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An EPR study of the interactions between heme and flavin in yeast flavocytochrome b_2

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Abstract. E.P.R. experiments and spin-lattice relaxation time measurements have been performed on *Flavocytochrome* b_2 in the range 10 K to 100 K, to obtain information on the distance between the two prosthetic groups of the protein, flavin and heme. We have used the stabilization effect of pyruvate on the semiquinone form of the flavin, to compare the E.P.R. spectral shape and the relaxation properties of the radical when the heme is either in the ferrous form or in the ferric form. When the heme is ferric, no significant increase of the line broadening or enhancement of the relaxation rate of the radical can be detected in the range 10 K to 100 K. From these results, a minimum intercentre distance of 18 to 20 Å can be estimated.

Key words: Flavocytochrome b_2, EPR, dipolar interaction, semiquinone radical, spin-lattice relaxation

Introduction

In yeast mitochondria adapted to lactate, and in aerobic conditions, the oxidation of L-lactate into pyruvate is coupled to the successive reduction of two molecules of cytochrome c through a specific enzyme, flavocytochrome b_2 (EC 1.1.2.3). In this tetrameric protein, each polypeptide chain is folded into two independent domains: an L-lactate flavodehydrogenase unit, and a cytochrome unit where heme b_2 can transfer electrons to cytochrome c. The catalytic cycle involves two successive intramolecular electron transfers from flavin to heme, these two prosthetic groups being very likely associated within the same active site. At the present time, the available kinetic data concerning these intramolecular electron transfers cannot be fully interpreted, owing to the lack of structural information (Labeyrie et al. 1978).

Recent studies have shown that pyruvate, the product of the catalytic reaction, stabilizes the semiquinone form of the flavin, so that a high level of this paramagnetic species can be obtained in samples characterized by very different extent of reduction of the heme. This effect is associated with an important shift of the midpoint potential for the flavin reduced/flavin semiquinone couple (−70 mV), and for the flavin semiquinone/flavin oxidized couple (+90 mV) (Tegoni et al. 1986).

Previous attempts to detect magnetic interactions by E.P.R. between the semiquinone radical and the paramagnetic heme b_2 led to negative results (Capeillere-Blandin et al. 1975). However, the experiments were performed on samples prepared without pyruvate, so that the proportion of paramagnetic heme associated with the flavin radical was not sufficient to allow an unambiguous interpretation of the experimental results.

The use of pyruvate now enables the preparation of samples of flavocytochrome b_2 where for most molecules the semiquinone radical is associated with an oxidized heme, and others where the radical is essentially associated with a reduced heme. In the present work, we examine whether the magnetic properties of the radical are dependent upon the redox state of the heme. The E.P.R. spectral characteristics and the spin-lattice relaxation behaviour of the semiquinone radical have been studied between 7 K and 100 K, and no evidence for magnetic interactions has been found. This result indicates that the two centres are relatively far away from each other in the protein, and simple models suggest a minimum centre-to-centre distance of 18 to 20 Å.

Material and methods

Materials

Flavocytochrome b_2 from *Hansenula anomala* yeast was purified and stored as described in Labeyrie

et al. (1978) with the modifications detailed in Gervais et al. (1980). The resulting enzyme has a ratio A_{280}/A_{423} (reduced) = 0.51 and a standard molar activity at 30 °C of 1,000 s^{-1} as expressed in electron equivalent per prosthetic heme. The absorption coefficients used throughout this study are 30.9 $mM^{-1}\,cm^{-1}$ and 9.17 $mM^{-1}\,cm^{-1}$ (Tegoni et al. 1986) for *dithionite* reduced and oxidized forms at 556 nm (λ_{max} of the α band of the reduced form), i.e. 3.5 and 6.81 respectively for the difference, $\varepsilon_{570\,nm}$ minus $\varepsilon_{690\,nm}$.

Flavodoxin sample

Flavodoxin from *Desulfovibrio vulgaris* was kindly provided by Drs. V. Favaudon and J. Le Gall. The enzyme was stored as lyophilized powder. Anaerobic reduction of the oxidized form to the semiquinone form was carried out in 25 m*M* phosphate buffer, 50 m*M* ethylenediamine tetracetic acid, 10 µ*M* FMN, pH 7.0, by direct irradiation for five minutes in the E.P.R. tube under argon flow (quality 5.7), with a 75 watt lamp (20 cm distance) (Dubourdieu et al. 1975). Under these conditions the total conversion of the oxidized form to the semiquinone form was obtained. After irradiation the flavodoxin sample (897 µ*M*) was frozen under argon flow in liquid nitrogen.

