AFFINITY OF PROTOHEME FOR THE APOPROTEIN OF CYTOCHROME $\underline{b}_2$

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A comparison of the affinities of the apoproteins of cytochrome $\underline{b}_2$ (E.C. 1.1.2.3) and myoglobin for protoheme allows one to estimate a value of 10^{-15} M for the dissociation constant of the reconstituted hemoprotein of cytochrome $\underline{b}_2$. In the initial untreated enzyme the affinity is still higher. Evidence is given of a binding of extra heme after saturation of the functional sites; the chromophore thus formed has different spectral characteristics.

Morton (1) and Morton and Shepley (2) have been the firsts to show that the heme group could be dissociated from cytochrome $\underline{b}_2$*, a tetrameric flavohemoprotein of 235,000 daltons (3-6) extracted from yeast. Their preparations of apoprotein were able to recombine partially with hemin with restoration of a hemochrome spectrum. Recently, improved methods for obtaining this apoprotein have been developped; the recombination with a stoichiometric amount of heme was observed and an upper limit of the value of the dissociation constant, 10^{-8} M has been fixed (7).

The present communication reports on competition experiments aimed at a better determination of the dissociation constant of the hemoprotein complex.

Cytochrome $\underline{b}_2$ was extracted from dried yeast according to Spyridakis et al. (8) and horse heart myoglobin was obtained from Calbiochem. The apoproteins were prepared either by the acid-acetone or the guanidine hydrochloride-mercaptoethanol treatments described by Mevel et al. (7).

In a first step, the reassociation of hemin with the apoprotein of cytochrome $\underline{b}_2$ was carried out in the presence of low molecular weight molecules known as heme ligands. Fluoride, histidylhistidine and cyanide were tried. At pH 7, the cyanide ion is the strongest of these ligands and displaces the others.

* L-lactate cytochrome $\underline{c}$ oxido reductase (E.C. 1.1.2.3)

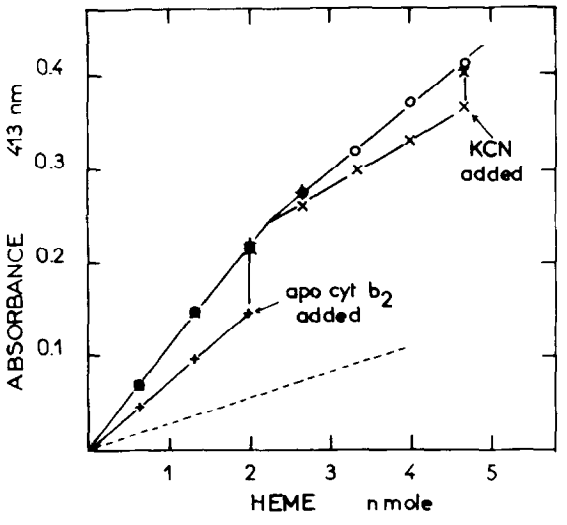

Fig. 1 : Competition between the apoprotein of cytochrome $\underline{b}_2$ and the cyanide ion for protoheme

(x) Titration of the apoprotein (2.15 nanomoles) with hemin, the arrow indicates a final addition of KCN (2.5 nM).
(o) Titration of the apoprotein with hemin in the presence of KCN (2.5 mM).
(+) Additions of hemin to a 2.5 mM KCN solution, the arrow indicates an addition of apoprotein.
The dashed line shows the increase of absorbance when free hemin is added to the buffer (phosphate 0.1 M, pH 8).

Fig. 1 presents the results of an investigation of the interactions of protoheme, cyanide and the apoprotein of cytochrome $\underline{b}_2$. When titrations of the protein with hemin are carried out in the presence or absence of cyanide, until the titration end point, the same absorbance changes are observed ; after the break, the slopes are different indicating that cyanide binds heme in excess. When the heme dicyanide complex is first formed and then the apoprotein added, the typical Soret peak of cytochrome $\underline{b}_2$ is reconstituted demonstrating the transfer of heme from the cyanide complex to the apoprotein. This could be expected since cyanide was found to have no effect on the visible spectrum and the enzymic activity of the holoenzyme.

Observing the action of cyanide at various stages of a reconstitution experiment afforded another mean to bring up the binding of non functional heme groups onto the reconstituted hemoprotein. It is seen in Fig. 2 that addition of cyanide does not affect significantly the absorption at 420 nm of the reconstituted hemoprotein. In contrast, alterations are observed when CN^- is allowed to react with hemin in the presence or absence of an identical amount of hemoprotein. One can notice that the absorbance increments are different as are the rates of reaction. The most plausible interpretation is that heme in

excess binds the hemoprotein at non specific sites to form weak complexes which are affected by the small ligand or a dialysis (7). This observation correlates the fact that, after the titration end-point, the slope of the titration curve differs from that obtained when hemin is added to the buffer (Fig.1).

