Thermodynamic and Electron Paramagnetic Resonance Characterization of Flavin in Succinate Dehydrogenase*

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Thermodynamic parameters of succinate dehydrogenase flavin were determined potentiometrically from the analysis of free radical signal levels as a function of the oxidation-reduction potential. Midpoint redox potentials of consecutive 1-electron transfer steps are $-127$ and $-31$ mV at pH 7.0. This corresponds to a stability constant of intermediate stability, $2.5 \times 10^{-7}$, which suggests flavin itself may be a converter from $n = 2$ to $n = 1$ electron transfer steps.

The pK values of the free radical (F$\text{H}^+$ \leftrightarrow \text{Fl}^*$) and the fully reduced form (F$\text{H}_2^+$ \leftrightarrow \text{Fl}^*$) were estimated as $8.0 \pm 0.2$ and $7.7 \pm 0.2$, respectively. Succinate dehydrogenase flavosemiquinone elicits an EPR spectrum at $g = 2.00$ with a peak to peak width of 1.2 mT even in the protonated form, suggesting the delocalization of the unpaired electron density.

A close proximity of succinate dehydrogenase flavin and iron-sulfur cluster S-1 was demonstrated based on the enhancement of flavin spin relaxation by Center S-1.

It is generally accepted that the succinate dehydrogenase molecule consists of two subunits: one flavo iron-sulfur subunit with $M_c$, of approximately 70,000 and one iron-sulfur subunit of 27,000. The flavo iron-sulfur subunit contains 4 Fe, 4 S, and one covalently bound FAD; the iron-sulfur subunit contains 4 Fe and 4 S (1–4). We have proposed the presence of two binuclear iron-sulfur clusters (Center S-1 and S-2) in the flavo iron-sulfur subunit and one tetranuclear cluster (Center S-3) in the iron-sulfur subunit based on the EPR and thermodynamic characteristics of these centers and on the correlation between enzymic activities toward various artificial electron acceptors and functional redox components which were studied with various types of soluble succinate dehydrogenase preparations, such as reconstitutively active BS-SDH, reconstitutively inactive B-SDH, and AA-SDH (2, 5, 6).

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The abbreviations used are: BS-SDH, butanol-solubilized enzyme with succinate preincubation; B-SDH, butanol-solubilized without succinate; AA-SDH, alkaline-solubilized from acetone powder of mitochondria or submitochondrial particles; EPR, electron paramagnetic resonance; UQ, ubiquinone.

In all the succinate dehydrogenase preparations we have so far reported, Center S-2 is EPR-detectable and its spin relaxation behavior seems to be highly sensitive to the molecular environment around the cluster (2, 5); a weak dipole-dipole interaction between Centers S-1 and S-2 was observed which was manifested as the relaxation enhancement of S-1 spins by S-2 or as either splitting or broadening of low temperature EPR spectra of fully reduced enzymes. The spatial relationship between Center S-1 and S-2 in different succinate dehydrogenase preparations has been discussed in detail in a preceding paper (5).

In this paper, we report thermodynamic parameters and EPR characteristics of the succinate dehydrogenase flavin and discuss a possible mechanism for flavin to convert the $n = 2$ electron transfer step to that of $n = 1$ in a mitochondrial primary dehydrogenase. A relatively short Center S-1 to flavin distance is implied by the spin-spin interaction we report, supporting our earlier assignment of cluster S-1 and S-2 in the flavo iron-sulfur subunit.

