Fluorescence and Phosphorescence of Yeast L-Lactate Dehydrogenase (Cytochrome b_2). Relative Orientations of the Prosthetic Heme and Flavin*

Jean-Loup Risler

ABSTRACT: The phosphorescence of yeast L-lactate dehydrogenase (cytochrome b_2) has been studied and compared to its fluorescence. It was already demonstrated that the tryptophan fluorescence is quenched only by flavin mononucleotide (FMN), not by heme. This study, on the contrary, showed that the protein phosphorescence is quenched only by the heme groups, not by FMN. This effect suggests the occurrence of a triplet–singlet energy transfer from tryptophans to heme. The critical transfer distances Trp → heme in reduced cytochrome b_2 were calculated to be 38 and 34 Å, respectively, for fluorescence and phosphorescence, whereas the molecular diameter of one protomer is about 40 Å. Thus, the reciprocal behavior of the tryptophan fluorescence and phosphorescence toward quenching by heme and FMN cannot be explained by too great a distance between chromophores and is best interpreted as being due to the perpendicularity of the emission oscillators of tryptophan. It is thus concluded that the FMN and heme planes must be roughly orthogonal.

Bakers' yeast cytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase, EC 1.1.2.3) is now known to be a tetrameric enzyme of molecular weight 235,000 bearing four hemes and four FMN prosthetic groups (Jacq and Lederer, 1970; Pajot and Groudinsky, 1970; Monteilhet and Risler, 1970). Unlike other hemoproteins such as hemoglobin, myoglobin (Truong *et al.*, 1967), peroxidase, and catalase (Weber and Teale, 1959), the deflavocytochrome b_2 (still possessing four hemes) exhibits a marked protein fluorescence (Iwatsubo and di Franco, 1968). In particular, it was shown by these last authors that, among the five tryptophans per protomer of 58,000, the fluorescence of one tryptophan was completely quenched, whereas that of the other four was not affected by the heme group. To explain this somewhat uncommon fact, two alternative explanations, based on Förster's (1959) theory of energy transfer, were considered (Labeyrie *et al.*, 1967). That is to say, either the tryptophans are far from the heme and close to the FMN, the two prosthetic groups being thus relatively far apart, or the fluorescence oscillators of tryptophans are parallel to the absorption oscillator of FMN and perpendicular to that of the heme.

The question is therefore raised as to whether the luminescence properties of cytochrome b_2 are determined by the distances between chromophores or by their mutual orientation. To solve this problem, we undertook a phosphorescence study of cytochrome b_2 and its derivatives, since it is known (Konev, 1967) that the phosphorescence oscillator of tryptophan is perpendicular to the indole plane, whereas the fluorescence oscillator lies in this plane. Therefore, if the orientation factor is of predominant importance the fluorescence and phosphorescence properties should be complementary. In fact, this complementary effect seems to be verified as will be shown.

Material and Methods

Type-II (DNA-free) cytochrome b_2 was obtained according to Symons (1965) from type-I crystals prepared following the method of Appleby and Morton (1954) as modified by Spyridakis *et al.* (1971). The solution was then desalted and oxidized by passing through a small column of Sephadex G-25 equilibrated in 0.1 M phosphate–1 mM EDTA (pH 7.0) and reduced, when necessary, by addition of a small amount of DL-lactate. The concentration, expressed in heme content, was estimated from the absorptivity of the reduced γ band (ϵ_{424} 183 mM^{-1} cm^{-1}) (Pajot and Groudinsky, 1970).

Deflavocytochrome b_2. The derivative without FMN—which still binds the hemes—was prepared by acid–sulfate treatment according to Baudras (1965). Recovery of 60% of the initial enzymic activity was usually obtained after FMN addition. For the reduced γ band, ϵ_{424} 181 mM^{-1} cm^{-1} was used.

Apocytochrome b_2. An apoprotein containing neither heme nor FMN, but which reversibly binds protoheme, was prepared according to Mével-Ninio *et al.* (1971) in the following way. A fresh enzyme solution was precipitated in 60% saturated ammonium sulfate (pH 7.0). The precipitate was dissolved in 6 M guanidine hydrochloride–0.1 M phosphate (pH 7.2) to a final concentration of 30–40 μM. The solution was allowed to stand for 1 hr at 0°, and then passed through a Sephadex G-25 column (1 × 15 cm) equilibrated in the same buffer, in order to remove the dissociated heme and flavin. The eluate was dialyzed at 4° for 60 hr against 50 mM DL-lactate, 20 μM EDTA, 0.1 M β-mercaptoethanol, and 0.2 M phosphate (pH 7.2), and then passed through a Sephadex G-25 column (1 × 20 cm) equilibrated in 0.2 M phosphate–20 μM EDTA (pH 7.2) to remove lactate and mercaptoethanol. The protein concentration was then estimated according to F. Labeyrie *et al.* (1970, personal communication) by comparing its tryptophan fluorescence in 6 M guanidine hydrochloride (excitation 295 nm) with that of free tryptophan added to this same solution, and assuming five tryptophans per heme, *i.e.*, per 58,000 g of protein.