Flavocytochrome b_2 samples

A suitable amount of an ammonium sulfate suspension of flavocytochrome b_2 was centrifuged for 10 min at 23,500 *g*. In order to eliminate ammonium sulfate and lactate without excessive dilution of the enzyme, the resulting pellet was directly dialysed against 100 m*M* phosphate buffer, pH 7.0 at 4 °C (2 h, with several changes).

After dialysis, the flavocytochrome b_2 solution was introduced into a Thunberg cell (optical path: 2 mm) by injection using Hamilton syringes. In order to obtain anaerobic conditions, argon gas was flushed through inox tubes into the plug of the Thunberg and into the E.P.R. tubes. Anaerobiosis was then established by a continuous argon flow over the solution (for 1 h).

Pyruvate (10 m*M* final) was added; the solution was kept in the dark to avoid the formation of an inactive photoadduct in the presence of pyruvate and oxygen (Blazy 1972).

Anaerobiosis was re-established and maintained by a continuous argon flow. During this operation the Thunberg vessel was maintained in an ice box.

Reduction of flavocytochrome b_2 was achieved at 18 °C by addition of a degassed L-lactate solution. Heme reduction levels (X) were estimated by absorbance measurements at 690 nm and 570 nm, using $X\% = 100\,(6.81 - \varepsilon_{app})/3.31$ (cf. Materials).

At suitable levels of heme reduction, 120 µl of the enzyme solution were taken using Hamilton syringes, and introduced into the E.P.R. tube, which was then frozen under argon flow in liquid nitrogen. For the two samples used in the present study, the concentration and the extent of reduction of the heme as determined by optical measurements are:

sample I: 0.39 m*M*, 100% heme reduced
sample II: 0.71 m*M*, 20% heme reduced.

E.P.R. spectra were recorded on a VARIAN E112 spectrometer. The samples were cooled with a gas-flow Air-Product system. The temperature was measured before and after each spectrum with a calibrated thermocouple (Chromel vs. Au-0.07% Fe) placed in an E.P.R. tube partially filled with water. To measure the extent of reduction of the heme, we compared the amplitude of the signal with that given by a sample fully oxidized by $Fe(CN)_6^{3-}$. The amount of semiquinone was obtained by double integration and comparison with a copper standard.

Methods

The relaxation time τ_1 of heme b_2 between 7 K and 21 K, and the relaxation time T_1 of the semiquinone radical between 10 K and 100 K were deduced from microwave saturation experiments. A modulation frequency of 1 kHz was used to avoid fast passage effects. In the case of heme b_2, the shape of the low field absorption derivative peak is nearly gaussian (Fig. 1), and τ_1 was obtained by the method of Gayda et al. (1979). The E.P.R. spectrum of the semiquinone radical is composed of several unresolved hyperfine lines (Erikson and Ehrenberg 1973), so that the relaxation processes could lead to complex saturation behaviour. We assumed that the saturated spectra could be approximated by the convolution product between an unsaturated spectrum and saturated lorentzian spin packets (Castner 1959). Saturation curves, representing the variation of the signal amplitude as a function of the component H_1 of the microwave field, were numerically computed for different half width ΔH_p of the spin packets. By comparison with the experimental saturation curves, we obtained the two parameters ΔH_p and $H_{1/2}$ which are related to T_1 by (Castner 1959):

$$H_{1/2} = \left(\frac{\Delta H_p}{\gamma T_1}\right)^{1/2}.$$

For temperatures higher than 40 K, the relaxation time of the heme was deduced from the relaxation broadening of the low field peak by the method of Bertrand et al. (1980).

Results

Redox state of the prosthetic groups

The results of the quantitative E.P.R. measurements performed at 20 K are reported in Table 1. The redox states thus determined correspond closely to the data obtained at room temperature (Tegoni et al. 1986), suggesting that the freezing process does not shift appreciably the thermodynamic equilibrium of the samples. From the model presented by Tegoni et al. (1986) it is possible to evaluate, for each sample, the ratio of the number of molecules containing radical and oxidized heme to those containing radical and reduced heme.

This ratio is less than 2×10^{-2} for sample I, and about 4.5 for sample II. These values depart sufficiently from unity to insure a non-ambiguous interpretation of the experimental results.