EXPERIMENTAL PROCEDURES

Potentiometric titration of the flavin-free radical of succinate dehydrogenase was conducted anaerobically as described by Dutton (8). The following redox-mediating dyes were used selectively to minimize interference from the dye $g = 2.00$ signals: 1-4-naphthoquinone, duroquinone, indigo disulfonate, indigo tetrasulfonate, 2-OH-1,4-naphthoquinone, phenosafranine, and safranine T; each at a final concentration of 50 $\mu$M. These dyes undergo redox changes in close to $n = 2$ processes and variations of the dye concentration from 10 to 500 $\mu$M did not affect the size of the measured $g = 2.00$ signal. In addition, dye signals can be distinguished from that of the flavin free radical associated with succinate dehydrogenase based on their slower spin relaxation rates similar to flavodoxin $g = 2.00$ signals as will be described later. Spin quantitation of the free radical signals at titration peaks were conducted by double integration of the $g = 2.005$ signal, using the flavodoxin free radical from Peptostreptococcus elsdenii as a standard (9). EPR samples were rapidly frozen in a 1:5 mixture of methylcyclohexane and isopentane at 80 K.

Redox titration data were simulated using supporting our earlier assignment of cluster S-1 and S-2 in the flavo iron-sulfur subunit.

RESULTS

Fig. 1 presents a potentiometric titration of the $g = 2.005$ signal of flavin free radical in the soluble succinate dehydrogenase preparation (BS-SDH) at pH 7.0. Peak to peak amplitude of the free radical signal was plotted as a function of $E_a$ (redox potential referred to the potential of a standard hydrogen electrode) of the enzyme solution. The bell-shaped titra-
EPR Studies of Succinate Dehydrogenase Flavin

Fig. 1. Potentiometric titration of the flavin free radical (g = 2.005 signal in succinate dehydrogenase. The enzyme (68.7 

\[ E_{m,2} = -79 \text{ mV} \]

\[ E_{m,1} = -127 \text{ mV} \]

\[ E_{m} = -31 \text{ mV} \]

free radical and the fully reduced form of the flavin were estimated. pK values of interest are pK_{F} (FIH \leftarrow FIH^-) and pK_{K} (FIH \leftarrow FIH^-). As seen in Fig. 2, the pK_{K} value can be obtained from the pH dependence of the midpoint potential (E_{m}) for the overall 2-electron transfer, namely from the oxidized to the fully reduced form of the flavin. These E_{m} values are obtained empirically from the E_{m} value of the peak position of the titration curves at different pH. The line in the figure is a theoretical curve with pK_{K} of 7.7. The straight lines with a slope of -60 mV and -30 mV/pH, respectively, which fit the curve in the lower and higher pH ranges, intersect at the pK_{K}. The pK_{K} does not affect the pH dependence of E_{m} values. Fig. 3 gives the maximum per cent free radical concentration of the potentiometric titrations as a function of pH. Below pH 7 the maximum free radical concentration remains unchanged and raising the pH above pH 7 leads to increasing maximum free radical concentration as seen in Fig. 3. The line drawn in the figure is a theoretical curve with a pK_{K} value of 7.95 and pK_{R} of 7.7. The computer-analyzed pH dependencies of the midpoint potentials of the first and second 1-electron transfer steps, namely E_{m1} and E_{m2}, are presented in Fig. 4. Below pH 7 the difference between E_{m1} and E_{m2}, i.e., -100 mV, is constant and in the higher pH range the difference is

Fig. 2. Determination of pK_{R} from the pH dependence of E_{m} values. E_{m} values were obtained directly from the redox potentials giving titration peaks.

Fig. 3. pH dependence of the maximum per cent free radical concentration in potentiometric titrations. Spin concentration of g = 2.00 signal and the flavin concentration in potentiometric titrations as a function of pH.

Fig. 4. pH dependence of E_{m1} and E_{m2}, midpoint potentials of two 1-electron transfer steps of flavin in succinate dehydrogenase. Individual E_{m1} and E_{m2} at different pH were calculated from the data presented in Figs. 2 and 3.
gradually diminished; the free radical state is more stabilized. Fitting the theoretical curves gave optimal values of pK_a and pK_b as 7.7 ± 0.2 and 8.0 ± 0.2, respectively. Thus, at pH 7.0, about 90% of the free radical is expected to be in the neutral (protonated) form (FIH^-), while at pH 9.0 about 90% is in the anionic (deprotonated) form (FI^-). Fig. 5 presents the flavin-free radical in succinate dehydrogenase (BS-SDH) at pH 6.5 and 9.0 which were obtained by poising the enzyme at redox potentials of ~16 mV and ~150 mV, respectively. The radical gives a g 2.00 signal with a linewidth of 1.15 mT at both pH 6.5 and 9.0 where the free radicals are mostly in the protonated and deprotonated form, respectively. Both spectra are typical of flavins with wings both on the lower and higher field side (11, 12).