Protoporphyrin IX was prepared from the dimethyl ester (Sigma) by hydrolysis in 5 N HCl (Grinstein, 1947). The extinc-

* From the Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique, 91-Gif-sur-Yvette, France. *Received November 13, 1970.* This work has been supported in part by a grant from the Délégation Générale à la Recherche Scientifique et Technique. This work constitutes a portion of a thesis to be submitted to the Université de Paris, Faculté des Sciences d'Orsay, for completion of a Doctorat ès Sciences.

tion coefficient in 2.7 N HCl is $\epsilon_{408} = 262$ mM^{-1} cm^{-1} (Falk, 1964a).

Fluorescence and Phosphorescence. The protein emission was studied on a spectrofluorometer built in the laboratory (Iwatsubo and di Franco, 1965) and slightly modified for phosphorescence studies with a rotating shutter. The light source was a 450-W xenon arc, and emission was recorded through a selected EMI photomultiplier (9558 QA). The gratings used (Bausch & Lomb) were blazed at 300 and 500 nm, for fluorescence and phosphorescence measurements, respectively. The excitation and emission bandwidths were adjusted to 5 nm for all spectra with excitation wavelength set at 295 nm, so that only the tryptophans were excited. Phosphorescence lifetimes were obtained by rapidly shutting the excitation slit and recording the phosphorescence decrease on a rapid-scanning recorder, or directly by observing on a storage oscilloscope screen the phosphorescence transmitted through the rotating shutter. Plots of $\ln I = f(t)$ gave straight lines of slopes equal to $1/\tau$. The luminescence spectra were obtained at 77°K, either by dissolving the sample in 0.1 M Tris-HCl (pH 7.0) containing 0.5% sucrose by weight—which gives a poor glass—or in a concentrated sucrose solution (14 g of sucrose in 10 ml of Tris buffer) which gives a perfectly transparent glass with practically no background emission. Both techniques gave the same quantitative results.

Because of the very strong fluorescence of the quartz capillaries and Dewar flasks, we could not measure simultaneously the protein fluorescence and phosphorescence. We therefore adopted the following procedure: the protein fluorescence was measured at room temperature (22°) and compared to the fluorescence of tryptophan; then, the protein phosphorescence was recorded at 77°K and also compared to the phosphorescence of tryptophan under the same conditions (excitation was always at 295 nm). P and F being, respectively, the integrated phosphorescence and fluorescence emissions, the ratio P/F was arbitrarily set at 1.0 for free tryptophan and the relative P/F ratio of the protein was subsequently calculated. Since the quantum yields of the tryptophan fluorescence and phosphorescence vary with temperature, we preferred to employ P/F ratios rather than quantum yields; the conditions of the protein fluorescence measurements, on the one hand, and those of the protein phosphorescence, on the other hand, being precisely defined, the relative P/F ratios are a reproducible and sensitive way of comparing the native protein and its derivatives. Moreover, the use of P/F is also useful when one deals with phosphorescence quenching.

Results

We have limited ourselves to the influence of the two prosthetic groups on the tryptophan fluorescence and phosphorescence by choosing an excitation wavelength of the protein luminescence such that only the tryptophan and *not* the tyrosine absorbs, *i.e.*, by exciting at 295 nm.

Tryptophan. In order to verify the performance of our apparatus, we first studied the phosphorescence of free tryptophan; the features of the spectra obtained (Figure 1) are in agreement with those already published (Truong *et al.*, 1967; Konev, 1967). The phosphorescence lifetime was found to be 6.3 ± 0.3 sec, a value which is consistent with those reported by others (Weinryb and Steiner, 1968; Steiner and Kolinsky, 1968).

Holocytochrome b_2. The fluorescence of the holocytochrome b_2 is 70% quenched by FMN (Iwatsubo and di Franco, 1968) while the phosphorescence of the native protein is weak