Table 1. Characterization of the redox state of the two samples. Optical spectroscopy measurements were performed at room temperature, and E.P.R. measurements at 20 K, as explained in the text

Sample	Reduced heme %		% Semiquinone (E.P.R)
	optical spectroscopy	E.P.R.	
I	100	> 98	11
II	20	20	90

Spin-lattice relaxation of heme b_2

Figure 1 shows the E.P.R. spectrum of *flavocytochrome* b_2 recorded at 20 K. The g values of this typical low spin heme signal are:

$1.455 \pm 0.003 \quad 2.218 \pm 0.002 \quad 3.018 \pm 0.003.$

The temperature dependence of $1/\tau_1$ is shown in Fig. 2. The T^5 dependence observed between 7 K and 20 K is similar to that reported for other low spin hemes (Blum and Ohnishi 1980; Stapleton et al. 1980; Scholes et al. 1984). In the high temperature range, a curvature appears around 100 K. It should be noted that the relaxation time measured in spectral broadening experiments is in fact τ_2, which is proportional to τ_1 in this range of temperature. Although the proportionality factor is not expected to differ strongly from unity, it could explain the shift which is necessary to bring the low temperature and the high temperature data into agreement (Bertrand et al. 1982; Allen et al. 1982).

As in other metalloproteins, the relaxation is fast and is probably due to a two phonon relaxation process. The temperature dependence of $1/\tau_1$ reflects the density of the vibrational modes coupled to the spin system (Allen et al. 1982; Bertrand et al. 1982).

Spin-lattice relaxation of the semiquinone radical

By using the procedure described in the methods section, it was possible to simulate correctly the saturation of the radical signal, with values of the packet linewidth ΔH_p between 0.2 Gauss and 1 Gauss. No significant "anomalous saturation" effects (Hyde et al. 1970) were observed in the tem-

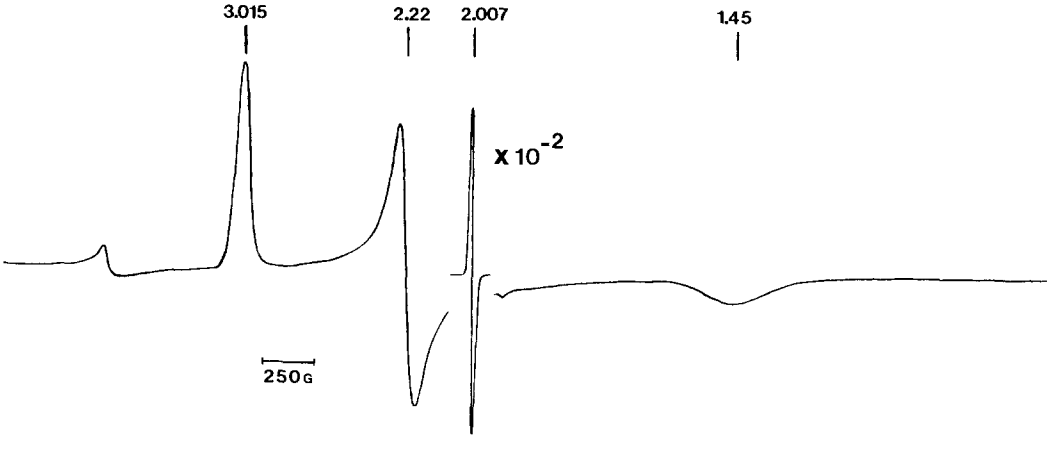

Fig. 1. E.P.R. spectrum of flavocytochrome b_2 (sample II) at 20 K. Experimental conditions: modulation frequency 100 kHz, modulation amplitude: 20 G, microwave power: 2 mW, microwave frequency: 9.297 GHz. The signal of the semiquinone radical is partially saturated under these conditions

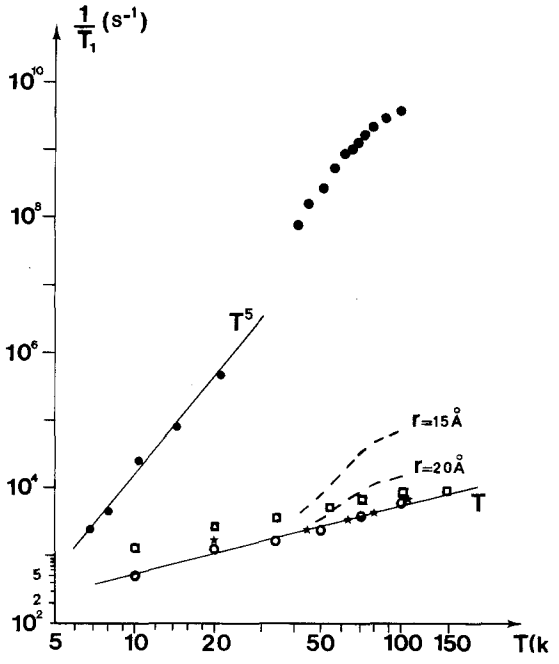

Fig. 2. Temperature dependence of the spin-lattice relaxation time of: ● heme b_2, ○ semiquinone radical of sample I, □ semiquinone radical of sample II, * flavodoxin semiquinone. The full lines represent fits with simple power laws, whereas the dashed lines are calculated from Eq. (2) of the text