Previously, Beinert et al. (13) reported a high level of the free radical state (about 80% at pH 7.8) in reconstitutively inactive succinate dehydrogenase preparations upon reduction with succinate. When reconstitutively active BS-SDH was reduced with succinate at different pH values we obtained much lower maximum free radical concentrations, namely, about 10% below pH 7 and 20% even at pH 9 (Fig. 6). In order to understand the widely different amounts obtained of the flavin free radicals in the succinate-reduced dehydrogenase preparations, we analyzed possible redox states of succinate dehydrogenase components when the dehydrogenase was poised with a succinate and fumarate couple which delivers 2 electrons at a time. The high potential iron-sulfur protein type iron-sulfur cluster, Center S-3 becomes extremely labile toward oxidants in the soluble state and is EPR-detectable only in the reconstitutively active form (6, 14, 15). Scheme 1 gives possible redox states of succinate dehydrogenase components upon 2-electron reduction in two different succinate dehydrogenase systems: System A retains active Center S-3; System B contains only inactive Center S-3. Intermolecular redox equilibration is assumed to be much slower than the intramolecular equilibration. In case A, two different redox states (S-1 reduced, S-3 oxidized, flavin free radical) and (S-1 oxidized, S-3 reduced, flavin free radical) are the states which give rise to free radical signals among a total of six different redox states. In case B, (S-1 reduced and flavin free radical) is the only state which gives rise to the free radical signal among three different redox states obtainable. The maximum free radical concentration at different pH values can be calculated for these two cases using E_n1 and E_n2 values of the flavin as reported above and E_n values of 0 mV and 60 mV for Center S-1 and S-3, respectively. This is plotted in Fig. 6 with a dashed line for case B and a dotted-dashed line for case A. High free radical concentrations reported for the reconstitutively inactive succinate dehydrogenase preparations (13) fits very well with case B. Data on BS-SDH fit rather well to the calculated curve in the pH range below 8, but gives slightly higher radical concentrations above pH 8 (Fig. 6). This is explicable from the partial inactivation of Center S-3 even in the reconstitutively active BS-SDH. It is worth pointing out that if we titrate Center S-1 and flavin in succinate dehydrogenase preparations where no Center S-3 is reactive, the titration with a succinate/fumarate couple gives the same redox titration curve in the pH range below 8, but gives slightly higher radical concentrations above pH 8 (Fig. 6). This is explicable from the partial inactivation of Center S-3 even in the reconstitutively active BS-SDH. In order to study spatial relationships between flavin and

![Fig. 5. EPR spectra of BS-SDH at pH 6.5 and 9.0. The enzymes were potentiometrically poised at ~16 mV and ~150 mV, respectively, for pH 6.5 and 9.0 systems. The former was dissolved in 7 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and 43 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.5) at the final flavin concentration of 63.9 μM and the latter was dissolved in 50 mM N-tris(hydroxymethyl)methylethanesulfonic acid buffer at 30.9 μM. EPR sample temperature, 213 K; input microwave power, 100 microwatts, modulation amplitude, 6.3 × 10^-4 tesla.]

![Fig. 6. pH dependence of g = 2.00 signal size in succinate reduced succinate dehydrogenase. Theoretical curves for case A (---) and case B (----) of Scheme I.]

