome b_2 is present in totality in this colored fraction as a hemoprotein of molecular weight of about 11,200 $\pm$ 700 (Labeyrie *et al.*, 1966). The collected red fraction is precipitated with ammonium sulfate up to 80% saturation; the precipitate is dissolved in water then readjusted to pH 7, dialyzed against water, and lyophilized. It is perfectly soluble even in distilled water and fairly stable.

Values are found for $\epsilon_{413\,m\mu}$ which widely scatter around the mean value of 112 mM^{-1} cm^{-1}. The maximum value being 128 mM^{-1} cm^{-1}, which approaches the value determined for the noyau cytochromique b_2 s. It is possible that in certain preparations of noyau cytochromique t the heme binding is slightly modified, thus giving a smaller ϵ.

The experiments presented are almost entirely carried out with the noyau cytochromique b_2 s and the value of ϵ taken as a basis for the concentration measurements is 125 mM^{-1} cm^{-1}. Analogous experiments were carried out on the noyau cytochromique b_2 t giving the same results.

Both these noyau cytochromique b_2 behave as homogenous solutions upon Sephadex columning in 0.1 M phosphate buffer (pH 7.2). However, upon electrophoretic migration on polyacrylamide gel at pH 7, three bands are always observed with the noyau cytochromique b_2 s. As for the noyau cytochromique t, three bands of comparable intensities and having the same mobilities as the noyau cytochromique b_2 s are generally noted with the exception of several preparations where only two or even one band were observed (Labeyrie *et al.*, 1966).

Apoprotein of noyau cytochromique b_2 s or t is prepared from either noyau cytochromique s or t as follows. A solution (150 μl) of the hemoprotein dissolved in distilled water (1–10 mg/ml) is added to 4 ml of acid–acetone (0.1 ml of 11 N HCl in 50 ml of acetone) with agitation at $-15°$; the precipitate is collected a few minutes later by centrifugation. The pinkish deposit is dissolved by addition of 150 μl of distilled water and the same treatment is repeated for complete dissociation of heme. The final white precipitate is vacuum dried at 0° (3 min) in order to remove any trace of residual acetone, then dissolved in 150 μl of 10 mM phosphate buffer (pH 7). The solution which appears perfectly clear is, however, centrifuged at 0° and 37,000*g* for 10 min. The apoprotein, thus prepared, is kept either in an ice bath or frozen at $-20°$ and under these conditions it appears to be stable.

Protohematin solutions were freshly prepared by dissolving some crystals of hemin chloride in 0.1 N NaOH. Dilutions were made just before use in 100 mM phosphate buffer (pH 7.0); the concentrations are deduced from absorbancy measurements in 0.1 N NaOH with $\epsilon_{385\,m\mu}$ 60.3 mM^{-1} cm^{-1} (see below). The experiments were carried out with a commercial sample (Calbiochem) except for some verifications with a sample of hemin chloride prepared (*cf.* Fisher, twice crystallized) and kindly given to us by Dr. R. Banerjee.

Measurements of Protohematin Concentrations. During the course of our work it was necessary to be able to calculate the concentration of heme in different solvents. In order to have precise relative values of the ϵ mM^{-1} cm^{-1}, the latter were deduced from spectra of freshly prepared solutions of hematin diluted in these different solvents. The following results were obtained by using as a basis the iron content measured, *cf.* Lorber (1927); in parentheses are given the values found in the literature expressed in units of mM^{-1} cm^{-1}; in 0.1 N NaOH, $\epsilon_{385\,m\mu}$ 60.3 (57.5, *cf.* Hogness *et al.*, 1937); in NaOH pyridine (reduced hemochromogen is prepared, *cf.* Appleby and Morton, 1959b), $\epsilon_{419\,m\mu}$ 154 and $\epsilon_{557\,m\mu}$ 30.2 (34.8, *cf.* Paul *et al.* (1953), or 32.0, *cf.* de Duve, 1948); in acid–acetone, $\epsilon_{385\,m\mu}$ 100 (88.5, *cf.* Lewis, 1954); and in 0.2 M KCN (freshly prepared), $\epsilon_{545\,m\mu}$ 11.1 (11.1, *cf.* Hogness *et al.*, 1937). The values given are those of Calbiochem sample; the corresponding values for the