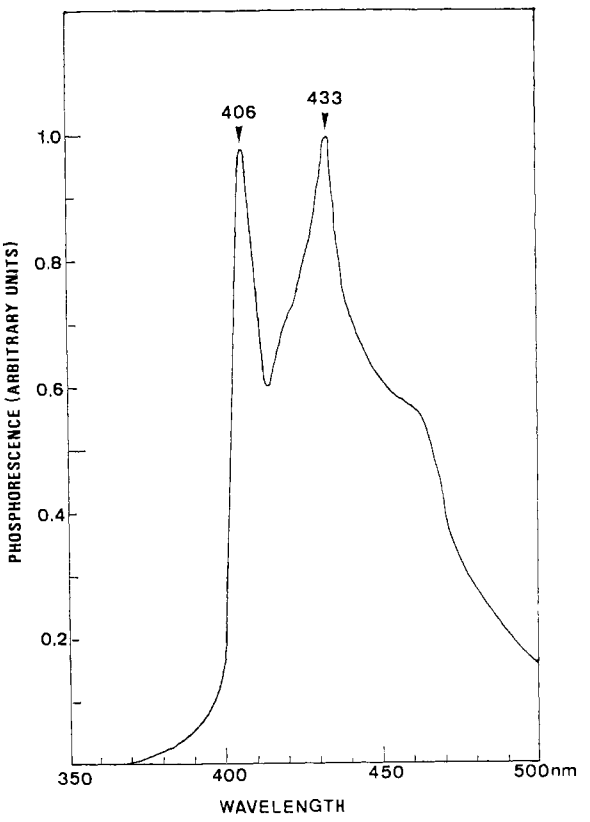


FIGURE 1: Phosphorescence spectrum at 77°K of tryptophan. Tryptophan was first dissolved in 0.1 M Tris-HCl–1 mM EDTA (pH 7.0) containing 0.5% sucrose by weight, and then brought to liquid nitrogen temperature. Excitation 295 nm. The spectrum is not corrected for photomultiplier response and grating transmission.

(Figure 2, curve b). The relative P/F ratio was found to be 0.4 (P/F arbitrarily set at 1.0 for free tryptophan) and the phosphorescence lifetime was 3.8 ± 0.3 sec, when in either the oxidized or reduced state. The 406-nm phosphorescence band is relatively lower in hemoproteins because of reabsorption of the emitted light by the strong heme Soret band. Corrections were calculated to account for this effect. At the protein concentration employed (3–5 μM) and a capillary radius of 1 mm, of the measuring cuvet, the corrections on the integrated phosphorescence spectra were of the order of 5–10%.

Deflavocytochrome b_2. The tryptophan phosphorescence of the FMN-free enzyme, which still binds the hemes, was about three times greater than in the holocytochrome b_2 (Figure 2, curve a), but the P/F ratio and the phosphorescence lifetime remained, respectively, 0.4 and 3.8 sec, regardless of the oxidation state of the heme. In Figure 2 (insertion) is also presented an oscilloscopic tracing of the decrease in phosphorescence of the deflavoenzyme, together with a plot of $\ln I = f(t)$. It can be seen that this plot is linear in the time scale used, thus a single lifetime is assumed. Since there are four emitting tryptophans per heme (Iwatsubo and di Franco, 1968), this suggests that these tryptophans are affected roughly in the same way.

Apocytochrome b_2. The fluorescence spectrum of the apoprotein showed a shift of the maximum from 355 nm in 6 M guanidine hydrochloride (pH 7.2) to 325 nm in 0.2 M phosphate (pH 7.2). Mention should be made that ultracentrifuge sedimentation determinations (Mével-Ninio *et al.*, 1971) performed on the same apocytochrome in 0.15 M phosphate (pH 7.2) gave a value of $s_{20,w}$ of about 7 S, a value quite comparable

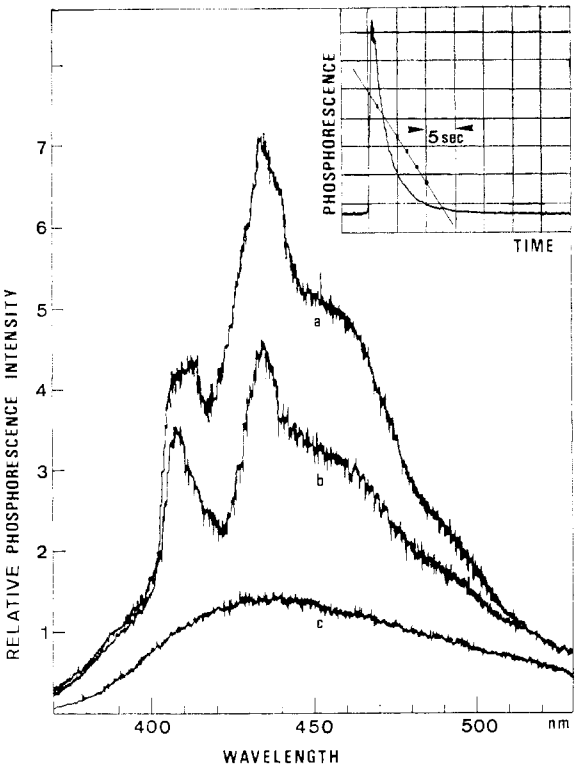


FIGURE 2: Phosphorescence spectra of (a) 3.8 μM deflavocytochrome b_2, (b) 5.5 μM holocytochrome b_2, and (c) solvent emission. Excitation 295 nm. Insertion: phosphorescence lifetime of the deflavocytochrome b_2, obtained by oscilloscopic recording of the light transmitted through the rotating shutter. Same conditions as in Figure 1.