**A. SDH with Center S-3**

- S-1^r oxidation, S-3^r flavin, S-1^ox flavin, S-3^ox flavin
- 2e transferred

**B. SDH without Center S-3**

- S-1^ox flavin, S-3^ox flavin
- 2e transferred

**SCHEME I. Possible redox state of succinate dehydrogenase components poised with succinate and fumarate couple (n = 2) electron donor. A and B correspond to reconstitutively active and inactive succinate dehydrogenase (SDH), respectively.**
Center S-1 in succinate dehydrogenase, we compared the power saturation behavior of the g = 2.00 signals of the flavin free radical of succinate dehydrogenase with that of flavodoxin at two different temperatures. In flavodoxin, no transition metal ion is present in the vicinity of the flavin free radical (7). A saturation parameter, $P_{1/2}$, was determined according to the quantitative procedure reported by Blum and Ohnishi (16). $P_{1/2}$ is the input microwave power level at which the saturation condition is satisfied as discussed in Ref. 16. The $P_{1/2}$ value of succinate dehydrogenase flavin is significantly higher than that of flavodoxin both at 193 and at 233 K. It is interesting to see that the spin relaxation of the flavodoxin free radical is insensitive to the temperature difference of 40 K in the above temperature range, whereas the $P_{1/2}$ value of the succinate dehydrogenase flavin free radical signal is doubled by raising the temperature from 193 K to 233 K. This indicates that in the succinate dehydrogenase molecule, spin relaxation of the flavin free radical signal is enhanced by nearby Center S-1 spins. In order to test this S-1 effect, flavin spin relaxation was examined after complete destruction of Center S-1 by lowering the pH of the enzyme to 4 and then returning to neutral pH. As seen in Table I, spin relaxation of the succinate dehydrogenase flavin approaches that of the flavodoxin free radical in the absence of S-1 spins. These results suggest a small distance between flavin and at least one of the binuclear iron-sulfur clusters, namely Center S-1. Center S-2 is not paramagnetic under the experimental conditions required for samples shown in Table I.

**DISCUSSION**

The present study shows that succinate dehydrogenase flavin has a free radical formation constant of $K = 2.5 \times 10^{-2}$ at neutral pH which is a far more stable intermediate redox state than the typical $n = 2$ redox components, such as a free ubiquinone/ubiquinol couple in a hydrophobic milieu ($K = 10^{-15}$) (17), or the NAD$^+$/NADH couple ($K = 10^{-11}$) (18). This indicates that succinate dehydrogenase flavin itself may function as a good converter from an $n = 2$ to an $n = 1$ electron transfer process. The flavin accepts electrons from succinate in a 2-electron step: the flavosemiquinone is stable enough to permit the reduction of iron-sulfur clusters of the dehydrogenase in sequential 1-electron steps, although the detailed mechanism of the latter steps is not yet completely known. It is therefore unnecessary to postulate simultaneous reduction of two iron-sulfur clusters by flavin as an $n = 2$ to $n = 1$ step-down mechanism (19).

Recently, Albracht proposed that succinate dehydrogenase contains only one binuclear (Center S-1) and one tetranuclear iron-sulfur cluster (Center S-3) per molecule based mostly on the nondetectability of the Center S-2 EPR signal in a succinate-cytochrome c reductase preparation (20) as well as in succinate dehydrogenase which was isolated from Complex II and retains almost full reconstitutive activity in a highly pure form (4, 21). We have confirmed these experimental observations (3). Even in these two systems, spin relaxation of Center S-1 is dramatically enhanced by fully reducing the enzyme with dithionite. Albracht (20) interpreted this spin relaxation enhancement as being caused by a protein conformational change induced by the reduction of the flavin to the fully reduced state. The enhancement of the S-1 spin relaxation is caused by an $n = 1$ redox component (Center S-2) with midpoint potential of approximately $-400$ mV in soluble succinate dehydrogenase preparations (2, 6).

