ELECTRON-NUCLEAR DOUBLE RESONANCE IN MELANINS

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ABSTRACT Electron-nuclear double resonance (ENDOR) signals from matrix protons interacting with the stable free radicals of "A"- and "B"-type melanins have been observed as a function of pH. In all samples the single line is similar in width and unusually narrow. The ENDOR reduction varies by more than a factor of 10, indicating a large sensitivity of relaxation properties of melanin to sample type. Signals were observed over a wide range of experimental conditions with good signal-to-noise ratio, establishing feasibility for further more detailed ENDOR studies. Incubation in D_2O resulted in little change, indicating that the free radical is well buried or protected. No resolved hyperfine structure was seen, consistent with the generally accepted view that melanin is a heterogeneous polymer.

INTRODUCTION

Melanin is the only biological polymer known to contain free radicals in its natural condition. It has been proposed that these paramagnetic sites are directly involved in redox regulation (1, 2), in the physiology of vision (3, 4), and in radiation protection (5, 6). It has also been suggested that the free radical can serve as a probe or natural label, even if it is inert, permitting the use of electron spin resonance (ESR) spectroscopy to investigate the structure and function of melanin (7). Functions of melanin that have been proposed and that might in principle be studied in this way, in addition to those in references 1–6, include photoprotection (8–10) and trace metal binding (11–13). For these reasons, it is appropriate to carry out investigations into the properties of the free radical of melanin.

The most complete ESR study of melanins is the work of Blois et al. (14). The spectrum is a single rather featureless line resembling that of a randomly oriented π-electron free radical of the semiquinone type. Because of the relatively low information content in the ESR spectrum, we decided to carry out electron-nuclear double resonance (ENDOR) experiments on melanin. In the ENDOR technique, the ESR signal is observed and the sample is simultaneously irradiated with a second radio-frequency source corresponding to the precession frequency of nuclei that are coupled to the paramagnetic center. The second source is swept in frequency and when reso-

nance is achieved, a change in ESR signal height is observed. This technique permits the determination of magnetic couplings to protons in the vicinity of the unpaired electron and thus inferentially yields information on the structure of the free radical. It has been used a few times to determine couplings in unordered solids where the ESR spectrum showed no resolved structure (15, 16).

MATERIALS AND METHODS

The ESR spectrometer of the ENDOR apparatus used here employs 100 kHz field modulation, incorporates a microwave bridge and cavity of conventional design, and permits observation of either the ESR absorption or dispersion. Neither the frequency nor the amplitude of the nuclear radio-frequency source is modulated in this equipment. The radio-frequency output from a 100-W broad-band amplifier is applied to a single-turn loop in the cavity, resulting in a maximum field of 1–2 G in the rotating frame (17).[1] ENDOR and ESR experiments were conducted at 4.2°K using a liquid helium cold-finger Dewar flask, and at 30°K using a boil-off technique. Survey experiments indicate a substantial decrease in ENDOR signal intensities at higher temperatures. We believe in the present experiment that $1/T_{2e} > \gamma_e H_m > \omega_m > 1/T_{1e}$, where T_{1e} and T_{2e} refer to the electron relaxation times, ω_m is the field modulation frequency in radians per second, γ_e is the electron gyromagnetic ratio, and H_m is the magnetic field modulation in gauss.

Two types of melanin were used, designated A and B. The technique of Plumer and Kopac (18) was used to prepare the A type from the choroid of bovine eyes. This form has protein complexed to the melanin and is considered relatively unchanged from melanin as it exists in cells. The B melanin was prepared from A by incubation in 6 M HCl at 90°C for 150 h in sealed tubes, and then washing in distilled water until the pH was approximately neutral (5–6). Protein is effectively absent in B-type samples. ESR samples were prepared from these materials by mixing H_2O or D_2O suspensions with solutions containing HCl or KOH. The deuterated water suspensions were incubated for 1 wk at 4°C. The D_2O was 99.8% pure and HCl and KOH were analytical grade. Samples were stored at 77°K.

RESULTS AND DISCUSSION

All samples exhibit microwave power saturation at incident powers in the range of 0.2–2 mW. The dispersion ESR line shapes are indicative of rapid-passage effects (19).

A single ENDOR line was observed at the "free proton frequency" (Fig. 1). ENDOR intensities tended to be greater in B- than A-type samples and also to increase when the pH was adjusted to high values. We believe the large variation in ENDOR signal intensities between melanins prepared in different ways arises primarily from differences in relaxation properties. Because so many properties of melanins hardly seem to change, the observed variation of the ENDOR signal intensity is specially significant. It may lead to a basis for classification of melanins and to a better understanding of structural differences among melanins.

