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STUDIES ON THE MOLECULAR ORGANIZATION OF CYTOCHROMES *P*-450 AND b_5 IN THE MICROSOMAL MEMBRANE

PETER R. RICH *, DAVID M. TIEDE and WALTER D. BONNER, Jr.

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)

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Summary

1. The relative orientations of the heme groups of cytochromes *P*-450 and b_5 in the microsomal membrane have been studied by the technique of electron paramagnetic resonance. The results show that the heme plane of cytochrome *P*-450 lies in the same plane as the membrane surface, whereas the cytochrome b_5 heme plane has a random orientation.

2. No significant broadening or change in relaxation properties of the g_z component of low spin cytochrome *P*-450 occurred when cytochrome b_5 was reduced by redox poising. It is concluded that there is little or no paramagnetic coupling between the heme groups of the two species.

3. The results favor a model in which no tight complex between cytochromes *P*-450 and b_5 is present, the species being independent and interacting only by random molecular collisions or via other intermediate species.

Introduction

The microsomal fraction isolated from a wide variety of tissues and organisms has been shown to contain cytochrome *P*-450. This is a terminal oxidase operative in a chain containing flavoprotein and possibly cytochrome b_5 [1,2]. A novel heme configuration causes the *P*-450 hemoprotein to display unusual and characteristic optical and EPR properties and these features have been well described. The native ferric enzyme exists in low spin (g_x = 1.93, g_y = 2.25, g_z = 2.40) and high spin (g_y = 3.3, g_z = 8.1) forms [3,4] which are in

* Present address: Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.
Abbreviation: MOPS, morpholinepropane sulfonic acid.

equilibrium [5]. Cytochrome b_5 is a low spin cytochrome with $g_z = 3.05$, $g_y = 2.22$ and $g_x = 1.41$ at pH 7 [6,7].

A recent application of EPR has been developed to determine orientation of membrane-bound paramagnetic species [8,9]. It involves the measurement of angular dependence with respect to magnetic field of the magnitudes of the vectorial components of the EPR signals seen in samples of stacked membranes. The technique has been used successfully to determine component orientations in cytochrome oxidase vesicles [8,10,11], submitochondrial particles [11], chromatophores of photosynthetic bacteria [12,13] and adrenal cortical mitochondria [14].

In this work we have employed the technique to determine the heme orientations of the microsomal cytochromes *P*-450 and b_5. Investigations of both the low spin and high spin species of oriented, ribosome-depleted rat liver microsome samples revealed that the plane of the heme group of the cytochrome *P*-450 lies approximately parallel to the plane of the membrane surface. This orientation is in contrast to that of mitochondrial respiratory cytochromes [11], but is similar to that of adrenal cortical mitochondrial cytochrome *P*-450 [14]. The cytochrome b_5 heme plane, however, is relatively unoriented, which implies a rather 'floppy' attachment of this pigment to the microsomal membrane surface.

Further studies, performed to investigate a possible paramagnetic interaction between the cytochromes b_5 and *P*-450 heme groups, demonstrated that they lacked a strong interaction since the presence of oxidized cytochrome b_5 did not effect the relaxation properties or signal broadness of low spin cytochrome *P*-450.

The results indicate that the cytochromes b_5 and *P*-450 are independent species and do not form a tight complex with each other.

Methods

Preparation of microsomes. A microsomal fraction was prepared from rat liver by the standard technique of differential centrifugation [15]. After its initial precipitation (100 000 × g_{av} for 30 min), the microsomal pellet was washed by resuspension and recentrifugation (100 000 × g_{av} for 30 min) in ice-cold 10 mM MOPS and 100 mM KCl at pH 7.0, to remove attached ribosomes and any contaminating hemoglobin.

Orientation of membranes. This was achieved essentially as described by other workers [8—14]. The washed pellet was resuspended to approximately twice its original volume in ice-cold 50 mM potassium phosphate with 50 mM KCl at pH 7.0 and several drops were placed on a small sheet of mylar. The sample was dried overnight in a dessicator at 4°C and 92% relative humidity (maintained with a saturated solution of K_2HPO_4). After 24 h the sample appeared brittle, translucent and dry, and was ready for analysis. Several strips (approx. 2 mm wide × 25 mm length) were cut from the sheet and placed in an EPR tube with all strips lying in the same plane.