perature range 10 K–100 K. The temperature dependence of $1/T_1$ represented in Fig. 2 is very similar for samples I and II of flavocytochrome b_2 and for the flavodoxin from *D. vulgaris*. To our knowledge, very few relaxation measurements have been performed at low temperature for radicals in proteins. One may mention a recent study on the radical Fe^{2+}–NO in myoglobin, where a similar T law has also been observed (Muench and Stapleton 1985).

Discussion

The dipolar interactions between two paramagnetic centres may induce static (spectral changes) as well as dynamic (shortening of the relaxation times) effects. We consider first the static effects. The E.P.R. spectrum of the semiquinone radical of sample II (80% ferriheme) of flavocytochrome b_2 is shown in Fig. 3. Within the experimental accuracy, the spectrum from sample I (~ 100% ferroheme) appears identical. The difference between the linewidths of the two spectra which could be deteted is smaller than about 1 Gauss. This value can be taken as an upper limit for an eventual broadening due to dipolar interactions in sample II.

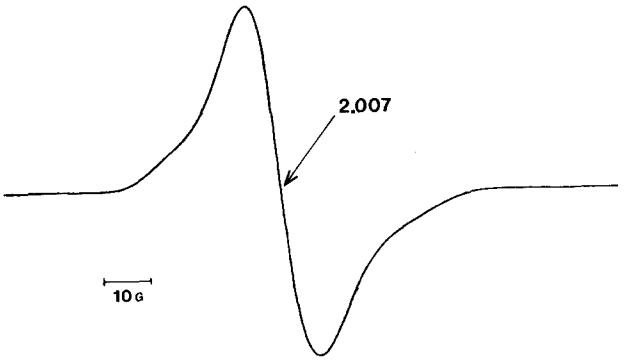

Fig. 3. E.P.R. spectrum of the semiquinone radical of sample II at 20 K. Experimental conditions: modulation frequency: 100 kHz, modulation amplitude: 10 G, microwave power: 0.2 μW

When two paramagnetic centres characterized by isotropic $\tilde{g}_1$ and $\tilde{g}_2$ tensors are separated by a large distance r, a good estimation of the line-broadening experienced by species 1 is given by:

$$\Delta H \sim \frac{g_2 \beta}{r^3}, \qquad (1)$$

where β is the Bohr magneton. In our case, the situation is more complicated for the following reasons:

i) the unpaired electron of the radical is delocalized over the isoalloxazine ring of the flavin;
ii) the $\tilde{g}$ tensor of heme b_2 is anisotropic;
iii) the E.P.R. signal of the radical is broadened by numerous unresolved hyperfine lines.

To obtain an estimation of the minimum distance between the two prosthetic groups, we used a simplified procedure which is expected to preserve the essential characteristics of the real situation:

i) We consider the mean distance between the iron atom of the heme and the centre of the electron spin distribution of the flavin. This distribution is essentially weighted on nitrogens N(5) and N(10) and carbons C(6) and C(8) (Erikson and Ehrenberg 1973).

ii) The $\tilde{g}$ tensor of the heme is replaced by an isotropic one characterized by the "most probable" g value: $g_2 = 2.22$.

iii) The E.P.R. signal of the radical is represented by a single lorentzian line characterized by a g value and a peak to peak linewidth identical to that of the semiquinone signal (Fig. 3).

Under these conditions, a numerical simulation of the dipolar broadening of the flavin semiquinone spectrum, based on the expressions presented by Smith and Pilbrow (1974) indicates that a minimum intercentre distance of 18 Å is required for a broadening of 1 Gauss.

It is interesting to note that a much larger distance, equal to 27 Å, would have been obtained by simply using Eq. (1). Concerning this point, one may mention the results reported for the system (cytochrome-bacteriochlorophyll) in the photosynthetic bacterial reaction centre: a minimum distance of about 25 Å was deduced from an E.P.R. analysis of the dipolar broadening on the basis of Eq. (1) (Tiede et al. 1978), whereas X-ray diffraction experiments have shown that the intercentre distance is 21 Å (Deisenhofer et al. 1984). The discrepancy could be due in part to the use of Eq. (1), which may yield overestimated distances, as mentioned above.