The ENDOR lines are unusually narrow compared with matrix ENDOR widths of π-electron radicals in organic hosts. One possible interpretation is that the regions immediately surrounding the paramagnetic centers are rather deficient in protons. The

[1] Hoffman, B. M., J. L. Peterson, and T. G. Brown. Single crystal ENDOR of metalloproteins. Manuscript in preparation.

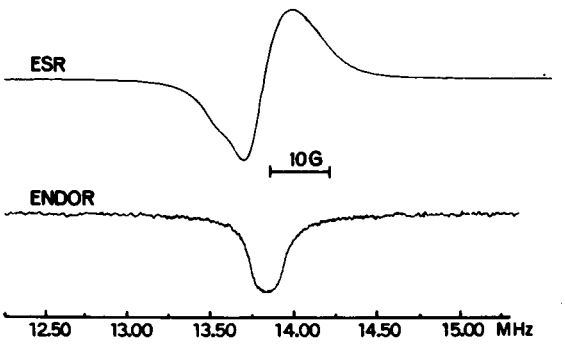

FIGURE 1 ESR and ENDOR signal of A-type melanin in 0.1 M KOH water suspension. T = 4.2°K, microwave power = 3 mW, modulation amplitude = 1.25 G, RF power set at −3 dB.

widths do suggest an unusual structure, and an understanding of this observation becomes a goal for future studies. We note that the observed line widths are greater than expected from proton-proton dipolar interactions. Incubation in D_2O decreased line widths by about 20%. See Table I. This rather small change indicates that the paramagnetic centers are well buried and protected in melanin particles. This is in agreement with the interpretation of Grady and Borg (20).

The absence of any hint of resolved structure or shoulders in the wings of the observed ENDOR lines suggests a heterogeneity of trapping sites (16, 21). This is consistent with the generally accepted model of Nicolaus (22) that melanin is a heterogeneous polymer.

ENDOR signal intensities as a function of the portion of the ESR line observed during scan of the radio frequency are shown in Figs. 2 and 3 for various conditions

TABLE I
SUMMARY OF ENDOR DATA ON MELANINS

		4.2°K		30°K	
		ENDOR reduction	ENDOR line width	ENDOR reduction	ENDOR line width
		%	*MHz*	%	*MHz*
B + H_2O	pH 5	0.8	0.17	1.0	0.18
B + D_2O		2.5	0.16	2.4	0.14
B + HCl	(1 M)	5	—	4.3	0.15
B + KOH		—	—	30*	0.28
A + H_2O	pH 6	Weak	—	0.8	0.15
A + D_2O		Very weak	—	0.2	—
A + H_2O	pH 1	Weak	—	0.5	—
A + D_2O		—	—	0.3	—
A + H_2O	pH 13	4.3	0.24	3.2	0.17
A + D_2O		—	—	2.1	0.14

*This sample saturates at unusually low microwave powers. This large ENDOR effect could be associated with an admixture of dispersion into the observed ESR signal.

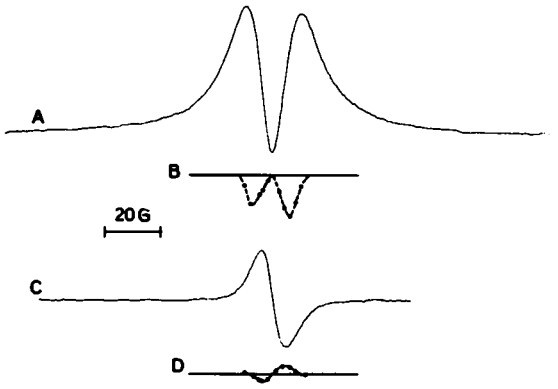

FIGURE 2 ESR dispersion (*A*) and absorption (*C*) signals of A-type melanin in 0.1 M KOH D_2O suspensions. Traces (*B*) and (*D*) show the magnetic field dependence of the ENDOR signal intensity. T = 30°K, Microwave power = 3 mW, modulation amplitude = 1.25 G, RF power set at −3 dB, radio frequency = 13.85 MHz. Gains for traces (*A*) and (*C*) are the same; gains for (*B*) and (*D*) are 10 times higher.

and samples. There are substantial differences and in some cases, particularly when observing the wings of the ESR lines, no ENDOR signals could be observed even though the ESR intensities seemed quite adequate. These results indicate a spectral variation of relaxation parameters in the ESR display. This could be a consequence of a superimposed contribution to the ESR spectrum from a radical or radicals with unfavorable relaxation properties for ENDOR. Such a superposition, particularly at high pH, has been suggested by Grady and Borg (20) and Sarna (13).