EPR measurements. All spectra were obtained with a Varian E109 spectrometer (Varian Associates). The temperature of the samples for EPR measurements was controlled with a variable temperature cryostat (Air Products Model

LTD-3-110). Temperature was monitored with a carbon resistor placed in the helium flow directly below the sample and g values were corrected by reference to a weak pitch standard.

Redox poising. Samples were poised in an anaerobic vessel under a constant flow of argon gas as described by Dutton [16]. Potentials were measured with a platinum electrode against a calomel standard. Redox mediators are given in the text.

Results

Fig. 1A illustrates a typical EPR spectrum of a sample of KCl-washed rat liver microsomes. Conditions were optimized so that both the low spin cytochrome b_5 (g_z = 3.04) and the low spin cytochrome P-450 (g_x = 1.92, g_y = 2.25, g_z = 2.41) could be detected in the same spectrum. In Fig. 1B, the identity of the g = 3.04 peak as that of cytochrome b_5 is confirmed in that 1 mM NADH was added to the microsomes in the presence of oxygen. It is well known by optical methods that only cytochrome b_5, and not cytochrome P-450, becomes reduced under these conditions [17]. It can be seen that only the g = 3.04 peak was lost, which strongly indicated that this peak belonged to ferric cytochrome b_5.

The lack of paramagnetic interaction between the heme groups of cytochromes P-450 and b_5

It was noted that aerobic NADH reduction produced a small amount of a second cytochrome P-450 species, seen as a peak around g = 2.5, a trough around g = 1.9 and a distortion of the g_y = 2.25 component (Fig. 1B). It was of interest to determine whether this was a paramagnetic phenomenon caused by

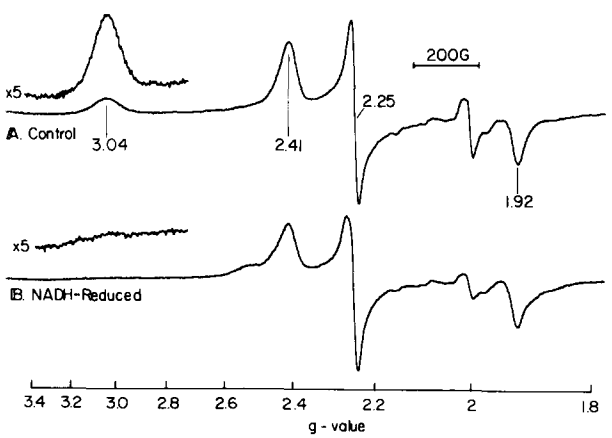

Fig. 1. EPR spectra of rat liver microsomes in the presence and absence of NADH. Rat liver microsomes were resuspended to approx. 30 mg protein/ml in 50 mM KCl and 50 mM potassium phosphate at pH 7.0. One sample (top trace) was transferred to a quartz EPR tube and frozen rapidly in liquid nitrogen. 1 mM NADH was added to a second identical sample (bottom trace) and this was also immediately frozen in liquid nitrogen. Conditions of EPR measurements were: modulation amplitude, 20 G; modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.182 GHz; temperature, 17 K.

interaction with, or absence of, ferric cytochrome b_5, or whether it was a secondary effect of NADH reduction. To investigate this, we exploited the difference in redox potentials of cytochrome b_5 (E_{m7} of around 0 mV) [18] and *P*-450 ($E_{m7} \simeq -300$ mV) [19,20,5] and poised a sample of microsomes anaerobically and in the presence of redox mediators [16] at pH 7 such that cytochrome *P*-450 was fully oxidized but cytochrome b_5 was either oxidized (E_h = +85 mV) or reduced (E_h = −130 mV). Under these conditions, no such second species of cytochrome *P*-450 was formed and the low spin cytochrome *P*-450 EPR spectra appeared identical under both conditions. For example, line width measurement of the g_z component of low spin cytochrome *P*-450 gave a $\Delta H_{1/2} = 62 \pm 1$ G under both conditions. Hence we conclude that the formation of the second cytochrome *P*-450 species is not caused by a paramagnetic interaction with cytochrome b_5 and must be a secondary effect of NADH reduction.