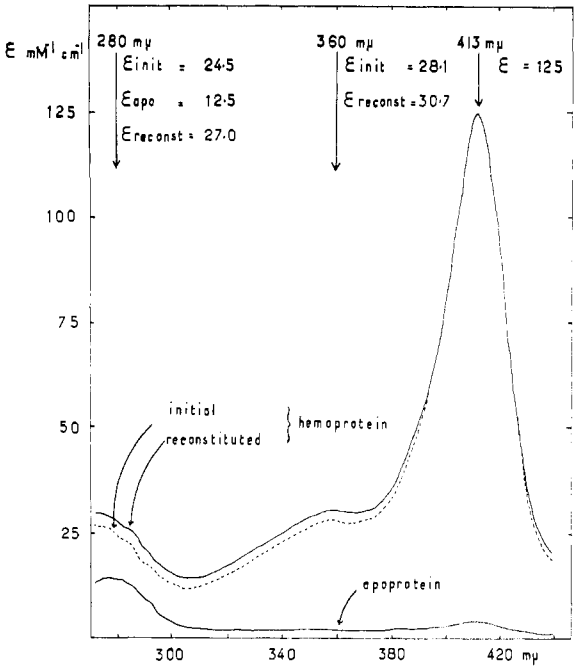

FIGURE 1: Spectra of the reconstituted hemoprotein compared with that of the apoprotein and the initial noyau cytochromique b_2. Conditions: 0.1 M phosphate (pH 7.5, 22°). The initial noyau cytochromique b_2 concentration (5.3 μM) is calculated using $\epsilon_{413\ m\mu}$ 125 mM^{-1} cm^{-1}. The reconstituted hemoprotein concentration (5.3 μM) is determined by heme titration. The ε data at the top of the figure were determined more precisely by using the slope of the absorbancy plots following Figure 5.

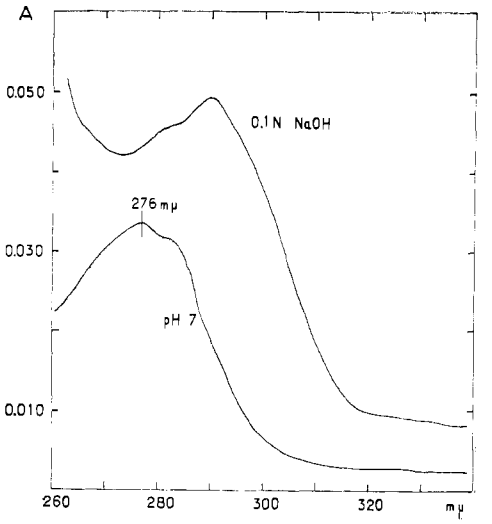

FIGURE 2: Ultraviolet absorption spectra of the protein moiety of the noyau cytochromique b_2 in neutral and alkaline medium. Apoprotein t (2.8 μM) in 0.1 M phosphate buffer (pH 7) or in 0.1 N NaOH. Spectra were recorded with Cary 14 spectrophotometer on the absorbancy scale 0–0.1.

sample prepared by Dr. Banerjee differ not more than 1% except for the α-reduced pyridine hemochromogen at 557 mμ where 5% higher was found. These determined values were taken as a basis for heme concentrations measurements since they are in accordance with values of Hogness *et al.* in KCN, the medium which is known to give the better reproducibility.

Fluorescence Spectrophotometry. The spectrofluorophotometer used for all fluorescence measurements was built by one of us (M. I.) and its description is given in detail by Iwatsubo and di Franco (1965). A high-pressure xenon arc light source (Osram, 450 w) and a selected EMI photomultiplier (9558 QA) were used for excitation and for emission measurements, respectively. All spectra were obtained using a 5-mμ band width for the excitation wavelength and 8-mμ band width for the emission wavelength. Quantum yields were calculated following a method established by Weber and Young (1964).