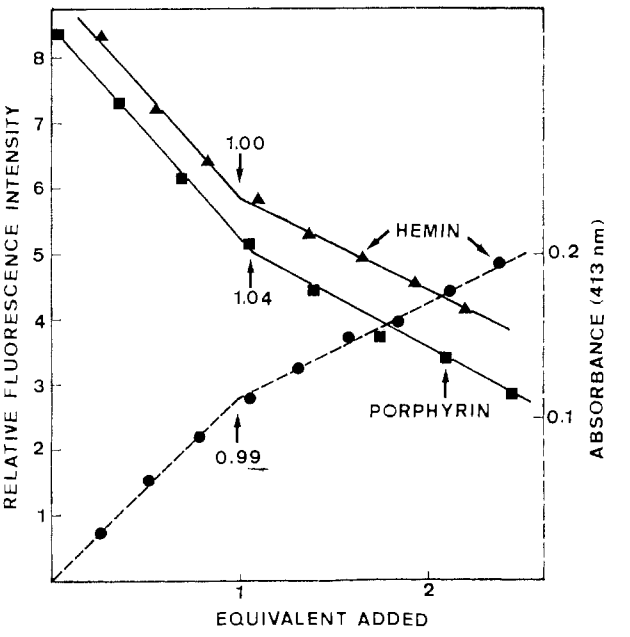


FIGURE 3: Binding of protoheme and protoporphyrin to apocytochrome b_2; the binding was followed by absorbance increase in the Soret band (413 nm) and by protein fluorescence quenching (excitation 295 nm, observation 325 nm). The concentration of the apoprotein was 1.41 μM. Conditions: 0.1 M Tris-HCl–1 mM EDTA (pH8.0), 25°.

to that of the holocytochrome b_2. These studies also showed that the binding of heme to the apoprotein did not affect the association state of the protein. From these remarks, we can conclude that the apocytochrome b_2 was in a "native" state.

A stoichiometric binding of protoheme to the apoenzyme has been observed by Mével-Ninio *et al.* (1971) as seen by both the absorbance increase in the Soret band and the protein fluorescence quenching (Figure 3). In a similar manner, we could observe that a stoichiometric binding of protoporphyrin to the apoprotein also occurs (Figure 3). The fluorescence properties of both the free and protein-bound protoporphyrin have been investigated, and it is shown in Figure 4 that they are markedly different. By mounting two polarizers, after the excitation slit and before the emission one, we could also measure the fluorescence polarization of protoporphyrin (excitation 510 nm, observation 628 nm) which was shown to increase from less than 1% with free porphyrin to 27% in the presence of apocytochrome b_2. This result, and those presented in Figure 4, indicate that protoporphyrin is tightly bound to the protein moiety. Nevertheless, we did not observe any sensitized fluorescence at 628 nm upon excitation at 295 nm. This is an indication of the lack of resonance transfer from tryptophans to heme (or porphyrin), and we shall discuss this point later on. It is worth noting that we did not observe any phosphorescence of the protoporphyrin, either free or bound to the protein, under our experimental conditions.

The relative P/F ratio of the apoenzyme was found to be 1.0 and the phosphorescence lifetime 6.3 sec, both of these values being the same as for free tryptophan.

Discussion

Let us first briefly recall the theory of resonance energy transfer developed by Förster (1959), which is valid for a $S \rightarrow S^1$ or a $T \rightarrow S$ transfer. The critical transfer distance, that is the distance at which the probability of energy transfer equals the probability of deexcitation by all other modes, is given by

$$R_0^6 \propto \phi K^2 J(\nu)$$

ϕ is the donor emission quantum yield; K^2 is an orientation factor which is maximum when the donor emission and acceptor absorption oscillators are parallel, and zero when they are perpendicular; $J(\nu)$ is an overlap integral defined by

$$J(\nu) = \int F_D(\nu)\epsilon_A(\nu)\frac{d\nu}{\nu^4}$$

where $F_D(\nu)$ is the donor spectral emission normalized to unity, and $\epsilon_A(\nu)$ the decadic molar extinction coefficient of the acceptor.

Some critical transfer distances involving tryptophan as a donor of fluorescence are available from the literature; in particular, R_0 was calculated to be 26 Å for the transfer Trp → oxidized FMN (Karreman *et al.*, 1958) and 37 Å for the transfer Trp → heme in carboxymyoglobin (Stryer, 1960). We can therefore calculate R_0 for the transfer Trp → heme in deflavocytochrome b_2 by comparing the overlap integral of this last system and those reported above. We thus found, for reduced deflavocytochrome b_2, values of 36 and 39 Å, respectively, by

[1] Abbreviations used are: S → S, singlet–singlet; T → S, triplet–singlet; T → T, triplet–triplet.

using the data of Karreman *et al.* (1958) and Stryer (1960). We shall therefore take for R_0 a mean value of 38 Å.

