Thermodynamic and EPR Characteristics of a HiPIP-type Iron-Sulfur Center in the Succinate Dehydrogenase of the Respiratory Chain*

(Received for publication, September 24, 1975)

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In addition to the two species of ferredoxin-type iron-sulfur centers (Centers S-1 and S-2), a third iron-sulfur center (Center S-3), which is paramagnetic in the oxidized state analogous to the bacterial high potential iron-sulfur protein, has been detected in the reconstitutively active soluble succinate dehydrogenase preparation. Midpoint potential (at pH 7.4) of Center S-3 determined in a particulate succinate-cytochrome c reductase is +60 ± 15 mV. In soluble form, Center S-3 becomes extremely labile towards oxygen or ferricyanide plus phenazine methosulfate similar to reconstitutive activity of the dehydrogenase. Thus, even freshly prepared reconstitutively active enzyme preparations show EPR spectra of Center S-3 which correspond approximately to 0.5 eq per flavin; in particulate preparations this component was found in a 1:1 ratio to flavin. All reconstitutively inactive dehydrogenase preparations so far examined give rise to no Center S-3 spectra or, if any, highly modified. These observations indicate that Center S-3 is an innate constituent of succinate dehydrogenase and plays an important role in mediating electrons from the flavoprotein subunit to most probably ubiquinone and then to the cytochrome chain.

The present paper describes EPR and thermodynamic characteristics of a HiPIP-type iron-sulfur center (designated as Center S-3) in various soluble succinate dehydrogenase preparations (cf. Table I in Ref. 1), which differ in their content of non-heme iron and acid-labile sulfide, and in their ability to reconstitute with purified cytochrome b5c1 complex or the alkali-treated submitochondrial particles (both of which are free from the dehydrogenase) for the reformation of antimycin A-sensitive succinate-cytochrome c reductase (6) and the succinate respiratory chain, respectively (cf. Ref. 1 and references cited therein).

Upon removal of the dehydrogenase from the mitochondrial membrane, Center S-3 becomes extremely labile toward oxygen or toward ferricyanide; rapid diminution of Center S-3 signals accompanies the parallel decline of the reconstitutive activity of the enzyme, but not of the artificial dye reductase activity as conventionally employed. These observations indicate that this center plays an important role in electron transfer in the following sequence: succinate → iron-flavoprotein subunit (Fp) → iron-sulfur protein subunit (Ip) (proposed to contain Center S-3) → most probably ubiquinone and eventually to the cytochrome system (the arrows symbolize the flow of electrons).
of electron or hydrogen). A preliminary report of this investigation has appeared (7).

**EXPERIMENTAL PROCEDURES**

All enzyme preparations and experimental methods used in this investigation were described in detail in the preceding paper (1).

**RESULTS**

Typical EPR spectra of the iron-sulfur center S-3 in particulate succinate-ubiquinone reductase (Complex II) and in a soluble reconstitutively active succinate dehydrogenase (BS-SDH) are presented in Fig. 1. As shown in a solid line, Center S-3 in the reductase preparation exhibits a spectrum which is centered around \( g = 2.01 \) with a peak to peak width of approximately 23 gauss, in agreement with previously reported characteristics of the HiPIP-type iron-sulfur centers detected in Complex II (2) and in Complex III (8).

The iron-sulfur center S-3 in the soluble BS-SDH preparation (broken line) reveals somewhat different line shape due to a contribution from the overlapping signals having peaks about 20 gauss away from those of Center S-3 detected in the particulate reductase. These additional signals appear to arise from slightly modified Center S-3, because less active BS-SDH preparations give rise to a more intensified peak at \( g = 2.03 \) and 2.00 relative to central \( g = 2.01 \) signal of the unmodified Center S-3 (7).

