EPR STUDIES ON A HIPIP TYPE IRON-SULFUR CENTER IN THE SUCCINATE DEHYDROGENASE SEGMENT OF THE RESPIRATORY CHAIN

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SUMMARY -- In addition to the two species of ferredoxin-type iron-sulfur centers (Centers S-1 and S-2), a Hipip-type iron-sulfur center (Center S-3) has been detected in the reconstitutively active soluble succinate dehydrogenases. $E_{m7.4}$ determined in a particulate, antimycin A sensitive succinate-cytochrome c reductase is +60 ± 15 mV. This center is extremely labile towards oxygen in a manner similar to the reconstitutive activity of the dehydrogenase. Even freshly prepared reconstitutively active enzyme shows a considerably diminished content of Center S-3 relative to flavin and displays a partly modified spectra. All reconstitutively inactive dehydrogenases give rise to a highly modified or no Center S-3 spectra at all. These observations indicate that Center S-3 is a constituent of succinate dehydrogenase and plays a role in the physiological function of the enzyme, i.e. transferring electrons most probably to ubiquinone.

In our previous communication (1), the presence of a distinct iron-sulfur center (designated as Center S-2) in addition to the classical "g = 1.94" species (2) (designated as Center S-1) in the succinate-ubiquinone (UQ) reductase segment of the respiratory chain was reported. Recently Beinert and his coworkers (3) demonstrated the presence of a third iron-sulfur center of the so-called Hipip type (high potential iron-sulfur protein) in a particulate succinate-UQ reductase preparation ("Complex II" ref. 4). However, the location of this Hipip-type center in the respiratory chain remained

uncertain because of the following two observations: (i) EPR signals arising from this iron-sulfur center were undetectable in the soluble succinate dehydrogenase preparations which contain either 8 or 4 non-heme iron atoms per flavin (3), and (ii) a Hipip-type iron-sulfur protein isolated by Ruzicka and Beinert (5) possesses a molecular weight of about 100,000 daltons which is even slightly larger than that of the whole succinate dehydrogenase molecule.

The present communication reports a Hipip-type iron-sulfur center [designated here as Center S-3 or $(Fe\text{-}S)_{S\text{-}3}$] in various soluble succinate dehydrogenase preparations (6, 7) which differ in their content of non-heme iron and acid labile sulfide, and in their ability to reconstitute succinate-cytochrome c reductase with the purified cytochrome $b\text{-}c_1$ complex (8). This iron-sulfur Center S-3 displays resonance absorbance only in the reconstitutively active succinate dehydrogenase preparations but not in the reconstitutively inactive enzymes. This finding underscores an important role for the center in the physiological function of succinate dehydrogenase, namely transfer of electrons from succinate to the cytochrome system. The accompanying paper characterizes iron-sulfur Centers S-1 and S-2 in various succinate dehydrogenases.

Materials and Methods

Nine different preparations of succinate dehydrogenase, as shown in Table I, were used in the studies reported in this and the accompanying paper. Except for AS-SDH which was prepared from succinate-UQ reductase ("Complex II" (4)), all others were prepared from the Keilin-Hartree preparation of bovine heart as described previously (6, 7). AA-SDH was made according to Bernath and Singer (8) from acetone powder of the heart muscle preparation instead of mitochondria. The flavin:Fe:S ratios and other properties of these preparations are summarized in Table I. The particulate, antimycin A sensitive succinate-cytochrome c reductase was prepared as before (9). The methods of the assays were the same as previously reported (6, 7). Potentiometric