We also studied the relaxation behavior of low spin cytochrome *P*-450, as manifested in a power saturation profile at fixed temperature, in the presence or absence of an oxidized paramagnetic cytochrome b_5. The redox state of the cytochrome b_5 was controlled by redox poising and was checked by the presence or absence of the $g = 3.04$ peak. It can be seen that the power saturation profile of low spin cytochrome *P*-450 was unaffected by the redox state of cytochrome b_5 (Fig. 2).

The orientation of the cytochromes b_5 and P-450 heme planes in the membrane

Fig. 3 illustrates the EPR spectra of cytochrome *P*-450 in an oriented sample of salt-washed rat liver microsomes. The angles refer to the angle between the

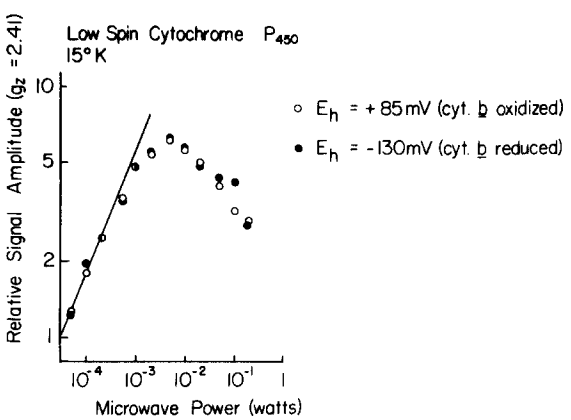

Fig. 2. Power saturation profiles of the $g = 2.40$ component of low spin cytochrome *P*-450. Samples of rat liver microsomes were resuspended to approx. 20 mg/ml in 50 mM KCl and 50 mM potassium phosphate at pH 7.0. The whole was made anaerobic with argon gas and an anaerobic redox titration was performed as described by Dutton [16] in the presence of 25—50 μM each of the following mediators (E_m at pH 7.0 given in parentheses): *N*-ethyl phenazonium ethosulfate (+55 mV), *N*-methyl phenazonium methosulfate (+8 mV), diaminodurol (+250 mV), pyocyanine (—40 mV), duroquinone (+10 mV) and 2-hydroxy-1,4-naphthoquinone (—125 mV). Samples at appropriate potential were anaerobically injected into quartz EPR tubes and frozen rapidly. Conditions of EPR measurement were as in Fig. 1 except that temperature was 15 K, and microwave power was varied at this constant temperature.

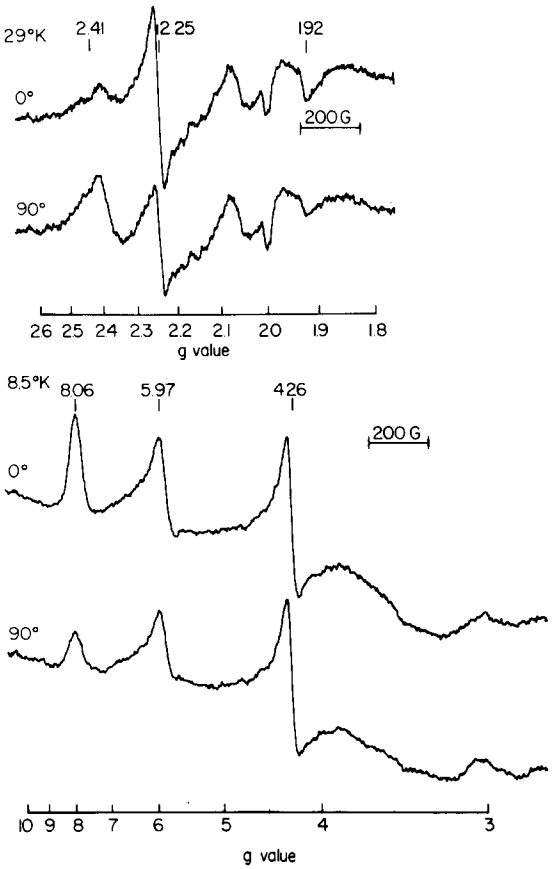

Fig. 3. EPR spectra of oriented multilayers of rat liver microsomes. Samples of oriented multilayers of rat liver microsomes were prepared as described in the text. Conditions of EPR measurement were: low spin cytochrome *P*-450 (top traces), modulation amplitude, 20 G; modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.108 GHz; temperature, 29 K; high spin cytochrome *P*-450 (bottom trace), modulation amplitude, 20 G; modulation frequency, 100 kHz; microwave power, 10 mW, microwave frequency, 9.108 GHz; temperature, 8.5 K. The angles refer to the angle between the membrane plane and the direction of the magnetic field.

