EPR STUDIES ON TWO FERREDOXIN-TYPE IRON-SULFUR CENTERS IN RECONSTITUTIVELY ACTIVE, INACTIVE, AND REACTIVATED SOLUBLE SUCCINATE DEHYDROGENASES

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SUMMARY -- Two distinct iron-sulfur centers, S-1 and S-2 are present in both reconstitutively active and inactive soluble succinate dehydrogenase preparations in approximately equivalent concentrations to that of bound flavin. The midpoint potentials at pH 7.4 of these centers are -5 ± 15 mV and -400 ± 15 mV, respectively. EPR characteristics of Center S-2, observed above 6° K, are not significantly different in the active and inactive dehydrogenases. At lower temperatures, however, major line shape modifications of Center S-2 spectra are observed in the reconstitutively inactive dehydrogenases, but neither in the active dehydrogenase nor in the particulate preparations. This phenomenon may reflect spin-spin interaction between Centers S-1 and S-2. Chemical reactivation of the reconstitutively inactive preparations abolishes this resonance modification and restores the normal line shape. This is a demonstration of another close correlation between a physical property and reconstitutive activity of succinate dehydrogenase.

INTRODUCTION

The presence of two distinct iron-sulfur centers, designated as Centers S-1 and S-2, was previously demonstrated in the succinate-ubiquinone (UQ) reductase segment of the respiratory chain (1). The field position of EPR signals arising from these two centers are similar; however, their EPR line shapes and temperature profiles are different. The midpoint potentials at pH 7.4 ($E_{m7.4}$) are 0 ± 15 mV and -260 ± 15 mV, respectively, when one measures them in particulate preparations such as succinate-UQ reductase or antimycine A sensitive succinate-cytochrome c reductase. In the preceding paper (2), the third iron-sulfur component of succinate dehydrogenase

(Center S-3) was compared in various succinate dehydrogenase preparations and the essentiality of the unmodified Center S-3 for reconstitutive activity was demonstrated. The present communication characterizes Centers S-1 and S-2 in various soluble succinate dehydrogenase preparations, as summarized in Table I in the preceding paper. The spin-spin interaction, probably between Center S-1 and Center S-2 was observed in the reconstitutively inactive dehydrogenases, but neither in the reconstitutively active and chemically reactivated dehydrogenases nor in the particulate preparations indicates the importance of structural integrity around the active centers of the dehydrogenase molecule for enzymatic function.

MATERIALS AND METHODS

Preparations of reconstitutively active or inactive succinate dehydrogenases used in this study are summarized in Table I of the preceding paper (2). The soluble cytochrome $\underline{b}$-$\underline{c}_1$ complex and the reconstituted succinate-cytochrome $\underline{c}$ reductase were prepared as previously described (3). All other experimental conditions were the same as described in the preceding paper (2).

RESULTS AND DISCUSSION

Figure 1 shows the redox titration of iron-sulfur Centers S-1 and S-2 in the reconstitutively active succinate dehydrogenases (BS-SDH and AS-SDH). The $E_{m7.4}$ values of Centers S-1 and S-2 in these soluble enzymes have been found to be -5 ± 15 mV and -400 ± 15 mV, respectively. The midpoint potential of Center S-1 remains essentially unchanged during the solubilization and isolation of the enzymes, while $E_{m7.4}$ of Center S-2 shifts to the more electronegative value by about 140 mV. Center S-2 shows an $E_{m7.4}$ of -260 ± 15 mV in the particulate preparations. Similar midpoint potentials are obtained in succinate dehydrogenase which contains the same ratio, FAD:Fe:S = 1:8:8, but which is reconstitutively inactive, $\underline{i.e.}$ B-SDH. The presence of two distinct iron-sulfur centers, S-1 and S-2, is also apparent in the succinate dehydrogenase preparations having ratios of 1:6:4