Center S-3 in Complex II or in succinate-cytochrome c reductase has a very short relaxation time, thus EPR signal is detectable only at temperatures below 15 K (at a microwave power setting of 1 milliwatt). The signal intensified as a linear function of 1/T as the temperature was lowered to 7.5 K. In a soluble reconstitutively active (BS-SDH) preparation, the central (unmodified) Center S-3 signal exhibits a similar temperature profile to that of Complex II. In contrast, EPR signals arising from modified Center S-3 are detectable at higher temperatures and saturate readily within this temperature range (at 1 milliwatt of power). It can be clearly seen in Fig. 2, where less active BS-SDH preparation was used, more modified spectra of Center S-3 were observed. Thus overlapped spectra can be resolved based on their different temperature and power profiles.

When a succinate dehydrogenase molecule is detached from the mitochondrial membrane, Center S-3 becomes extremely labile towards oxygen or other oxidizing agents, similar to the reconstitutive activity of the enzyme. As exemplified in Fig. 3, unmodified Center S-3 signal diminishes dramatically by increasing ferricyanide concentration to above 0.1 mM. The system the enzyme was kept reduced in the presence of added succinate throughout the isolation and purification; at least 50 mM succinate was required for oxidizing Center S-3. But significant S-3 signals.

In Fig. 4 the decay of reconstitutive activity of freshly prepared BS-SDH and concomitant loss of the Center S-3 EPR spectra is presented as a function of incubation time in contact partially modifying this center. Thus, experimentally, as a compromise 100 \( \mu \)M ferricyanide was added to assure that S-3 was completely oxidized (Fig. 1). Analogous to the S-3 signals, the initial (<45 s) enzymic activity of the reconstitutively active BS-SDH, but not the particulate reductase, has been found also very labile to both ferricyanide and phenazine methosulfate (9, 10).

In Fig. 4 the decay of reconstitutive activity of freshly prepared BS-SDH and concomitant loss of the Center S-3 EPR spectra is presented as a function of incubation time in contact with 100 \( \mu \)M ferricyanide and 10 \( \mu \)M phenazine methosulfate. Enzymes were rapidly frozen after 1-min incubation at room temperature. EPR operating conditions were: modulation frequency, 100 kHz; modulation amplitude, 5 gauss; microwave frequency, 9.12 GHz; time constant, 0.3 s; scanning rate, 200 gauss/min; microwave power, 0.5 milliwatt; sample temperature, 8.1 K. Solid line. Center S-3 in Complex II and dotted line, BS-SDH. Spectrum in dotted line was recorded at 4 times higher gain than that of solid line spectrum.

![Fig. 1. EPR spectra of Center S-3 in particulate succinate-ubiquinone reductase (Complex II) and in soluble reconstitutively active succinate dehydrogenase (BS-SDH). Concentrations of enzymes used were 19 mg of protein/ml of 3.7 nmol of flavin/mg of protein (Complex II) and 13.3 mg of protein/ml of 3 nmol of flavin/mg of protein (BS-SDH). Complex II was oxidized with 150 \( \mu \)M ferricyanide in the presence of 50 \( \mu \)M phenazine methosulfate, while BS-SDH was oxidized with 100 \( \mu \)M ferricyanide and 10 \( \mu \)M phenazine methosulfate. Enzymes were rapidly frozen after 1-min incubation at room temperature. EPR operating conditions were: modulation frequency, 100 kHz; modulation amplitude, 5 gauss; microwave frequency, 9.12 GHz; time constant, 0.3 s; scanning rate, 200 gauss/min; microwave power, 0.5 milliwatt; sample temperature, 8.1 K. Solid line, Center S-3 in Complex II and dotted line, BS-SDH. Spectrum in dotted line was recorded at 4 times higher gain than that of solid line spectrum.](http://www.jbc.org/)
absorbance of Center S-3. Spectrum B demonstrates that reconstitutively inactive dehydrogenases exhibit EPR toward the cytochrome b,c, complex (6). None of the inher-

preparations which differ in their content of non-heme iron and EPR operating conditions were the same as in Fig. 3. Were also plotted as a function of the aging time of the debydrogenase.