From this value, the critical transfer distance Trp → heme in phosphorescence (for a T → S transfer) can be estimated from the relation

$$\frac{R_{0P}^6}{R_{0F}^6} = \frac{\phi_P J(\nu)_P}{\phi_F J(\nu)_F}$$

where P and F refer to phosphorescence and fluorescence, respectively, the same orientation factor being assumed in both cases. The ratio $J(\nu)_P/J(\nu)_F$ was evaluated graphically, ϕ_P/ϕ_F is 0.25 at 77°K (Weber and Teale, 1965; Bishai *et al.*, 1967) so that R_{0P} is 34 Å for reduced deflavocytochrome b_2. This value of R_{0P} may seem high in view of the low phosphorescence quantum yield, but in fact the very high absorptivity of the heme Soret band causes the overlap integral $J(\nu)_P$ to be large, which compensates the decrease in quantum yield.

We can now compare these values of R_{0F} and R_{0P} to the molecular dimensions of the protein. From X-ray diffraction studies (Monteilhet and Risler, 1970) and electron microscopy data (L. Benedetti *et al.*, unpublished results; Burgoyne *et al.*, 1967), it is now clear that a molecule of cytochrome b_2 (235,000) is composed of four protomers, each bearing one heme and one FMN, and each having a spheroidal shape with a diameter of 40–45 Å. This value compares well with the critical transfer distances Trp → heme in fluorescence and phosphorescence.

The fluorescence studies performed by Iwatsubo and di Franco (1968) on holocytochrome b_2 and its deflavo derivative (hemoprotein) showed that there was no S → S energy transfer from tryptophans to heme since the protein fluorescence quenching is very low and does not depend on the reduction state of the heme in spite of the marked difference between its reduced and oxidized spectra. This is clearly supported by the fact that we have not detected any sensitized fluorescence of the protein-bound protoporphyrin upon excitation at 295 nm. Cytochrome b_2 thus appears as a unique hemoprotein in which the tryptophan fluorescence is not quenched by the heme and, considering the critical transfer distance calculated above, this cannot be explained by too great a distance between tryptophans and heme. It may thus be concluded that the orientation factor is responsible for this lack of fluorescence quenching.

The phosphorescence studies presented above have shown, in both the holocytochrome b_2 and its deflavo derivative, a quenching of the tryptophan phosphorescence (P/F = 0.4) and a marked (40%) reduction in its lifetime, whereas in the apoprotein this phosphorescence was identical with that of free tryptophan. Thus, the presence of the heme group considerably alters the protein phosphorescence. In fact, we cannot obtain direct evidence that this quenching is due to energy transfer from tryptophan to heme, since we could not measure the P/F ratio of the acceptor; protoheme is self-quenched and protophorphyrin phosphorescence was not detected in our apparatus. Moreover, $J(\nu)_P$ increases only by 9% when the heme is reduced, a value which is of the order of experimental uncertainty. It may thus be possible that the phosphorescence properties of the heme proteins, different from that of the apoenzyme, could arise from the influence of the environment of the indole residues. In this case, a conformational change affecting the tryptophan environment would occur upon binding of heme to the apoprotein.

Indeed, different values of the P/F ratios of tryptophan have been observed in papain (Weinryb and Steiner, 1970), trypsin

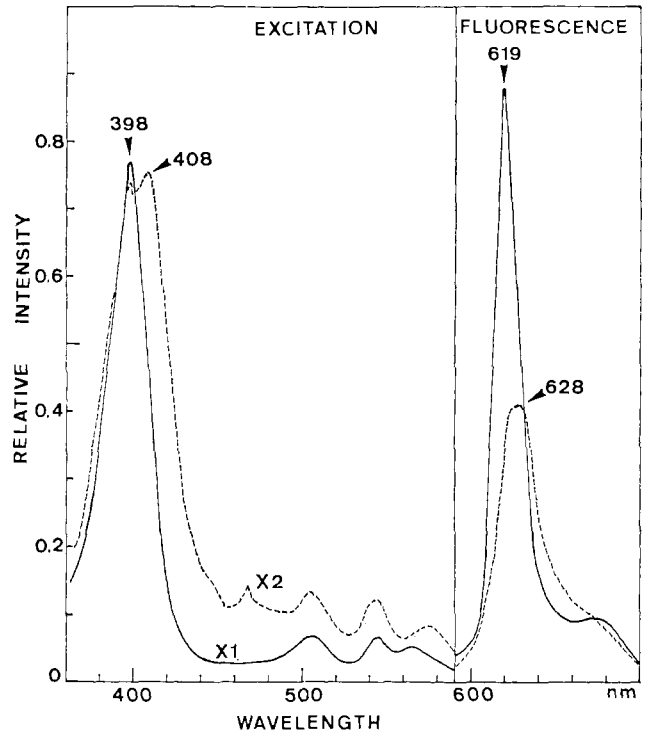


FIGURE 4: Fluorescence excitation spectra of free (——) protoporphyrin (observed at 619 nm) and protein-bound (---) protoporphyrin (observed at 628 nm), and fluorescence emission spectra of the same species. The excitation spectrum of the protein-bound porphyrin has been multiplied by a factor of 2. Same conditions as in Figure 3.