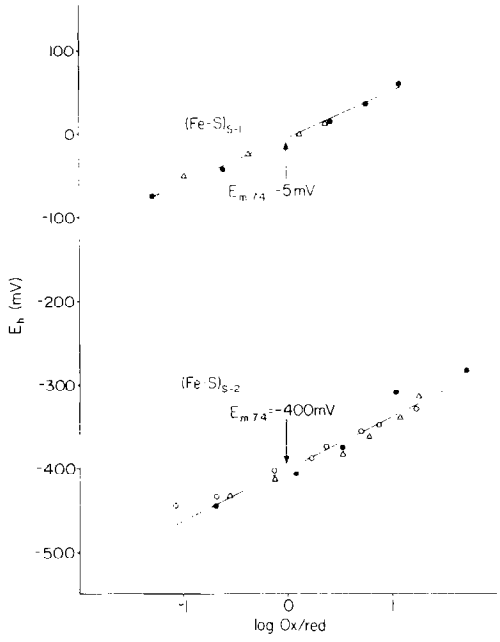

FIGURE 1. Nernst plots of the redox titration of Centers S-1 [$(Fe\text{-}S)_{S-1}$] and S-2 [$(Fe\text{-}S)_{S-2}$] of soluble succinate dehydrogenases. Reconstitutively active succinate dehydrogenase (4 nmoles flavin/mg protein) (BS-SDH) from the heart muscle preparation (HMP), and (8 nmoles flavin/mg protein) for AS-SDH from Complex II were used. For the individual titrations, the protein concentrations were: 5.1 mg/ml for both $(Fe\text{-}S)_{S-1}$ and $(Fe\text{-}S)_{S-2}$ titrations (● —— ●); 3.0 mg/ml for $(Fe\text{-}S)_{S-2}$ titrations (o —— o); 5.8 mg/ml for $(Fe\text{-}S)_{S-1}$ and 2.9 mg/ml for $(Fe\text{-}S)_{S-2}$ titrations (Δ—— Δ). Redox mediators for the $(Fe\text{-}S)_{S-1}$ titration: 62.5 μM phenazine methosulfate, 62.5 μM phenazine ethosulfate, 25 μM duroquinone, 6.3 μM pyocyanine, 25 μM 2-hydroxy-naphthoquinone and 6 μM resorufin. Redox mediators for the $(Fe\text{-}S)_{S-2}$ titration: 62.5 μM phenazine ethosulfate, 6.3 μM pyocyanine, 6 μM resorufin, 25 μM 2-hydroxy-naphthoquinone, 77.5 μM phenosafranine, 94 μM benzyl viologen and 133 μM methyl viologen. EPR conditions used were microwave frequency 9.1 GHz with a 12.5 gauss modulation amplitude; time constant, 0.3 sec; scanning rate, 500 gauss per min. Temperature and microwave power for the titrations of $(Fe\text{-}S)_{S-1}$ and $(Fe\text{-}S)_{S-2}$ were 27° K and 20 mW, and 7° K and 5 mW, respectively.

(CN-SDH) and 1:4:4 (AA-SDH).

The temperature dependence of the EPR signals arising from Centers S-1 and S-2 in these different soluble succinate dehydrogenase preparations is very similar to that observed in the particulate preparations (cf. Ref. 1). Center S-1 signals can be observed even at 77°K, but readily show power saturation at temperatures below 20°K. Center S-2 signals are clearly seen only at temperatures below 20°K (cf. also Ref. 4).