Peak heights at g = 2.02 from the base-line of the lower magnetic field ferricyanide and 10 PM phenazine methosulfate and rapidly frozen. Assay of the succinate oxidixed/min/mg of protein at about 22O. Samples for EPR measurement were simultaneously taken out and oxidized with 100 PM ferricyanide in the presence of 43 CAM phenazine methosul-

The enzyme was oxidized with the concentration of ferricyanide as indicated (Le. 0.1 mM, 0.2 mM, and 0.3 mM) in the presence of 104 Mm phenazine methosulfate. EPR operating conditions were: microwave frequency, 9.14 GHz; microwave power, 1 milliwatt; scanning rate, 250 gauss/min; sample temperature, 8.4 K. Other conditions were the same as in Fig. 1.

with air at 0°. The peak height at g = 2.02 and reconstitutive activity decayed in parallel, with half-time of approximately 35 min in either case. On the other hand, more than about 70% of artificial dye reductase activity (measured as electron transfer from succinate to 2,6-dichlorophenolindophenol mediated by phenazine methosulfate) remained even after both Center S-3 signals (7, 11) and reconstitutive activity (see p. 182, Ref. 12) were practically lost. EPR-detectable Centers S-1 and S-2 showed less than 30% decrease in the time period of the exper-

iment (125 min) for the measurement of decay of Center S-3 signals.

Fig. 5 compares EPR spectra of succinate dehydrogenase preparations which differ in their content of non-heme iron and acid-labile sulfide, as well as in their reconstitutive activity toward the cytochrome b-c, complex (6). None of the inherently reconstitutively inactive dehydrogenases exhibit EPR absorbance of Center S-3. Spectrum B demonstrates that B-SDH, which was prepared by the same procedure as BS-SDH except without succinate in the preincubation mixture, exhibits no resonance absorbance of Center S-3. This preparation, however, contains 8 atoms of non-heme iron and 8 mol of acid-labile sulfide per flavin, the same as the reconstitutively active BS-SDH. These observations further indicate that even a subtle change of the molecular configuration around Center S-3 results in a loss of the reconstitutive activity simultaneously with the EPR spectrum of this HiPIP-type iron-sulfur center. Center S-3 is stable in the particulate reductase but very labile in the soluble enzyme. This characteristic resembles the reconstitutive property of succinate dehydrogenase, which is very stable in particulate preparations but extremely labile once solubilized (12, 13 and references cited therein). Double integration of Center S-3 spectra obtained with particulate preparations (such as succinate-cytochrome c reductase and Complex II) shows that this center is present in a 1:1 ratio to the flavin, in agreement with previously reported results (2). In the soluble enzyme (BS-SDH) about one-half of the Center S-3 remains EPR-active (Table I). This is evidently due to an inactivation of this center during the purification and subsequent manipulation, since Center S-3 is extremely labile, as is the reconstitutive activity of the dehydrogenase (cf. Figs. 3 and 4).

Fig. 6 represents a potentiometric oxidation-reduction titra-
tion of Center S-3 conducted with particulate antimycin A-sensitive succinate-cytochrome c reductase (6). Midpoint potential measured at pH 7.4 was obtained as +60 ± 15 mV. The midpoint potential of Center S-3 has been reported to be +120 mV in mitochondria (14). This difference in values serves to emphasize again the sensitivity of Center S-3 to its environment. Center S-3 is classified as a HiPIP-type iron-sul-
fur center (15), because this center is found to be paramagnetic in the oxidized state, similar to bacterial high potential iron-sulfur protein (4) in contrast with ferredoxin-type iron-sulfur centers which are paramagnetic in the reduced state. Thus, this nomenclature does not refer to the oxidation-reduction
**A HiPIP-type Iron-Sulfur Center in Succinate Dehydrogenase**