Results

Preparation of the Apoprotein. The preparation of the apoprotein, as described in Methods, allows the separation of about 97% of the heme; most of this heme is eliminated in the course of the first acid–acetone treatment. As will be presented later on, the hemoprotein can be reconstituted by addition of heme to this apoprotein.

The recovery of the protein is generally elevated (more than 85%) even at low protein concentrations (1 mg/ml); our best preparation gave 97% recovery. This value is calculated from the ratio of the amount of apoprotein (known by titration with hematin) to the amount of initial hemoprotein treated.

The comparison of the absorption spectra of the initial and reconstituted hemoproteins (after addition of an equivalent of hematin to the apoprotein) shows a complete coincidence in the visible region and only a slight difference in the ultraviolet region where the reconstituted protein absorption is stronger (Figure 1).

Spectral and Fluorometric Studies of the Apoprotein. The absorption spectrum of the dissolved apoprotein at neutrality (Figure 2) indicates a maximum in the ultraviolet region at 276 mμ with a calculated absorbancy coefficient ε 12.2 mM^{-1} cm^{-1}; a small absorbance, near 413 mμ, is associated with a small residual amount of bound heme (~3%). From the spectrum in 0.1 N NaOH (Figure 2) the number of tyrosine and tryptophan residues is estimated following a method of Holiday (Beaven and Holiday, 1952). After having corrected for an important diffusion and for the residual heme, values around 5 for tyrosine and 1–1.5 for tryptophan were found for both the s and t apoproteins. Better determinations were obtained by difference spectra between noyau cytochromique b_2 s (10,800*g* protein) in 0.1 N NaOH and hematin in 0.1 N NaOH at an equivalent concentration, giving four tyrosines (4.20 and 4.15) and one tryptophan

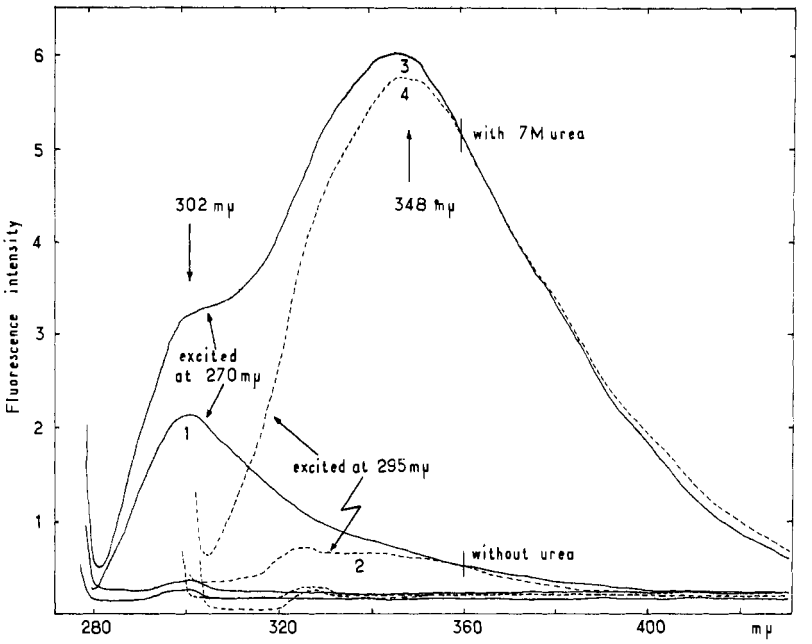

FIGURE 3: Fluorescence emission spectra of the apoprotein in phosphate buffer and in urea. Fluorescence measurements were all made at 18° and a concentration of 6.2 μM apoprotein. Curves 1 and 2: apoprotein s in 0.1 M phosphate (pH 7.3) excited at 270 and 295 mμ, respectively. Curves 3 and 4: apoprotein in 7 M urea dissolved in 0.1 M phosphate (pH 7.3) excited at 270 and 295 mμ, respectively. The four lower spectra are the phosphate and urea references.