Fig. 3 A shows a change from ENDOR enhancement to ENDOR reduction as the observing magnetic field setting is increased, with the largest signal obtained near the cross-over of the derivative ESR line. Similar phenomena have previously been reported by Eriksson and Ehrenberg (23), and must depend on details of relaxation processes not yet fully understood.

We have investigated the mechanisms for ENDOR in the present experiment. Two models for ENDOR have been developed that might in principle be applicable: the packet-shifting model of Feher (24) modified for powders by Hyde et al. (15), and the

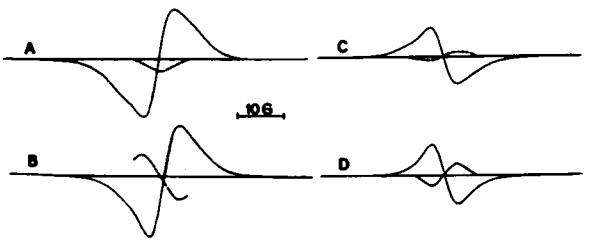

FIGURE 3 ESR signals (solid lines) of B melanin in neutral H_2O (*A*, *C*) and D_2O (*B*, *D*) suspensions. Dotted lines show the magnetic field dependence of the ENDOR signal intensity (gain 10 times higher). T = 4.2°K for (*A*, *B*) and 30°K for (*C*, *D*), microwave power = 3 mW, modulation amplitude = 1.25 G, RF power set at −3 dB, radio frequency = 13.86 MHz.

depolarization model of Lambe et al. (25). Characteristic differences between these mechanisms are: (*a*) The ENDOR decay time when the nuclear radio-frequency power is turned off should be T_{1e} (i.e. equal to the electron spin-lattice relaxation time) in the packet-shifting model and T_{1N} (the nuclear spin-lattice relaxation time) in the depolarization model. (*b*) Packet shifting requires that the ESR line be inhomogeneously broadened, and only couplings that are greater than $1/T_{2e}$ can be seen. These requirements are not necessary in the depolarization model. (*c*) Packet shifting should dominate at low paramagnetic concentrations (approx. 10^{16} centers/cm^3), while depolarization ENDOR requires higher concentrations (approx. 10^{18} centers/cm^3). (*d*) When using magnetic field modulation, the absorption ESR line intensity increases if packet shifting dominates, and it decreases if depolarization dominates (24). (In the absence of field modulation, both models predict an increase in the pure absorption ESR signal height. Packet shifting yields an increase in dispersion while the depolarization model predicts a decrease [25].)

Concentrations of free radicals in the melanin samples were of the order of 10^{18} per g of dried substance. The ESR lines had a high homogeneous content but were inhomogeneously broadened primarily by the anisotropy of the g-tensor, secondarily from dipolar and contact interaction with protons and/or nitrogen and quite possibly from overlap of signals of a heterogeneity of paramagnetic trapping sites. ENDOR recovery times were of the order of seconds, which permits the conclusion that nuclear relaxation determines the recovery times. The ENDOR signals represented, in all cases, a decrease in the ESR intensity (Fig. 2). ENDOR-induced ESR lines resemble qualitatively the shapes shown by Terhune et al. (26). All of these observations support the conclusion that the depolarization model is dominant in melanins observed under the conditions of the present experiment. We believe that this finding will be useful in the design of future ENDOR experiments on melanin.

Of probably greatest significance in this work is the simple observation that ENDOR can be detected in melanin with good signal-to-noise ratio over a wide range of experimental conditions. In general, matrix ENDOR is easier to detect than ENDOR of strongly coupled nuclei in an unordered solid. It is a well-established experimental procedure to optimize instrumental settings on the matrix ENDOR in an initial search with ENDOR apparatus. This has been done here. It now becomes reasonable to consider more elaborate ENDOR investigations. Among these are modulation schemes that yield displays from nuclei and/or electrons that have relaxation times falling in predetermined ranges, more careful searches for evidence of stronger couplings which may yet permit structural determination of the site or sites, and tailoring of electron and/or nuclear relaxation times by introduction of suitable paramagnetic metals to the surface of melanin.

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