**Table 1**
Relative spin concentration of Center S-3 in particulate and soluble preparations

| Preparation                          | Spin concentration per flavin |
|--------------------------------------|------------------------------|
| Succinate-cytochrome c reductase     | 0.9–1.0                      |
| Succinate-ubiquinone reductase       | 0.9–1.0                      |
| Reconstitutively active succinate dehydrogenase | 0.25–0.50*               |
| BS-SDH                               |                              |
| Reconstitutively inactive succinate dehydrogenase | 0                       |
| B-SDH                                |                              |
| Reconstitutively inactive succinate dehydrogenase | 0                       |
| AA-SDH                               |                              |

*See Table 1 of Ref. 1.

*Dependent mostly on the batches of BS-SDH. These EPR samples were mostly made in Albany, New York and then shipped to Philadelphia in liquid nitrogen for the EPR experiment.

**Fig. 6. Oxidation-reduction titration of Center S-3 in succinate-cytochrome c reductase.** Concentration of antimycin A-sensitive succinate cytochrome c reductase was 13.8 mg of protein/ml of approximately 1 nmol of flavin/mg of protein. Potentiometric titration was performed anaerobically in the presence of the following oxidation-reduction dyes: 50 µM diaminodurene, 50 µM phenazine methosulfate, 58 µM phenazine ethosulfate, 17 µM duroquinone, 17 µM pycocyanine, 7 µM resorufin, 33 µM 2-hydroxynaphthoquinone, and 67 µM phenosafranine. Signal heights (at E<sub>m</sub>) measured from the base-line of low magnetic field was plotted as a function of oxidation-reduction potential relative to the standard hydrogen electrode. EPR operating conditions were: microwave frequency, 9.14 GHz; modulation amplitude, 10 gauss; microwave power, 10 milliwatts; time constant, 0.3 s; scanning rate, 000 gauss/min; sample temperature, 15 K.

**DISCUSSION**

The present investigation has demonstrated that Center S-3 is the most labile component of the three distinct iron-sulfur centers in the soluble reconstitutively active succinate dehydrogenase. Center S-3 can thus be considered to be originally present in the dehydrogenase molecule in a 1:1 ratio to the flavin, in spite of the observed apparently lower ratio in the isolated system as described.

Rapid decay of the Center S-3 EPR signal parallels fast decline of the reconstitutive activity of the dehydrogenase. It must be emphasized that unmodified resonance absorbance of Center S-3 was never detected in all inherently reconstitutively inactive enzyme preparations, even in BS-SDH, which was prepared exactly the same way as BS-SDH except with no succinate preincubation and contains 8 non-heme iron atoms and 8 mol of acid-labile sulfide per flavin, similarly to the reconstitutively active BS-SDH. These results indicate that even a subtle conformational change around the active center of this HiPIP-type component renders the enzyme reconstitutively inactive. It explains conclusively why some investigators (2) observed that Center S-3 was absent in soluble succinate dehydrogenase preparations containing 4 atoms of iron and 4 mol of sulfide or 8 atoms of iron and 8 mol of sulfide per flavin. These investigators used 8-iron, 8-sulfide enzymes which were extracted by perchlorate (16) but without succinate preincubation, thus obtaining a reconstitutively inactive enzyme (type 3, according to their nomenclature) as in the case of B-SDH.

Recently Vinogradov et al. (10) reported the existence of two ferricyanide-reactive sites in BS-SDH which exhibit different K<sub>m</sub> values for K<sub>f</sub>F<sub>e</sub>(CN)<sub>4</sub>, namely 3 mM and 250 mM, respectively. The "low K<sub>m</sub> ferricyanide site" simulates the lability toward oxygen and ferricyanide as the HiPIP-type signal of the dehydrogenase presented in this paper. Vinogradov et al. (16) designated this labile K<sub>f</sub>F<sub>e</sub>(CN)<sub>4</sub> reacting site as "site 2," which may correspond to Center S-3 according to our nomenclature of iron-sulfur centers in the succinate dehydrogenase molecule (8, 17). In their system, succinate was used as a reductant, and Center S-3 remained oxidized even before the K<sub>f</sub>F<sub>e</sub>(CN)<sub>4</sub> reaction. However strong the circumstantial evidence presented by Vinogradov et al. (10), the weight of the final conclusion seems to lie more on the results summarized in Fig. 4 than anything else.