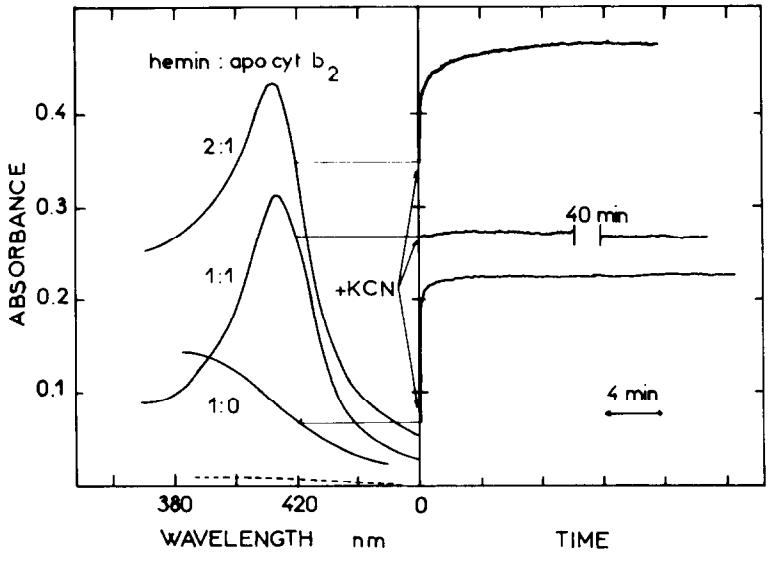

Fig. 2 : Effect of the addition of cyanide at various stages of a titration. On the left hand site are shown parts of the initial spectra and on the right hand side, the evolution of the absorbance at 420 nm following an addition of KCN (22 mM).
A - free hemin (2.6 µM).
B - Reconstituted hemoprotein of cytochrome $\underline{b}_2$ (2.6 µM).
C - A mixture of 2 equivalents of hemin and 1 of apoprotein (2.53 µM).
(0.5 M NaCl, 0.05 M phosphate, pH 7).

In a second step, an attempt was made to see whether it was possible to observe a heme transfer between a native hemoprotein and the apoprotein of cytochrome $\underline{b}_2$. Horse metmyoglobin, the dissociation of which has been studied by Banerjee (9), was added to an apoprotein solution ; the heme exchange was manifested by slow spectral changes. After three hours, the wavelength of the Soret peak had shifted from initial 409 nm to 413 nm, characteristic of the cytochrome $\underline{b}_2$ hemochromophore. Addition of dithionite displaced the maximum toward 425 nm. Fig. 3 allows one to compare the spectra of the reconstituted hemoprotein of cytochrome $\underline{b}_2$, metmyoglobin and of a mixture of metmyoglobin and the apoprotein of cytochrome $\underline{b}_2$ after a period of time. A complete transfer has not been observed with apoprotein concentrations up to three times that the metmyoglobin, the affinities of the two proteins for protoheme appear to be of the same order of magnitude.

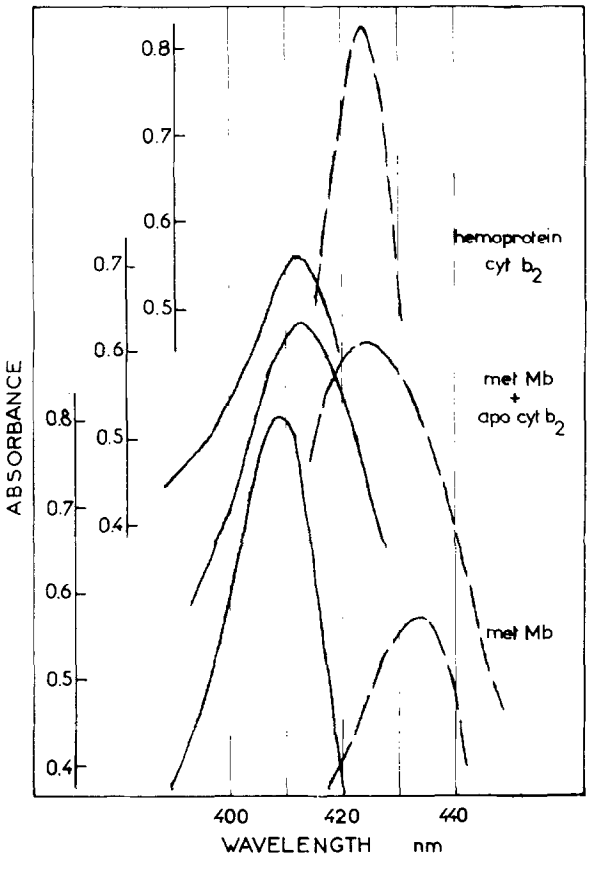

Fig. 3 : Heme transfer between metmyoglobin and the apoprotein of cytochrome $\underline{b}_2$. The full lines correspond to spectra of the oxidized forms, the dashed lines to the reduced by dithionite forms. The middle traces show the Soret peaks of a mixture of the apoprotein of cytochrome $\underline{b}_2$ (7.5 µM) and horse metmyoglobin (5 µM) after 3 hours of incubation in 0.2 M NaCl, 0.2 M phosphate, pH 7. For reference, the peak positions of the reconstituted hemoprotein of cytochrome $\underline{b}_2$, upper traces, and metmyoglobin, lower traces, are given.