We now turn to the dynamic effects of the dipolar interactions. It is possible to express in closed form the changes of the relaxation times T_1 and T_2 induced by dipolar coupling with a fast relaxing neighbour, only in the case of isotropic $\tilde{g}$ tensors. The expressions include several terms which depend on the angle θ between the inter-centre axis and the applied magnetic field (Brudwig et al. 1984). One may reasonably assume that the main contribution comes from the term involving in the denominator the difference $\Delta\omega$ between the resonance angular frequencies of the two centres. The relaxation time is then given by:

$$\frac{1}{T_1} = \left(\frac{1}{T_1}\right)_0 + \frac{(1-3\cos^2\theta)^2\,\beta^4\,S(S+1)\,g_1^2\,g_2^2}{6\,\hbar^2\,r^6} \cdot \frac{\tau_2}{1+\Delta\omega^2\,\tau_2^2}, \quad (2)$$

where $(T_1)_0$ represents the value of T_1 in the absence of dipolar interactions, and τ_2 the spin-spin relaxation time of the fast relaxing species.

This expression can be used to estimate the magnitude of the effects expected in our case, by taking for $(T_1)_0$ the spin-lattice relaxation time of the radical in sample I, for τ_2 the relaxation time of the heme deduced from the line broadening experiments, and by replacing g_2 by 2.22 and the orientation factor $(1-3\cos^2\theta)^2$ by its average value 0.8 (Ohnishi et al. 1982). The temperature dependence of $1/T_1$ given by Eq. (2) is represented by the dashed curves in Fig. 2 for different values of r. It appears that a minimum distance of about 20 Å is required to account for the values of T_1 measured in sample II. Thus the minimum intercentre distances given by the two independent methods used in the present study are very similar.

The dynamic effect of the dipolar interactions may also induce a shortening of the radical spin-spin relaxation time T_2, which then results in a significant deviation of the temperature dependence of the signal amplitude with respect to the Curie law

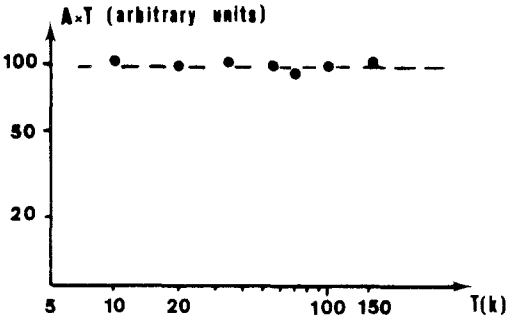

Fig. 4. Temperature dependence of the peak to peak amplitude A of the semiquinone radical of sample II, measured under nonsaturating conditions

(Leigh 1970). The absence of any appreciable deviation in sample II (80% ferriheme) confirms that the dipolar effects are very weak (Fig. 4).

Conclusion

E.P.R. experiments and spin-lattice relaxation time measurements have been performed at low temperature on flavocytochrome b_2, to obtain information on the distance between the flavin radical and the heme. Using the stabilization effect of pyruvate on the semiquinone form of the flavin, we have compared the E.P.R. spectral shape and the relaxation properties of the radical when the heme is either in the ferrous form or in the ferric form. When the heme is ferric, no significant increase of the line broadening or enhancement of the relaxation rate of the radical, due to dipolar interactions, were observed in the range 10 K to 100 K. From an analysis of the results, which include a numerical simulation of the dipolar broadening effect and T_1 measurements for the heme between 10 K and 100 K, a minimum distance between the two centres of 18 to 20 Å can be estimated. This evaluation is in agreement with the preliminary results obtained by X-ray diffraction experiments (S. Mathews: Personal communication).

It thus appears that *long range* intramolecular electron transfers can take place within milliseconds in flavocytochrome b_2 (Tegoni et al. 1984a and b). Similar or even faster long range kinetics have already been observed in proteins or protein complexes (Deisenhoffer et al. 1984; Peterson-Kennedy et al. 1984; Ho et al. 1985; King et al. 1985). However, it should be noted that the driving force ΔE^0 is very weak in the case of flavocytochrome b_2, being equal to about 40 mV for the transfer from the reduced flavin, and about 0 mV for the transfer from the semiquinone flavin (Tegoni et al. 1986). According to electron transfer theories, the activa-

tion energy E_a is related to the free energy variation ΔE^0 and the reorganization energy Δ by (Marcus and Sutin 1985):

$$E_a = (\Delta - \Delta E^0)^2 / 4\,\Delta \,. \qquad (3)$$

It would be very interesting to measure E_a in order to evaluate Δ for these two electron transfers. Moreover, the shift of ΔE^0 induced by the presence of pyruvate could be used to test the validity of Eq. (3).

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