From the work of Davis and Hatefi (16) and Hanstein et al. (18) it is known that succinate dehydrogenase is composed of two subunits of molecular weights of about 70,000 and 27,000. The large subunit, designated F<sub>µ</sub>, contains covalently bound (acid-nonextractable) FAD, and 4 non-heme iron atoms and 4 mol of acid-labile sulfide. From the oxalacetate binding studies (19, 20) it has been demonstrated that this inhibitor exerts its effect by binding with the active site sulfhydryl groups in the large subunit F<sub>µ</sub>. Hence, all machinery necessary for the primary oxidation of succinate is located in this subunit. Furthermore, the enzyme extracted from the acetone powder of mitochondria or submitochondrial particles by the procedure of Singer and his co-workers (21) (AA-SDH) contains only 4 atoms of iron and 4 mol of sulfide per flavin and is also active toward artificial electron acceptors. As reported in the preceding paper (1) both ferredoxin-type iron-sulfur centers S-1 and S-2 are detectable in AA-SDH preparations while no Center S-3 signal whatsoever was detected in this enzyme. Thus both Centers S-1 and S-2 seem to have a 2-iron, 2-sulfide structure similar to adrenodoxin or spinach ferredoxin (22), and are located in the F<sub>µ</sub> subunit.

The other subunit (designated as I<sub>µ</sub>, (7, 18), i.e., the remaining 4 iron atoms and 4 mol of sulfide, is necessary for transfer of electrons from F<sub>µ</sub> to ubiquinone and the cytochrome system. Major evidence for this arises from the fact that only...
the enzymes which contain 8 non-heme iron atoms and 8 mol of acid-labile sulfide per flavin are capable of reconstitution. No investigators have succeeded so far in separating and then reconstituting these individual subunits to directly demonstrate their functional role. Only the most drastic procedures (16, 18) cleave the two subunits, and the resulting components are denatured and inactive even toward artificial acceptors; hence, the definitive experiments of recombination of native Fp and Ip subunits have yet to be performed. However, a close parallelism between the reconstitutive activity of BS-SDH and a decrease of the center S-3 signal (Fig. 4), as well as a disappearance of the second or the so-called low Kf ferricyanide reacting site (10), strongly indicates that S-3 resides in the smaller subunit Ip and plays a role in transferring electrons from Fp to ubiquinone.

Here a dilemma exists superficially, since soluble succinate dehydrogenase (BS SDH) does not directly react with ubiquinone. However, the recent proposal by Mitchell (23), for example, can resolve the difficulty, as the active species accepting the electron (hydrogen) from the dehydrogenase actually may be a semiquinone. Support for the involvement of the semiquinone species of ubiquinone in the respiratory chain comes from some recent work performed by Ingledew et al. (14, 24) and Ruzicka et al. (25).

Other experimental evidence substantiating the notion that Center S-3 is located in the smaller subunit Ip and transfers electrons from Fp to, most probably, ubiquinone is the effect of cyanide reaction on iron-sulfur centers of succinate dehydrogenase (BS-SDH) and on the electron-transferring activity to the artificial electron acceptors and to the respiratory chain. The reaction of cyanide with succinate dehydrogenase appears to occur via several intermediate steps; the initial iron-sulfur complex is located in the smaller subunit Ip, and plays a role in transferring electrons from Fp to ubiquinone.

Note Added in Proof—A HiPIP-type iron-sulfur center with a midpoint reduction potential of -420 mV was recently found in Azotobacter vinelandii (27).

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J. Biol. Chem. 1976, 251:2105-2109.

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