TABLE I: Fluorescence Quantum Yields of the Apoprotein.[a]

	$\langle q \rangle_{protein}$	$\langle q \rangle_{tyrosine}$	$\langle q \rangle_{tryptophan}$	$q_{0\,tyrosine}$*	$q_{0\,tryptophan}$**
Apoprotein in 0.1 M phosphate (pH 7.3, 18°)	0.028*	0.017	0.012	0.030	0.025
Apoprotein in 7 M urea–0.1 M phosphate (pH 7.3, 18°)	0.117**	0.023	0.094	0.045	0.208

[a] $\langle q \rangle_{protein}$ was determined from the area under the emission spectrum (Figure 3) when excited at 270 mμ. $\langle q \rangle_{tryptophan}$ was determined from the area under the emission spectrum (Figure 3) of tryptophan excited at 295 mμ corrected by an appropriate factor to coincide at 360 mμ where the tryptophan is the only emitter, with the spectrum excited at 270 mμ. $\langle q \rangle_{tyrosine}$ is given by the difference of the two precedent areas (protein less the tryptophan). All of these curves are compared to either tyrosine* (q = 0.21) or tryptophan** (q = 0.20) having the same absorption at 270 mμ as the protein solution studied. The reference was selected in order to compensate for a slight difference in the response of our fluorescence spectrophotometer in the region of 300 and 350 mμ. The q_0 values are calculated by using the tryptophan and tyrosine areas as determined above, taking into account not the total absorbance of the protein, but the calculated participation of each. For this calculation, the following data are used: 1 mole of tryptophan and 4 moles of tyrosine/mole 11,000 g of apoprotein; the millimolar absorption coefficient for each of these amino acids being at 270 mμ: 5.3 for tryptophan and 1.2 for tyrosine.

(1.04 and 1.06). These values are to be compared to the values of 13.8 and 3.4/unit carrying one heme group of cytochrome b_2, *i.e.*, 77,000*g* protein (Appleby *et al.*, 1960).

Following Weber and Young (1964), there are several ways to express the fluorescence quantum yield of a protein. The total protein fluorescence quantum yield $\langle q \rangle_{protein}$ (defined as total quanta emitted per total quanta absorbed) is measured by comparison of the areas under the emission spectra excited at 270 mμ of the apoprotein, which has a maximum at 302 mμ (Figure 3), and free tyrosine in water, respectively, both solutions having the same absorbance at 270 mμ. The values found were $\langle q \rangle_{protein}$ = 0.028 for the apoprotein in phosphate buffer