Analysis of the titration data presented in Fig. 1 indicates that a midpoint potential ($E_m$) of $-81$ mV would be obtained for a titration monitoring the fully reduced form of flavin, and $-77$ mV for a titration of the oxidized form, both with $n$ value close to 2. Neither midpoint potential nor $n$ value for the flavin reduction support the hypothesis proposed by Albracht (20) and the enhancement of S-1 spin relaxation seems to be consistent with the cross-relaxation of S-1 via S-2 spins (2, 5, 6). As we reported previously, spin relaxation of Center S-2 greatly depends on the microenvironment of the active center. We interpret the nondetectability of Center S-2 in these intact systems as being due to either (i) an extremely short relaxation time for S-2 spins or (ii) the spin coupling between S-1 and S-2 is such that we do not see signals for more than one spin per molecule in the fully reduced enzyme of these intact preparations.

The presence of two binuclear and one tetranuclear iron-sulfur clusters in the succinate dehydrogenase molecule has also been demonstrated by an independent procedure, namely, iron-sulfur core extrusion and core displacement as reported by Coles et al. (4).

Ackrell et al. (22) conducted a potentiometric titration of the activation process of the enzyme in the presence of excess oxalacetate; oxalacetate binds tightly to succinate dehydrogenase in the oxidized state, inactivating the dehydrogenase (22). In this method an oxidation-reduction component was titrated with midpoint potential $E_{m/2}$ value between $-60$ and $-90$ mV in an $n = 2$ process, suggesting the titration of the

![FIG. 7. Power saturation behavior of the flavin free radical in succinate dehydrogenase and flavodoxin at two different temperatures.](image)

**TABLE I**

| Samples                  | $P_{1/2}$ values |
|--------------------------|------------------|
| A. Flavodoxin            | 0.20             |
| B. BS-SDH poised at $-78$ mV | 0.78             |
| C. B-SDH reduced with succinate | 0.80             |
| D. Acid-treated BS-SDH poised at $-70$ mV | 0.23             |

T. Ohnishi, H. Blum, C. A. Yu, and L. Yu, unpublished data.
succinate dehydrogenase flavin. This is in good agreement with the titration of the fully reduced form of flavin.

In this paper we have demonstrated the close proximity between succinate dehydrogenase flavin and Center S-1 from the enhancement of flavin spin relaxation by S-1 spins (Fig. 7 and Table I). We previously reported that the distance between the S-1 and S-2 clusters is approximately 10 Å (2, 5); a high potential iron-sulfur protein-type cluster (Center S-3) and a ubiquinone pair (23, 24) which is the specific electron acceptor of succinate dehydrogenase are also considered to be located within about 15 Å of each other (24, 25). As shown in Fig. 7 this paper, the saturation parameter (P1/2) of succinate dehydrogenase flavin is 2.0 milliwatts at 233 K, indicating that there is an iron-sulfur cluster in the near vicinity of the flavin. This is a binuclear rather than tetranuclear cluster, since intact Center S-3 is present in BS-SDH and absent in B-SDH and destruction of S-1 and S-2 causes the flavin relaxation to slow markedly. The very short relaxation time (P1/2 > 100 milliwatts at 233 K) observed with the g = 2.00 signal of ubisemiquinone is consistent with its location close to the tetranuclear cluster, Center S-3 (24–26). These results further strengthen our earlier topographical assignment of two binuclear clusters S-1 and S-2 in flavin-iron-sulfur subunit and Center S-3 tetranuclear iron-sulfur cluster in the iron-sulfur subunit.

Using the previously reported lifetime broadening of the Center S-1 EPR spectrum (27), we can estimate the T1 of Center S-1 as about 10−8 s at 230 K. The flavin power saturation curve in the presence of reduced Center S-1 has a P1/2 of about 2 milliwatts, which corresponds to a cross-relaxation time of the order of 10−8 s. Since no broadening or splitting of the iron-sulfur EPR spectrum due to the flavin semiquinone can be observed at low temperature, we can place an upper limit of <5 G on any coupling between the two. We thus expect a flavin-iron-sulfur distance of at least 12 Å.