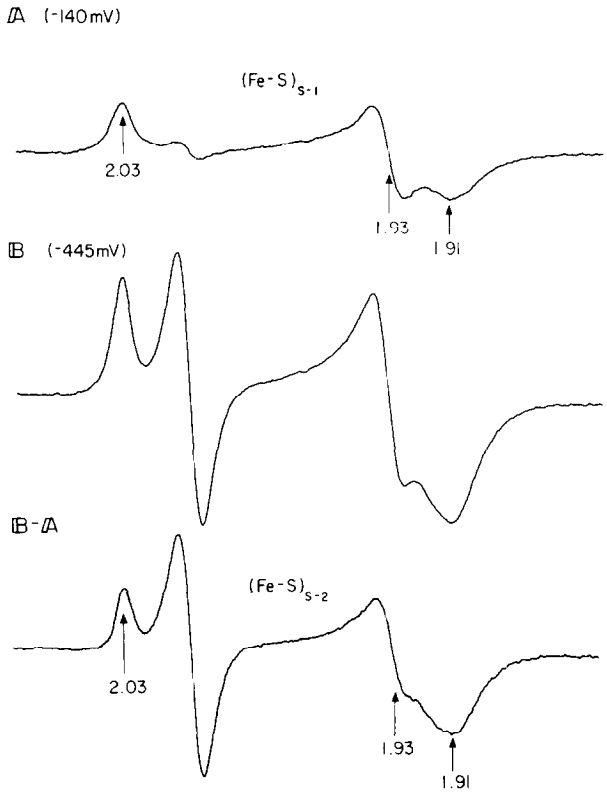

FIGURE 2. EPR spectra of Centers S-1 and S-2 in BS-SDH. Center S-2 spectrum was obtained as the difference of spectra A and B, which were poised at -140 mV and -445 mV, respectively. The difference spectrum B-A was obtained by a Nicolet Signal Averager (NIC-1074). Concentration of BS-SDH was 20 mg protein/ml of 3 nmoles flavin/mg protein. In the present titration, all the redox mediating dyes were present, which were added either for titration of Center S-1 or S-2, separately in Fig. 1. EPR operation conditions used were modulation amplitude, 5 gauss; microwave frequency, 9.13 GHz; time constant, 0.3 sec; scanning rate, 200 gauss/min; microwave power, 0.5 mW; temperature, 9.8° K.

Because of the large difference in their midpoint potentials, individual EPR spectra of Centers S-1 and S-2 can easily be obtained. EPR spectra of BS-SDH poised at -140 mV and -445 mV are presented in Fig. 2. At an E_h of -140 mV, Center S-1 is almost completely reduced, while Center S-2 remains entirely oxidized; this gives signals for Center S-1 occurring at $g_z = 2.03$, $g_y = 1.93$, and $g_x = 1.91$. At the indicated temperature and microwave power setting the Center S-1 signal is considerably saturated but the line shape still remains unmodified. These EPR conditions were

chosen to obtain spectra of Centers S-1 and S-2 under the same EPR condition. At an E_h of -445 mV, both Centers S-1 and S-2 are reduced. In this way, a difference spectrum (B-A) yields the Center S-2 spectrum under the non-saturated condition. As illustrated by spectra A and C, Centers S-1 and S-2 give rise to resonance signals at the same field positions, but with different line shapes.

In order to examine the relative concentrations of Centers S-1 and S-2 in these enzymes, double integration of the individual EPR spectra was performed under non-power-saturated conditions. The integrated value for Center S-2 was corrected for the partial contribution of Center S-1 by employing two dehydrogenase samples; one fully reduced with dithionite and the other poised at about E_h of -150 mV (not reduced with succinate). As shown in Table I, the concentrations of both Centers S-1 and S-2 were nearly

TABLE I

RELATIVE SPIN CONCENTRATIONS OF CENTERS S-1 AND S-2*

Preparation succinate dehydrogenase**	Center S-1	Center S-2
BS-SDH	0.8 - 0.9	0.8 - 1.0
B-SDH	0.8 - 0.9	0.8 - 0.9
AA-SDH	0.8 - 1.0	0.6 - 0.8

* EPR spectra of Center S-1 were recorded at 22.2° K with 1 mW microwave power; spectra of Center S-2 at 10° K with 0.5 mW power, and contribution from Center S-1 was corrected as described in the text. EDTA-Cu(II) at the given temperature and power was used as a standard for the double integration. Corrections were made for transition probability according to Aasa and Vänngard (7).

** Cf. Table I of the preceeding paper for the preparation and other properties.

stoichiometrically equivalent to flavin content in all these dehydrogenase preparations, in agreement with the double integration value obtained by DerVartanian et al. (5) and by Beinert et al. (4) for Center S-1. The latter group (4) reported that the concentration of Center S-2 averaged 0.2 ∿ 0.3 per flavin, however, their EPR conditions for double integration are still not available.

Two distinct iron-sulfur centers, S-1 and S-2, are present, even in the dehydrogenase (AA-SDH) which contains only 4 atoms each of iron and sulfur per flavin, although the S-2 concentration in this case appears to be slightly less than that of S-1. This fact indicates that both Centers S-1 and S-2 consist of 2 iron and 2 acid labile sulfur atoms, similar to adrenodoxin or spinach ferredoxin (6).