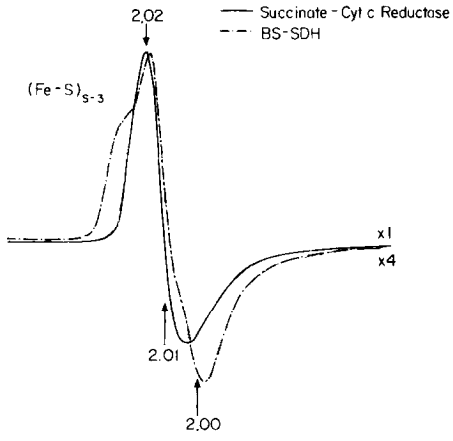

Fig. 1. Comparison of EPR spectra of Center S-3 from the particulate, antimycin A sensitive succinate-cytochrome c reductase and reconstitutively active soluble succinate dehydrogenase. Concentrations of enzymes used were 20 mg protein per ml of 1 nmole flavin/mg protein for the reductase and 20 mg protein per ml of 3 nmoles flavin/mg protein for BS-SDH. Both systems were oxidized with 100 μM $K_3Fe(CN)_6$ in the presence of 43 μM phenazine methosulfate. EPR operating conditions were: modulation amplitude, 5 gauss; microwave frequency, 9.14 GHz; time constant, 0.3 sec; scanning rate 200 gauss/min; temperature, 9.2° K; microwave power, 5 mw.

titrations were conducted according to Dutton (10) and Wilson et al. (11). EPR measurements were performed with a Varian E4 spectrometer and the temperature of the samples was controlled by means of a variable temperature cryostat (Air Products Model LTD-3-110) and monitored by a carbon register installed below the EPR sample tube. Double integration of EPR spectra was performed on a Nicolet Signal Averager (NIC-1074) using an EDTA-Cu(II) complex as a standard.

Results and Discussion

Figure 1 depicts typical EPR spectra of the iron-sulfur Center S-3 in the particulate, antimycin A sensitive succinate-cytochrome c reductase and in a soluble succinate dehydrogenase preparation (BS-SDH). As shown with solid line, Center S-3 in the particulate preparation exhibits a spectrum which is centered at g = 2.01 with a peak to peak width of about 25 gauss, in agreement with previously reported characteristics of the Hipip-type

TABLE I

A SUMMARY OF SUCCINATE DEHYDROGENASE PREPARATIONS*

Preparations	Solubilizing method and treatment	Prior succinate incubation	Reconstitutive activity	Flavin:Fe:S
1 BS-SDH	Butanol extraction at pH 9.1	+	+	1:8:8
2 AS-SDH	Alkali extraction (pH 10.6) of succinate-UQ reductase	+	+	1:8:8
3 B-SDH	Butanol extraction at pH 9.1	−	−	1:8:8
4 CN-SDH	Cyanide extraction	−	−	1:6:4
5 AA-SDH	Alkali (pH 10) extraction of acetone powder	−	−	1:4:4
6 BS-SDH-A	Air-inactivated BS-SDH	+	−	1:8:8
7 BS-SDH-CN	Cyanide-treated BS-SDH	+	−	∿ 1:6:4
8 B-SDH-R	Chemically reactivated B-SDH	−**	+	∿ 1:8:8***
9 CN-SDH-R	Chemically reactivated CN-SDH	−**	+	∿ 1:8:8***

* All preparations are active for artificial electron acceptors, such as phenazine methosulfate.

** Although no succinate is present during the solublization of the enzyme, it is necessary to have it in the reactivation mixture (7).

*** Upon isolation of the chemically reactivated enzyme it contains an excess of iron and sulfide, however it was shown (7) that only 8 Fe and 8 S are necessary for its reconstitutive activity.

iron-sulfur center detected in "Complex II" (3) and in "Complex III" (12). In contrast, the iron-sulfur Center S-3 in the reconstitutively active succinate dehydrogenase (broken line) reveals more complex line shape, due to the overlapping signals having peaks about 10 gauss away from those of Center S-3 in the particulate reductase. These additional signals appear to arise from a somewhat modified Center S-3, because less active BS-SDH preparations give a diminished g = 2.02 peak and intensified peaks both at g = 2.03 and 1.998. These two different species of EPR signals arising from unmodified and modified Center S-3 exhibit different temperature profiles, the latter being detectable at higher temperatures than the former. Double integration of the Center S-3 spectra from succinate-cytochrome c reductase