Another approach to estimation of the dissociation constant of the heme prosthetic group of cytochrome $\underline{b}_2$ consisted in preparing the two apoproteins apomyoglobin and apocytochrome $\underline{b}_2$, and observing the competition for added heme. When this was done, one observed first the appearance of a Soret maximum centered at 409 nm slowly shifting to 411 nm, half way between the absorption maxima of metmyoglobin and cytochrome $\underline{b}_2$. Fig. 4 shows titration curves of each apoprotein and a mixture of both with hemin at pH 7.3. With both proteins in almost equal concentrations, heme is distributed equally between the two indicating a similar affinity.

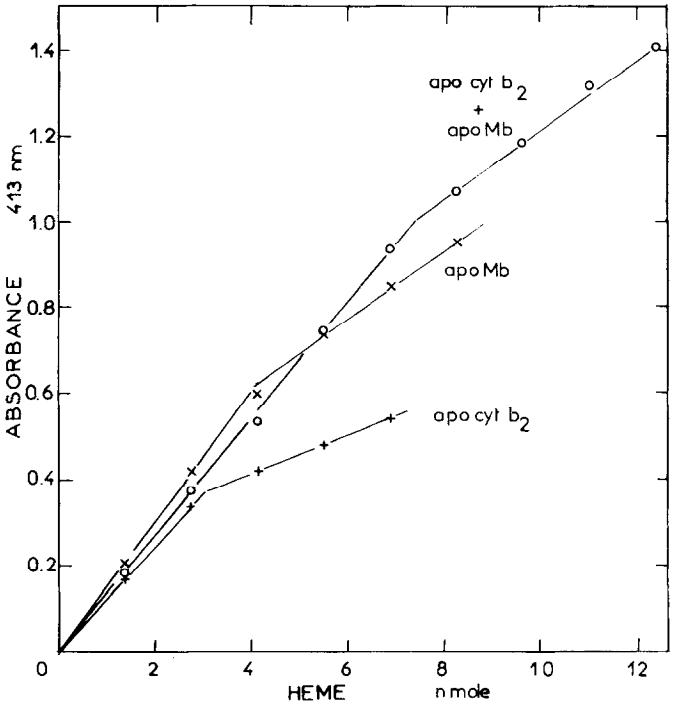

Fig. 4 : Competition between the apoproteins of cytochrome $\underline{b}_2$ and myoglobin for protoheme.

Apomyoglobin (4.06 nmole), apocytochrome $\underline{b}_2$ (3.03 nmole) and a mixture of both are titrated with hemin. After each addition, the spectrum is recorded off and on and the readings are made when the absorbance has reached a plateau for 5 minutes.

Banerjee (9) has defined the equilibrium constant for the dissociation of a hemoprotein to yield dimeric hemin and protein,

$$2\,HP \rightleftharpoons 2\,P + H_2$$

$$K = \frac{(P)^2 \quad (H_2)}{(HP)^2}$$

and has given a value $K = 10^{-15}$ M at 25° for metmyoglobin. A figure of the same order of magnitude applies to the reconstituted hemoprotein of cytochrome $\underline{b}_2$. The actual value, for untreated crystalline cytochrome $\underline{b}_2$ is still lower since it has been impossible to obtain any spectral evidence of a heme transfer when holoenzyme (5.5 µM) was incubated with apomyoglobin (17.3 µM) for three days, at pH 7.1. This was verified with the oxidized and reduced forms of cytochrome $\underline{b}_2$, confirming an unpublished observation by Baudras and Banerjee.

This communication reports a new case of heme transfer between proteins,

the possibility of such a phenomenon was suggested in 1957 by Ycas and Drabkin (10) and first observed by Rossi-Fanelli and Antonini when mixing horse apomyoglobin or apohemoglobin with aplysia myoglobin (11). The exchange of heme among hemoglobin and albumin in vivo was more recently reported by Bunn and Jandl (12). The use of heme exchange for studying certain properties of hemoproteins is thus possible as it was shown by Muller-Eberhard et al. who have investigated the role of heme in P 450 in removing it with apomyoglobin and hemopexin (13).

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