As previously reported in detail (cf. Ref. 8,9), antimycin A-sensitive succinate-cytochrome c reductase activity is restored by reconstituting the cytochrome b-c_1 complex with reconstitutively active succinate dehydrogenase (BS-SDH). The redox titration of Center S-2 was performed using the reconstituted succinate-cytochrome c reductase. The $E_{m7.4}$ value of Center S-2 increased from -400 mV ± 15 to -250 ± 20 mV, which was approximately the $E_{m7.4}$ value obtained originally in succinate-cytochrome c reductase before the removal of succinate dehydrogenase. If the reconstituted reductase was centrifuged down and suspended without washing, a significant fraction of Center S-2 exhibited an $E_{m7.4}$ value which was the same as that in soluble succinate dehydrogenase. However, this low $E_{m7.4}$ fraction was easily washed off from the reconstituted particles. These observations indicate that Center S-2 restores its original molecular environment in the reconstitution process. In contrast, reconstitutively inactive succinate dehydrogenases did not recombine with the cytochrome b-c_1 complex.

Figure 3 illustrates the EPR spectra of Center S-2 in three different succinate dehydrogenases at two different temperatures. At temperatures above 6° K, no significant difference in the line shape of Center S-2 is

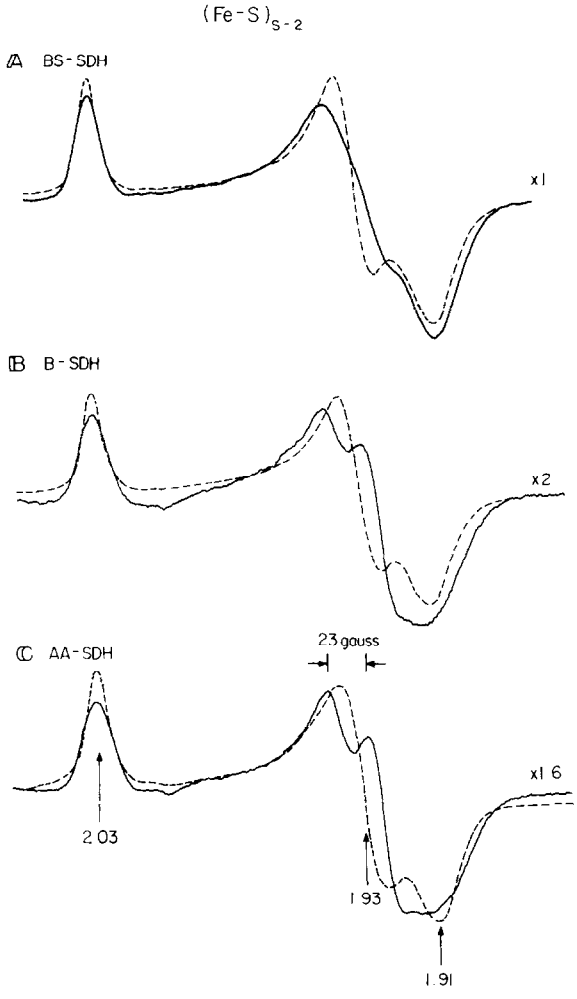

FIGURE 3. EPR spectra of Center S-2 in reconstitutively active (BS-SDH) and inactive (B-SDH and AA-SDH) succinate dehydrogenases. A. BS-SDH, 20 mg protein/ml of 3 nmoles flavin/mg protein, in 0.1 M K-phosphate buffer (pH 7.4). B. B-SDH, 11 mg protein/ml of 2 nmoles flavin/mg protein. C. AA-SDH, 30.2 mg protein/ml of 1.9 nmoles flavin/mg protein. In all cases, the dehydrogenase was fully reduced with a slight excess of solid dithionite. EPR spectra in solid lines were obtained at 4.7° K, while spectra in dotted lines were obtained at 8.5° K. Other EPR operating conditions for all spectra were microwave frequency, 9.13 GHz with a 5 gauss modulation amplitude and 10 mW power, time constant, 0.3 sec; scanning rate, 200 gauss/min.

observed among these enzymes (Spectra A-C in the dotted line). At temperatures below 5° K, the EPR line shape of reconstitutively inactive enzymes (B-SDH and AA-SDH) becomes modified (Spectra B and C in the solid line). The central resonance undergoes a splitting of approximately 23 gauss, while both

side signals become broadened simultaneously. We should emphasize that this line shape change occurs even in the dehydrogenase (AA-SDH) which contains only two iron-sulfur clusters (S-1 and S-2) and flavin (Spectrum C). The strong temperature dependence of the line shape alterations indicates that this change may arise from the spin-spin interaction between Centers S-1 and Center S-2 which may be at most 10 Å apart, closer distances are possible by consideration of angular factors (10). This line shape modification is observed only in the reconstitutively inactive dehydrogenase preparations, not in the reconstitutively active dehydrogenase nor in the enzymatically active particulate preparations. These observations indicate that, with prior incubation with succinate or dithionite, the structural integrity of the enzyme molecule remains intact during the solubilization and isolation process, resulting in a reconstitutively active dehydrogenase. In contrast, a conformational change occurs during the extraction process, which gives rise to a reconstitutively