In the absence of spin-spin interaction, a system containing two S = ½ species in a static magnetic field H0, will have four states, αα, αβ, βα, and ββ, which correspond to the possible orientations of each spin in the magnetic field ("up up," "up down" etc.). The Zeeman interactions can be represented

\[ H_{\text{Zeeman}} = (S_1 \cdot g_1 \cdot H_0 + S_2 \cdot g_2 \cdot H_0) \]

The energies of the four states are thus

\[ \frac{1}{2} (-g_1 H_0 + g_2 H_0), \frac{1}{2} (-g_1 H_0 - g_2 H_0), \frac{1}{2} (g_1 H_0 - g_2 H_0), \frac{1}{2} (g_1 H_0 + g_2 H_0). \]

If S1 has a rapid relaxation rate but S2 relaxation is slow, the αα ↔ αβ and βα ↔ ββ transitions will be difficult to saturate while αα ↔ βα and βα ↔ ββ will be easily saturable. The dipolar interaction between two spins S1 and S2 can be represented by the Hamiltonian

\[ H_{\text{dip}} = \frac{g_1 g_2 \beta r_s}{r} + \frac{g_1 g_2 \beta}{r} (S_1 \cdot \vec{r} \cdot S_2) \]

where β is the Bohr magneton and \( \vec{r} \) is the interspin vector. This is often decomposed as follows

\[ H_{\text{dip}} = (g_1^2 \beta / r^3) (A + B + C + D + E + F) \]

where A = S1 wide latter (1/3 cos θ)\( S_2 \), B = (1/3 cos θ)\( S_1 \cdot S_2 \), C, D, E, and F are composed of terms such as S1 \( S_1^\prime \), S1 \( S_2^\prime \), S1 \( S_2^\prime \), S2 \( S_2^\prime \), S1 \( S_2^\prime \), S1 \( S_2^\prime \) with only states which differ by at least one Zeeman splitting (g1βH or g2βH).

The exchange Hamiltonian is just

\[ H_{\text{ex}} = -2S_1 \cdot J \cdot S_2 \]

which has only terms in S1 \( S_1^\prime \), S2 \( S_2^\prime \), and S1 \( S_2^\prime \). The terms in S1 \( S_1^\prime \), in the dipolar and exchange Hamiltonians give rise to diagonal matrix elements; these contribute a first order splitting (of 2A for the dipolar terms) but do not mix the states. The terms in S1 \( S_1^\prime \) and S2 \( S_2^\prime \) connect the αβ and βα states. These states are mixed and two new states result

\[ \psi_t = c_2 \alpha + c_3 \beta \]

\[ \psi_s = c_2 \beta - c_3 \alpha \]

If the coupling terms are smaller than the energy difference between αβ and βα, (g1 − g2)βH0, will be nearly 1, and using perturbation theory \( c_1 \sim B'/(g_1 - g_2)βH0 \), where \( B' \) is the sum of the dipolar B and exchange terms. The S1 transitions thus have some S2 character even with relatively weak coupling if the separation between αβ and βα is smaller (g1 − g2). In the limiting case, αβ and βα are degenerate and the states are completely mixed (c = c2 = 1/√2).

The fast relaxation of S1 transitions must result from matrix elements from other sources which couple αα with βα and αβ with ββ. In the coupled system, there will be matrix elements of this type connecting αα with \( \psi_t \) and \( \psi_s \) with \( \beta \beta \) weaker by a factor of B’/(g1 − g2)βH0 than those connecting αα and ββ in the uncoupled system. The transition probability depends on the square of the matrix element connecting two states. Thus, we expect the predominantly S2 transition to acquire a new relaxation mechanism with a characteristic time \( 1/T_1 = 1/T_{1S_2}, (-B'/(g_1 - g_2)βH_0)^2 \).