(Nag-Chaudhuri and Augenstein, 1964), and lysozyme (Longworth, 1966) but the phosphorescence lifetime was always practically equal to that of free tryptophan. A systematic study of Konev (1967) on one-tenth of the proteins, either native or denatured, also showed variations in the P/F ratios concomitant with a remarkable constancy in the phosphorescence lifetime. It was also shown, in a number of oligopeptides containing tryptophan, that its phosphorescence lifetime remained practically unchanged (Weinryb and Steiner, 1968; Steiner and Kolinsky, 1968). It thus seems that the influence of the peptide chain, if any, exerts a change on the rate of internal conversion rather than on the rate of return to the ground state. In this respect, the 40% decrease in the phosphorescence lifetime of holo- and deflavocytochrome b_2 is much bigger than in all the studies reported above, and therefore can be reasonably explained by an energy transfer from tryptophans to heme. Moreover, if the decrease in both the values of P/F and τ was not due to energy transfer, we would be confronted with the following situation. (i) The critical transfer distances Trp → heme in fluorescence and phosphorescence are practically equal to the molecular dimensions; (ii) the fluorescence and phosphorescence oscillators of tryptophan are perpendicular to each other, whereas (iii) neither the fluorescence nor the phosphorescence of tryptophans would be quenched by the heme groups. This would be most contradictory, and we thus think that the phosphorescence quenching in cytochrome b_2 can be reasonably and logically explained by an energy transfer from tryptophans to heme.

Does this transfer come from one or two, or from the four emitting tryptophans? In fact, the full three-dimensional structure of cytochrome b_2 is not yet available, and therefore we do not know whether all the tryptophans are close to each other and whether they are all oriented in the same direction.

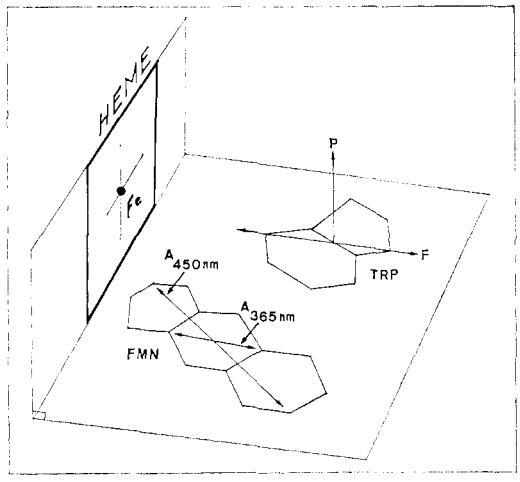


FIGURE 5: Tentative scheme showing the perpendicularity of the FMN and heme planes. This figure shows that (i) the fluorescence of tryptophan is quenched by FMN, not the phosphorescence and (ii) the phosphorescence of tryptophan is quenched by the heme, not the fluorescence. The relative positions of the chromophores are arbitrary.

However, the complete lack of fluorescence quenching by the heme group is extremely surprising and suggests the occurrence of a very particular spatial organization. We do not think that this lack of quenching could arise from a conformational change to a more expanded conformation in the deflavoprotein. In effect, if the distances and/or the orientations of tryptophans relative to heme were so strongly modified, upon removal of FMN, as to completely suppress the fluorescence quenching, these modifications would also affect the tryptophan phosphorescence quenching. This possibility can be eliminated since, as we have shown, the phosphorescence of the native and deflavoenzyme are identical. Also, it is important to note that we always observed a single phosphorescence lifetime in both the holo- and deflavocytochrome b_2, significantly different from that of free tryptophan; this lifetime is likely to be the mean value of several closely related lifetimes, which indicates that the tryptophans are affected roughly, if not exactly, in the same way by the heme groups, thus suggesting a similar distance and orientation relative to heme. This also implies that all the tryptophans are involved in energy transfer; should the phosphorescence of one or several indole residues be not quenched by the heme groups, their phosphorescence lifetime should be significantly higher and this would appear in the recordings of the phosphorescence decrease.

As far as the energy transfer from the tryptophans to the prosthetic flavin in the holocytochrome b_2 is concerned, a S → S transfer very likely occurs since the tryptophan fluorescence is 70% quenched by FMN (Iwatsubo and di Franco, 1968). On the contrary, there is obviously neither T → S nor T → T energy transfer from tryptophans to FMN since the phosphorescence properties (lifetime, P/F ratio) of the holocytochrome b_2 are identical with those of the deflavo derivative. Moreover, due to the calculated ratio $J(\nu)_P^{\text{FMN ox}}/J(\nu)_P^{\text{FMN red}}$ found to be 6.5, the tryptophan phosphorescence should increase considerably upon reduction of the prosthetic groups if energy transfer to FMN occurs. As it has been described above, this is not the case.