plane of the membrane surface and the direction of the magnetic field. The EPR absorptions of both low spin and high spin forms of the cytochrome *P*-450 showed considerable dependence upon the angular orientation of the stacked membrane planes to the applied magnetic field. The g_z (2.41) component of the low spin spectrum was found to have a maximal absorption when the membrane planes were positioned perpendicular to the magnetic field (90°C), and show a minimal absorption when the membranes were parallel to the field (0°C), with a ratio of 4—5 between these orientations; conversely, the low spin g_y (2.25) and g_x (1.92) absorptions were found to be maximal when the membranes were placed in a direction fully parallel to the magnetic field, and 2—3 times larger than the perpendicular orientation. A similar heme orientation is also found for the high spin species where the g_x (8.06) and g_y (3.4) components were found to be maximal in the parallel direction and

minimal in the perpendicular. We were unable to detect the broad, weak g_z component of the high spin species in these samples in either orientation. Within the experimental accuracy, these angular dependencies indicate that the cytochrome *P*-450 g_x and g_y EPR absorptions lie parallel (within 10°) to the plane of the membrane surface.

At 8.5 K, two other features of the spectrum were apparent; firstly, the overlapped g_x and g_y components of cytochrome *P*-420 which peak around $g = 5.97$ [3,21,22]. This species represents a denatured form of the cytochrome *P*-450 and was presumably formed at some stage during the sample preparative procedure. It has a decreased degree of orientation as judged by the smaller angular dependence of this signal, since the ratio between maximal and minimal absorptions (occurring at 0° and 90°, respectively) was less than two; secondly, the rather ubiquitous $g = 4.26$ 'ferric iron in a field of low symmetry' [23] signal. This is probably non-specifically bound iron and it acts as an internal control for the system in that the signal size was independent of the angle of the membrane plane with respect to magnetic field, i.e. its orientation of the membrane is random.

In order to test the generality of the membrane orientation of the cytochrome *P*-450, similar experiments were also performed with the cytochrome *P*-450 in microsomes isolated from a higher plant source, in this case tulip bulb tissue (*Tulip gesnerana* var. Darwin) which is a good source of plant microsomal cytochrome *P*-450 [5]. In this case, exactly analogous results were also

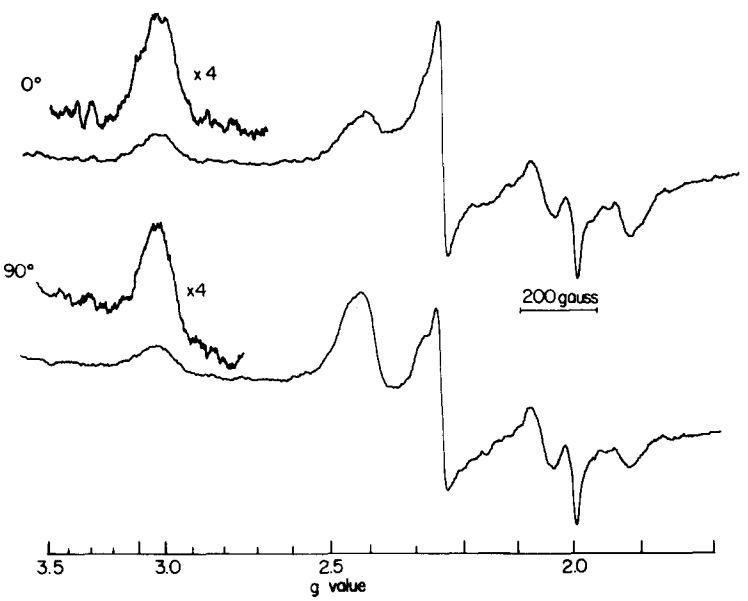

Fig. 4. The orientation of the heme plane of cytochrome b_5 in the microsomal membrane. An oriented sample of rat liver microsomes was prepared as described in the text. Conditions of EPR measurement were optimized so that both cytochrome *b*5 and low spin cytochrome *P*-450 EPR spectra could be seen in the same trace. Modulation amplitude, 20 G; modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.083 GHz; temperature, 17.5 K. The angles refer to the angle between the membrane plane and the direction of the magnetic field.