The terms in S1 \( S_2 \), in the dipolar and exchange Hamiltonians give rise to the first order splittings; clearly, these must be within the linewidth. The terms in S1 \( S_2 \) and S1 \( S_3 \) connect the αβ and βα states. This contributes to the observed splitting when the magnitude of the off diagonal terms is not much smaller than the separation of the basis states.

Since the g tensors of both the iron-sulfur cluster and flavin semiquinone are not very anisotropic and overlap, rather small dipolar or exchange couplings would be effective in mixing the iron-sulfur and iron-sulfur transitions through the S1 \( S_2 \) and S1 \( S_3 \) terms. The anisotropy of the iron-sulfur center, while not large in proportion to its magnetic moment, will lead to an angular dependent ΔE = g1 − g2. At some orientations, g1 = g2 and the iron-sulfur and flavin semiquinone transitions will be completely mixed. It will be difficult to saturate the "flavin" transition of molecules oriented in H0 so that g1 and g2 are nearly equal.

The majority of molecules will not have these special orientations. The onset of saturation will be determined by molecules which have |g1 − g2| nearly maximal or have the value of the B’ term near zero because of the (1−3 cos θ) dipolar angular dependence; the overall saturation behavior will be more characteristic of the average separation and average B’ value. A full description would require numerical integration over all orientations at each power. This would depend on the orientation of the g tensors with respect to each other and the F as well as the magnitudes of g and F. We can get some idea of the coupling strength necessary to account for the observed relief of saturation by considering molecules oriented so that g1 = g2 is close to the average.

The average separation of the two middle states ("αβ" and "ββ") of the four level system resulting from weak coupling of the two S = ½ spins is about 90 G. The iron-sulfur relaxation mechanisms should therefore enhance the flavin relaxation by

\[ 1/T_{1S_2} \approx (1/T_{1S_2})_{\text{B'}} (B'/90 \text{ G})^2 \]

where \( B' \) is the magnitude of the S1 \( S_2 \) and S1 \( S_3 \) terms in gauss; \( B' \approx 3 \text{ G} \) is sufficient to account for the observed relaxation through this mechanism.

If \( B' \) is due to dipolar terms, the flavin-iron-sulfur distance would be ~15 Å using a point dipole approximation. The
unpaired electron should be highly delocalized; the 15 Å distance would lie between the center to center and edge to edge distances because of the $r^{-3}$ weighting imposed by dipolar coupling. $B'$ can also have contributions from exchange coupling, but it is unlikely that substantial coupling would occur at much larger distances than this, since exchange interactions are believed to fall off roughly an order of magnitude per bond length. The separation is probably thus between 12 and 18 Å, but more sophisticated techniques will be necessary to sort out the system thoroughly.

From the comparison of EPR and optical properties of various flavo proteins, Massey and Palmer (9) demonstrated that neutral flavin free radical species, protonated at N(5), generally exhibit an EPR spectrum with a linewidth of 1.9 mT and anionic species a 1.5 mT linewidth. The exceptionally narrow linewidth of succinate dehydrogenase flavin (Fig. 5) was suggested to be due to the anionic form further narrowed due to covalent linkage to the apoprotein polypeptide at the 8α carbon position which eliminates the hyperfine coupling contribution from the 8-CH$_3$ group (28). The present study points out that both neutral and anionic flavo semiquinone of succinate dehydrogenase exhibit the same narrow linewidths of 1.2 mT, suggesting that the unpaired spin density distribution in succinate dehydrogenase flavo semiquinone is probably more delocalized than in an isolated succinate dehydrogenase flavin peptide preparation (29, 30) or in other flavin species (31, 32). On the other hand, equilibrium titration data do not exclude the possibility that the pH-dependent $E_r$ value for the flavin/flavin semiquinone couple is due to protonation of a neighboring amino acid residue of the succinate dehydrogenase protein concurrent with electron transfer to the flavin (see Ref. 33).

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