With the set of data presented in this paper, we are able to give a more precise picture of the relative orientations of heme, flavin, and tryptophans, since the positions of the various emission and absorption oscillators with which we are concerned are known; first, the two absorption oscillators of FMN corresponding to the 365-nm (which quenches the fluorescence) and 450-nm bands, lie in the isoalloxazine plane, making an angle of about 40° between them (Kurtin and Song, 1968); second, the γ (which quenches the phosphorescence) and δ absorption transition moments of protoheme are in the porphyrin plane (George and Griffith, 1961; Falk, 1964b) and third the fluorescence oscillator of tryptophan lies in the indole ring plane, whereas the phosphorescence oscillator is perpendicular to this plane (Konev, 1967). Thus, taking into account these data and ours, we arrive at the following interpretation: the indole planes are parallel to the isoalloxazine plane and perpendicular to the porphyrin ring (see Figure 5). In this case, the tryptophan phosphorescence is indeed quenched by the heme while the fluorescence is not. Moreover, the tryptophan fluorescence is quenched by FMN while the phosphorescence is not. It can therefore be concluded that the FMN and heme planes are roughly orthogonal.

As it was shown in the above discussion, the four emitting tryptophans in cytochrome b_2 probably present a great similarity in their distances and orientations relative to the heme, a fact which leads us to conclude that the FMN and heme planes are perpendicular. Of course, it would not be surprising if there did exist slight deviations between the different tryptophans, and what we are observing is actually a mean effect, thus making it impossible to give a precise value for the angle between heme and flavin. It is clear that the method we have described is best suited to proteins with low tryptophan contents, and we hope it may prove useful for elucidating relative orientations of chromophores in such proteins.

Acknowledgments

The author thanks Drs. F. Labeyrie and M. Iwatsubo for their constant help during the course of this work, Dr. P. Douzou for many helpful discussions, and A. di Franco for kindly correcting the manuscript.

References

Appleby, C. A., and Morton, R. K. (1954), *Nature (London) 173*, 749.

Baudras, A. (1965), *Biochem. Biophys. Res. Commun. 7*, 310.

Bishai, F., Kuntz, E., and Augenstein, L. (1967), *Biochim. Biophys. Acta 140*, 381.

Burgoyne, L. A., Dyer, P. Y., and Symons, R. H. (1967), *J. Ultrastructure Res. 20*, 20.

Falk, J. E. (1964a), *Porphyrins Metalloporphyrins 2*, 236.

Falk, J. E. (1964b), *Porphyrins Metalloporphyrins 2*, 85.

Förster, T. (1959), *Discuss. Faraday Soc. 27*, 7.

George, P., and Griffith, J. S. (1961), *in* Haematin Enzymes, Falk, J. E., Lemberg, R., and Morton, R. K., Ed., Oxford, Pergamon Press, p 139.

Grinstein, M. (1947), *J. Biol. Chem. 167*, 515.

Iwatsubo, M., and di Franco, A. (1965), *Bull. Soc. Chim. Biol. 47*, 891.

Iwatsubo, M., and di Franco, A. (1968), *in* Structure and Function of Cytochromes, Okunuki, M., Kamen, M. D., and Sekuzu, I., Ed., Baltimore, Md., Manchester, University Park Press, p 613.

Jacq, C., and Lederer, F. (1970), *Eur. J. Biochem. 12*, 154.

Karreman, G., Steele, R. H., and Szent-Györgyi, A. (1958),

Proc. Nat. Acad. Sci. U. S. 44, 140.
Konev, S. V. (1967), Fluorescence and Phosphorescence of Proteins and Nucleic Acids, New York, N. Y., Plenum Press, p 39.
Kurtin, W. E., and Song, P. S. (1968), *Photochem. Photobiol. 7*, 263.
Labeyrie, F., di Franco, A., Iwatsubo, M., and Baudras, A. (1967), *Biochemistry 6*, 1791.
Longworth, J. W. (1966), *Biopolymers 4*, 1131.
Mével-Ninio, M., Pajot, P., and Labeyrie, F. (1971), *Biochimie 53*, 35.
Monteilhet, C., and Risler, J. L. (1970), *Eur. J. Biochem. 12*, 165.
Nag-Chaudhuri, J., and Augenstein, L. (1964), *Biopolymers Symp. 1*, 441.
Pajot, P., and Groudinsky, O. (1970), *Eur. J. Biochem. 12*, 158.
Spyridakis, A., Naslin, L., and Labeyrie, F. (1971), *Biochimie 53* (in press).
Steiner, R. F., and Kolinsky, R. (1968), *Biochemistry 7*, 1014.
Stryer, L. (1960), *Radiat. Res., Suppl. 2*, 432.
Symons, R. H. (1965), *Biochim. Biophys. Acta 103*, 298.
Truong, T., Bersohn, R., Brumer, P., Luk, C. K., and Tao, T. (1967), *J. Biol. Chem. 242*, 2979.
Weber, G., and Teale, F. J. W. (1959), *Discuss. Faraday Soc. 27*, 134.
Weber, G., and Teale, F. J. W. (1965), *Proteins 3*, 485.
Weinryb, I., and Steiner, R. F. (1968), *Biochemistry 7*, 2488.
Weinryb, I., and Steiner, R. F. (1970), *Biochemistry 9*, 135.