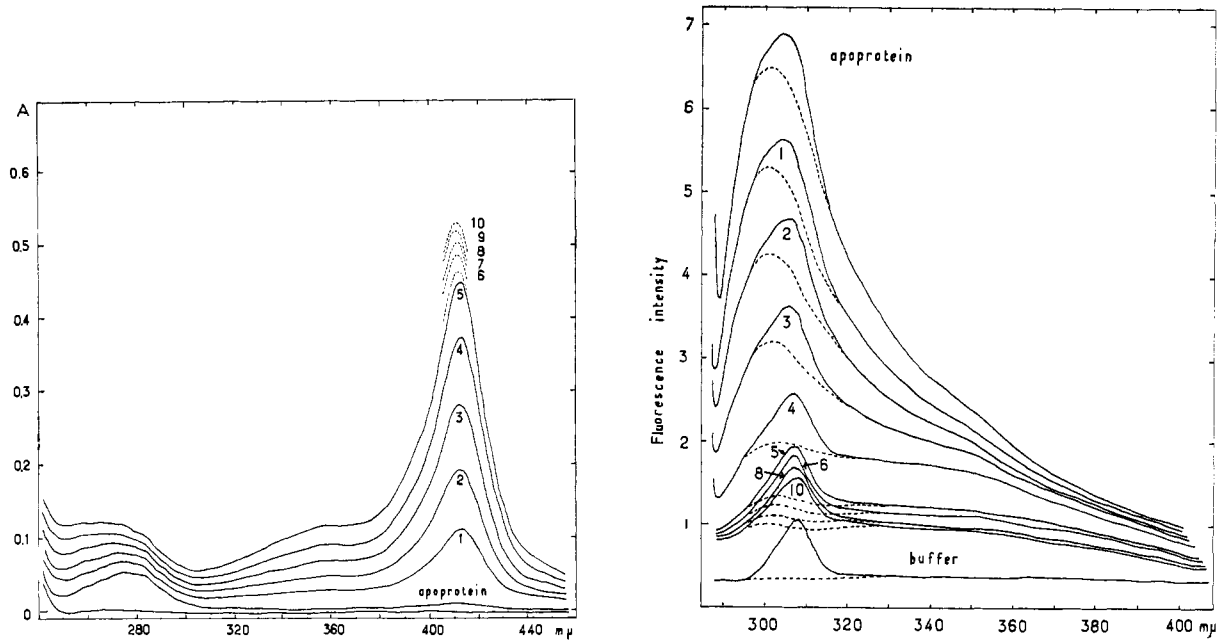

FIGURE 4: Absorption and fluorescence emission spectra following successive heme additions to the apoprotein. The apoprotein s was titrated in 0.1 M phosphate (pH 7.3) at 22°; each heme addition corresponds to 0.8 μM. The numbers on the figures indicate the number of such additions. (left) Absorption spectra, measured on a Cary 14, of the apoprotein s (residual bound heme 2.5%). The titrated concentration is 4.0 μM. (right) Fluorescence emission spectra measured with the same solution of apoprotein excited at 278 mμ. The dotted lines represent the spectra corrected for Raman emission.

and $\langle q \rangle_{\text{protein}} = 0.124$ for the same solution in the presence of 7 M urea.

The quantum yield of "protein fluorescence as tyrosine" defined as $\langle q \rangle_{\text{tyrosine}}$ = quanta emitted as tyrosine fluorescence per quanta absorbed by protein and the quantum yield of "tyrosine in the protein" $q_{0\ \text{tyrosine}}$ defined as quanta emitted as tyrosine fluorescence per quanta absorbed by tyrosine were determined and the results are given in Table I. In the latter case, the absorption of the phenylalanines (3–4/unit of noyau cytochromique b_2 having a molecular weight of 11,000 (F. Lederer, personal communication)) is neglected since its total contribution is less than 1%. As expected the $q_{0\ \text{tyrosine}}$ is small and remains so, even in 7 M urea. On the other hand, the tryptophan residues are strongly quenched in phosphate, but increase in urea to give a quantum yield equal to that of free tryptophan (Figure 3, Table I).

Reconstitution of the Hemoprotein. The reconstitution of the hemoprotein with protohematin is carried out as follows. Aliquots of a freshly prepared alkaline hematin solution are added (1) to a solution of apoprotein in 100 mM phosphate buffer (pH 7) and (2) to the same volume of the buffer without protein. Each time, the following spectra were taken: absolute spectrum against buffer (1 *vs.* buffer) (Figure 4, left), difference spectrum (1 *vs.* 2), and fluorescence emission spectrum excited at 278 mμ (Figure 4, right). At neutrality, the fixation equilibrium is extremely fast and of the order of $10^7\ \text{M}^{-1}$ sec^{-1} as estimated by stopped-flow measurement; whereas at acidic pH values slow fixation is observed (10^3–$10^4\ \text{M}^{-1}\ \text{sec}^{-1}$).

A typical titration curve read at 413 mμ (Figure 5) plotted against an increasing heme concentration consists of two straight-line portions, the former representing the quantitative association of heme to the apoprotein with essentially no free hematin in solution and the latter, the accumulative absorption of free hematin, which is parallel to a curve of free hematin in the same phosphate buffer and at the same wavelength.

The values of ε calculated from the initial linear increment of absorbances of the bound heme in function of the heme concentrations are given in Figure 1. At 413 mμ, the maximum of the γ-oxidized band, the ε found is in excellent agreement with the corresponding untreated hemoprotein. A similar accord is found for the apoprotein t where a mean value of 113 $\text{mM}^{-1}\ \text{cm}^{-1}$ is to be compared with the value of 112 $\text{mM}^{-1}\ \text{cm}^{-1}$ for the untreated hemoprotein.