obtained, showing approximately the same degree of orientation. The cytochrome *P*-450 g_x and g_y EPR absorptions occurred maximally when the membranes were positioned parallel to the magnetic field, and 2—3 times larger than the perpendicular orientation. The cytochrome *P*-450 g_x and g_y EPR absorptions occurred maximally when the membranes were positioned parallel to the magnetic field, and the g_z absorption occurred maximally in the perpendicular orientation.

We also studied the orientation of the cytochrome b_5 heme plane relative to the membrane. The results of such an investigation are illustrated in Fig. 4. In order to obtain good resolution of the less intense cytochrome b_5 EPR signal, higher concentrations of microsomal membranes were required, which tended to decrease the extent of the membrane orientation. However, in these samples the cytochrome *P*-450 g_z component showed at least a 3-fold ratio between maximal and minimal amplitudes, while the cytochrome b_5 signal showed essentially no dependence upon the angular positions of the membranes in the magnetic field, and maximal or minimal signal amplitudes could not be found for any orientation. It is concluded that, in contrast to the cytochrome *P*-450, the cytochrome b_5 is not specifically oriented on the microsomal membrane, and instead has some degree of freedom in its method of attachment.

Discussion

The technique of membrane orientation has been successfully applied to the microsomal cytochrome *P*-450 system. The results indicate a significant degree of orientation of the cytochrome *P*-450 heme groups, with the heme plane lying in the same plane as the membrane surface, as is the case with the adrenal cortical mitochondrial system [14]. This is deduced from the fact that the g_z vectorial component (likely to be perpendicular to the heme plane, cf. refs. 24 and 25) interacts with the magnetic field when the membrane is perpendicular to the applied magnetic field, whereas the g_x and g_y vectorial components (in the plane of the heme group) interact with the magnetic field when the membrane plane is parallel to the applied magnetic field. We are unable to tell at present whether the residual unoriented fraction of the signals represent disordered cytochromes *P*-450 or disordered membranes in the preparation.

The cytochrome b_5, however, appears to be relatively unoriented in these properties, and this suggests to us that it has a large degree of mobility of movement in terms of side to side 'rolling' on its membrane anchor. This latter deduction, taken in conjunction with the finding that there is little or no paramagnetic interaction between the heme groups of cytochrome b_5 and *P*-450, further suggests that the two hemoproteins do not form a tightly structured complex but instead are independent moieties. This picture is consistent with the generally held model of microsomal structure of hydrophobically buried cytochrome *P*-450 proteins which are relatively fixed, and mobile, surface-located cytochrome b_5 proteins, and is in contrast to the model of fixed multimolecular units of, for example, mitochondrial membranes. Although not altogether ruling about the possibility of direct electron transport between the hemoproteins by a rather inefficient means of random, low affinity, molecular collisions, this model suggests that a function of cytochrome b_5 is not, as

previously suggested, to directly donate a second electron to cytochrome *P*-450 [26]. However, at present we are unable to rule out an electron donation via one or more as yet unidentified intermediates.

In addition, preliminary experiments show that both the cytochrome *P*-450 and cytochrome b_5 EPR signals are altered by the addition of water-soluble paramagnetic ions, for example, nickel EDTA, which suggests that both the cytochromes b_5 and *P*-450 hemes lie within 20 Å of the membrane surface (for a further example and discussion of this distance probe, see ref. 27). Entirely analogous results for orientation and location have also been found on the cytochrome *P*-450 heme in adrenal mitochondria by Blum et al. [14].

The finding that NADH altered the physical properties of at least part of the cytochrome *P*-450 may possibly reflect an interaction of an NADH-reducible flavoprotein with the cytochrome *P*-450. This could be of relevance to studies on the precise electron transfer pathways between components in the microsomal membranes. Further work is necessary to clarify this observation.

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