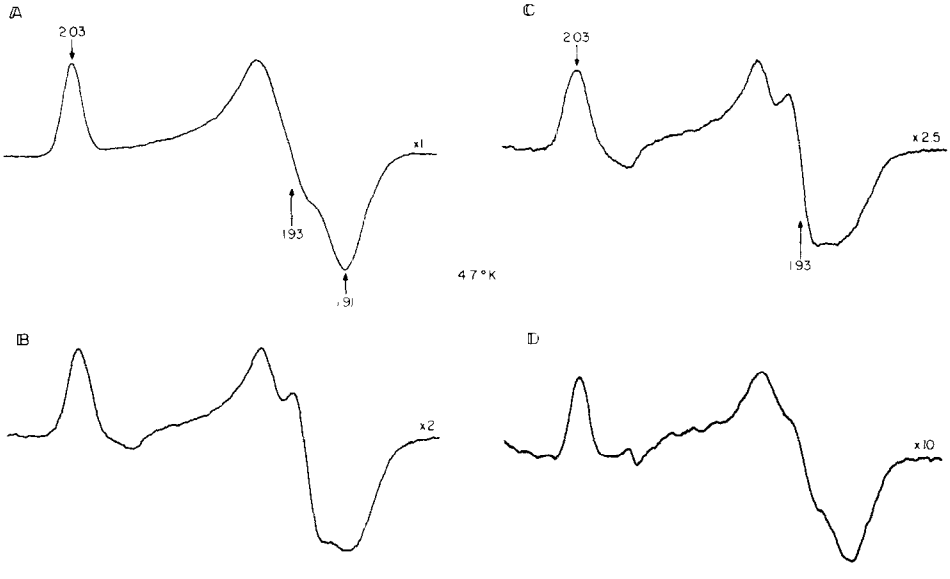

FIGURE 4. EPR spectra of Center S-2 in BS-SDH, BS-SDH-CN, CN-SDH, and CN-SDH-R. A. BS-SDH (20 mg protein/ml and 3 nmoles flavin/mg protein). B. BS-SDH-CN (21.9 mg protein/ml and 3.5 nmoles flavin/mg protein). C. CN-SDH (9.7 mg protein/ml and 5.3 nmoles flavin/mg protein). D. CN-SDH-R (2.7 mg protein/ml and 5.5 nmoles flavin/mg protein). Temperature of EPR samples was 4.7° K. All other experimental conditions are the same as in Figure 3 except that in spectrum D the time constant was 1 second and the scanning rate was 100 gauss/min.

inactive preparation in which the S-1 and S-2 clusters move closer to each other. Hence, spin-spin interaction is detected in these reconstitutively inactive preparations.

The observation that air- or cyanide-inactivated reconstitutively active dehydrogenase (BS-SDH-A; BS-SDH-CN, Figure 4, spectrum B) and the cyanide-dissociated dehydrogenase (CN-SDH, spectrum C) behave in the same manner as the reconstitutively inactive enzymes (B-SDH and AA-SDH) confirm the present conclusion. In addition, after the inactive enzyme preparations (B-SDH or CN-SDH, Figure 4, spectrum D) are chemically reactivated (9,11) by incubation with ferrous ammonium sulfate, sodium sulfide, and mercaptoethanol, the behavior of Center S-2 is largely reverted to that of a reconstitutively active preparation (Figure 4, spectrum A) and, concomitantly, the line shape modification virtually disappears. All these observations clearly indicate that a modification of the molecular structure occurs even during a brief exposure to air; the half time($t_{1/2}$) for inactivating the reconstitutive activity of succinate dehydrogenase is only about 45 min (Figure 2 in Ref. 2). The presence of succinate (or a suitable reducing agent) in the pre-incubation mixture before solubilization determines the structural conformation of the isolated dehydrogenase as discussed in detail previously (8). Similarly, chemical reactivation apparently restores or repairs the dislocated iron-sulfur clusters (cf. Ref. 9) in such a manner that their native configuration is regained.

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