As expected the fluorescence of the protein moiety (excited at 278 mμ) is quenched upon heme fixation (Figures 4 (right) and 5). Upon titration, the equivalent point is identical with that measured spectrophotometrically. In several of the early preparations of the apoprotein, the tryptophan fluorescence was initially almost completely quenched (as in Figure 4 (right)) but progressively increased over a period of 4 days (three to four times) during which time the tyrosine

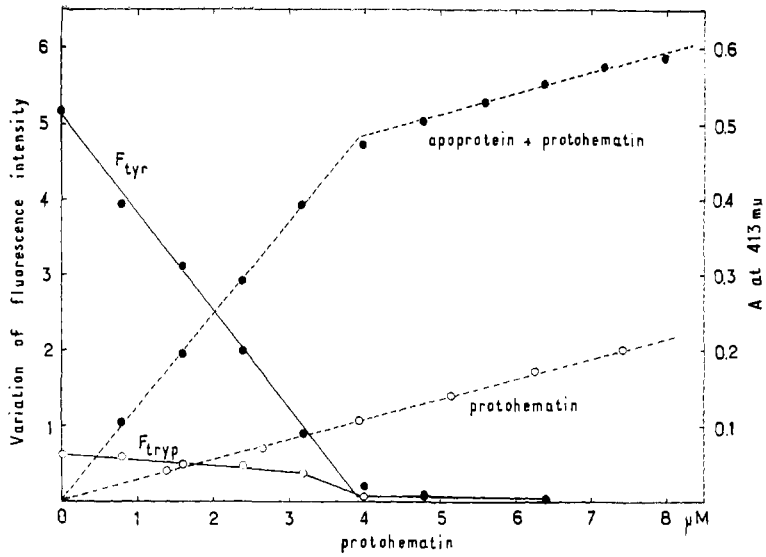

FIGURE 5: Titration curves of the apoprotein with heme, observed by spectrophotometric and spectrofluorometric measurements. Data from experiments, *cf.* Figure 5. Apoprotein s 4.0 μM in 0.1 M phosphate (pH 7.3, 22°). Absorbances measured against buffer at 413 mμ of (1) apoprotein plus protohematin (●---●) (the initial slope giving the ε value of the bound heme) and (2) protohematin (○---○). Fluorescence intensities (excited at 278 mμ) read at 302 mμ for tyrosine (●——●) and 360 mμ for tryptophan (○——○) are corrected for dilution and protohematin absorption and are substracted from curve 10 (Figure 4 (right)) and not from the buffer level, since the fluorescence between these two spectra is considered to be due to an impurity. It should be mentioned that the aspect of the $F_{tryptophan}$ curve varies from one preparation to another (for both s and t apoprotein), the break in the curve occurring when between 60 and 80% of the apoprotein is titrated. Correlation between the observed heterogeneity (see text) and this break is probable.

fluorescence remained constant. It should be noted, however, that even when the apoprotein shows a strong tryptophan fluorescence, a total quenching is, nevertheless, observed upon protohematin titration.

Discussion

It has been shown previously (Labeyrie *et al.*, 1966) that the heme–protein linkage is likely to be essentially the same in the cytochrome b_2 itself (mol wt 183,000) as in the low molecular weight derivative called noyau cytochromique b_2 (mol wt 11,000). So far, all the properties studied of the bound heme (visible absorption spectra and redox potential) are very similar. Thus the reversible resolution of the noyau cytochromique b_2 into its heme and apoprotein moiety, will afford the possibility of the elucidation of the residues implicated in the binding of the heme to the cytochrome b_2 (L-lactate dehydrogenase). However, examples such as hemoglobin, cytochrome *c*, and cytochrome b_5 have previously shown how difficult it is to acquire such information with certainty.