Analogs of Phosphoenolpyruvate. On the Specificity of Pyruvate Kinase from Rabbit Muscle*

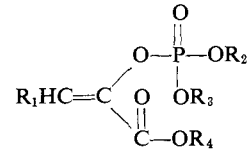
Jo Anne Stubbe† and George L. Kenyon‡

ABSTRACT: Several compounds with structures analogous to phosphoenolpyruvate were synthesized for the purpose of investigating the specificity of pyruvate kinase (EC 2.7.1.40). Contrary to the report of A. E. Woods *et al.* [*Biochemistry 9*, 2334 (1970)], it has been found that phosphoenol-α-ketobutyrate is a pseudosubstrate in the pyruvate kinase reaction. Phosphoenol-3-bromopyruvate was also shown to be a pseudosubstrate, but, within the limits of detection, phosphoenol-3-phenylpyruvate was inactive. The stereochemistries of the *E* and *Z* isomers of both phosphoenol-α-ketobutyrate and phosphoenol-3-bromopyruvate were assigned using nuclear magnetic resonance spectroscopy. (*Z*)-Phosphoenol-α-ketobutyrate has been shown to yield (3*R*)-[3-^{2}H]α-ketobutyrate stereospecifically when the enzymatic reaction was carried out in D_2O.

This corresponds to the addition of deuterium at C-3 on the 2-*si*, 3-*re* face of the (*Z*)-phosphoenol-α-ketobutyrate or its mechanistic equivalent in the enzyme-catalyzed reaction. This result confirms recent similar findings of Bondinell and Sprinson [*Biochem. Biophys. Res. Commun. 40*, 1464 (1970)].

In a recent paper Woods *et al.* (1970) reported the synthesis of several new homologs of phosphoenolpyruvate (PEP)[1] and presented evidence to show that none of these homologs, including phosphoenol-α-ketobutyrate, was active as a pseudosubstrate in the pyruvate kinase (EC 2.7.1.40) reaction. We have synthesized several analogs of PEP (**1**), including **2**–**9**.

We wish to present evidence to show that phosphoenol-α-ketobutyrate (**4**) is a relatively slowly reacting pseudosubstrate for pyruvate kinase, that phosphoenol-3-bromopyruvate (**7**) is even more reactive, but that no detectable reaction was observed with phosphoenol-3-phenylpyruvate (**9**) or the other analogs listed. We have also made stereochemical assignments for the *E* and *Z* isomers of both phosphoenol-α-ketobutyrate and phosphoenol-3-bromopyruvate using nmr spectroscopy and have shown that *Z*-phosphoenol-α-ketobutyrate gives (3*R*)-[3-^{2}H]α-ketobutyric acid stereospecifically[2] when the enzymatic conversion is carried out in D_2O. This confirms the stereochemical course for the protonation of PEP itself in the enzymatic conversion recently found by Rose (1970) using tritium–deuterium-labeling techniques.



1, R_1, R_2, R_3, R_4 = H
2, R_1, R_2 = H; R_3 = CH_3; R_4 = C_2H_5
3, R_1 = H; R_2, R_3 = CH_3; R_4 = C_2H_5
4, R_1 = CH_3; R_2, R_3, R_4 = H
5, R_1, R_2, R_3 = CH_3; R_4 = H
6, R_1, R_2, R_3, R_4 = CH_3
7, R_1 = Br; R_2, R_3, R_4 = H
8, R_1 = Br; R_2, R_3 = CH_3; R_4 = H
9, R_1 = C_6H_5; R_2, R_3, R_4 = H

* From the Department of Chemistry, University of California, Berkeley, California 94720. *Received January 8, 1971.* This work was supported by U. S. Public Health Service Grant AM-13529 from the National Institute of Arthritis and Metabolic Diseases. Portions of this work were presented at the Pacific Conference on Chemistry and Spectroscopy, San Francisco, Calif., Oct 6, 1970.

† National Institutes of Health Predoctoral Fellow, 1969 to present.

‡ To whom to address correspondence.

[1] Abbreviation used is: PEP, phosphoenolpyruvate.

[2] A high degree of stereoselectivity for this reaction has obviously not been established in this study since the specific rotation of our isolated sodium (*R*)-α-[^{2}H]propionate is considerably lower than the highest reported value (see Results). In light of the results of Rose (1970) and Bondinell and Sprinson (1970), we believe, however, that the reaction is a stereospecific one and that we partially racemized the asymmetric center during our oxidation and isolation procedures.