It should be noted that the preparations of noyau cytochromique b_2, obtained either by trypsic hydrolysis of cytochrome b_2 or "spontaneously" in the supernatant of recrystallization of the same protein, were both heterogenous on polyacrylamide migration (the same two or three hemoproteins bands) or on Sephadex G-100 in water, though they behave quite homogenously on Sephadex G-100 in saline medium (O. Groudinsky, unpublished results). The explanation of this heterogeneity and the variation in the number of bands has been discussed elsewhere (Labeyrie *et al.*, 1966) and is still not clearly understood. However it is likely that the differences between the molecular species involved are due to slight differences in amino acids content. Such a heterogeneity, linked to very small variations in terminal residues, has been observed by Strittmatter and Ozols (1966) on cytochrome b_5. It is interesting to note that many similarities (molecular weight, absorbance, and magnetic properties; Watari *et al.*, 1967) exist between this liver microsomal cytochrome and the noyau cytochromique b_2.

Also of interest is the fact that the same tryptophan content (one residue per mole of heme) and about the same tyrosine content (three to four residues per mole of heme) are found for cytochromes of approximately the same molecular weight, such as cytochrome *c* (horse heart muscle; Margoliash *et al.*, 1961), yeast iso I and iso II (Slonimski *et al.*, 1965)), cytochrome b_5 (Strittmatter, 1960; Strittmatter and Ozols, 1966), and noyau cytochromique b_2 as is shown in this paper from spectral measurements. Thus, it seems quite probable that the different electrophoretic components of the latter have the same tryptophan and tyrosine content.

The apoprotein moiety of the noyau cytochromique

b_3 is fluorescent while that of the initial hemoprotein is completely quenched. This fluorescence has the typical aspect of that of tyrosine, the contribution of the tryptophan being very low. The value obtained for tyrosine quantum yield ($q_0 = 0.03$) falls within the range usually found for other proteins (Teale, 1960; Weber and Young, 1964) since tyrosine is always quenched when integrated in the protein structure.

As for the tryptophan fluorescence, the value $q_0 = 0.025$ observed for apoprotein, appears exceptionally small as compared with data for other proteins without prosthetic groups; these are around 0.1 for trypsin, trypsinogen, and chymotrypsin; around 0.2 for ovalbumin and carboxypeptidase (Teale, 1960); as high as 0.57 for serumalbumin (Weber and Young, 1964); and 0.44 for glutamate dehydrogenase (Iwatsubo and di Franco, 1967a). The interpretation of the low tryptophan fluorescence of the apoprotein is not clear. However, during the denaturation of this apoprotein by urea, this fluorescence increases tenfold (until $q_{0\,\text{tryptophan}} = 0.20$) while the tyrosine fluorescence remains fairly constant. An analogous increase of tryptophan fluorescence was also observed during aging of some of the early preparations. As a general effect, urea seems to give a leveling of quantum yield values for many proteins toward 0.20 regardless of whether the values before treatment are higher or lower, as can be noted from data presented by Teale (1960) and others. The fact that the tryptophan is strongly quenched in the "native" apoprotein of noyau cytochromique b_2 and fluoresces after denaturation suggests the existence of a structured conformation of this molecule even after the removal of heme.

The addition of heme to apoprotein reestablishes the spectral and fluorescence properties of the initial hemoprotein. The fluorescence is quenched about 95%. This is a general phenomenon observed by Weber and Teale (1959) for a number of hemoproteins, and attributed to a resonance-transfer mechanism, that is to say, that the degree of quenching is a function of the distance between the heme and these aromatic groups, and more or less efficient following whether or not the planes of these two rings are parallel or perpendicular. As for the noyau cytochromique b_2 (mol wt 11,000) which is probably an entire chain and not a fragment obtained by breaking of peptide bonds of cytochrome b_2 (mol wt 183,000), the heme acts on all the tyrosines and tryptophans present. The comparison of the fluorescence of the flavocytochrome b_3 and of cytochrome b_2 (flavin-free apoenzyme) shows that since the latter is notably fluorescent, and as the former is not, the heme and the flavin do not act on the same ensemble of tyrosines and tryptophans (Iwatsubo and di Franco, 1967b). This suggests that these two prosthetic groups are either distant from each other and/or in orthogonal planes.

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