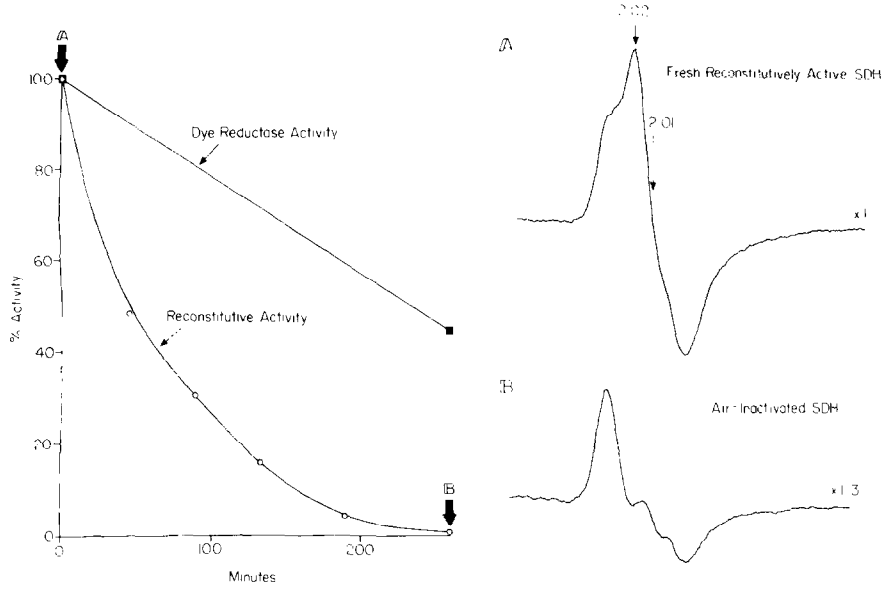

Fig. 2. The decay of BS-SDH with respect ot its reconstitutive and artificial (phenazine methosulfate) reductase activities in air at 0° C (the left side) and concomitant loss of the Center S-3 EPR spectra (the right side). Freshly isolated BS-SDH (18.5 mg protein/ml and 3.4 nmoles flavin/mg protein) in 50 mM K-phosphate buffer, pH 7.4 was aged at 0° C in air. The dehydrogenase solution was stirred, and every 15 min aliquots were withdrawn as illustrated. Reconstitutive and artificial activity assays were performed immediately. Initial activities measured at 23° C were 5.4 and 11.7 µmoles succinate oxidized per min per mg protein, respectively. The Center S-3 spectra of oxidized samples shown with arrows A and B were examined at the times shown on the left side. EPR operating conditions were the same as those described in Fig. 1.

shows that this center is present in a one to one ratio to the flavin, while in the isolated enzyme only about half of the Center S-3 remains EPR active. This is evidently due to a loss or inactivation of this center during the purification and subsequent manipulation, since Center S-3 has been found to be extremely labile as is the reconstitutive activity of the dehydrogenase. The initial (< 45 sec) enzymic activity of the reconstitutively active soluble succinate dehydrogenase (BS-SDH) but not particulate preparations has been found also to be very labile to both ferricyanide and phenazine methosulfate (J. Ketterman, Jr. and T.E. King, unpublished observation). As illustrated in Fig. 2, reconstitutive activity of BS-SDH in contact with air rapidly decreases even at 0° with a half-time of about 45 minutes. Concomitant with this loss of reconstitutive activity, the intact structure of Center S-3 is almost completely lost at time B of the figure. Air-inactivated enzymes exhibited only modified signals of Center S-3 (Fig. 2, Spectrum B).

Figure 3 compares EPR spectra of other succinate dehydrogenase preparations which differ in their content of non-heme iron and acid labile sulfide, as well as the reconstitutive activity with the cytochrome $\underline{b}$-$\underline{c}_1$ complex (9). None of the inherently reconstitutively inactive dehydrogenases exhibited 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 preincubation, exhibits no resonance absorbance of Center S-3. This preparation, however, contains 8 atoms of non-heme iron and 8 moles of acid-labile sulfide per flavin, the same as the reconstitutively active BS-SDH. These observations thus indicate that even a subtle change of the molecular configuration around Center S-3 results in a loss of the reconstitutive activity as well as the EPR spectrum of this particular Fe-S center. Center S-3 is stable in the particulate reductase but very labile in the soluble enzyme. This characteristic is identical to the reconstitution property of succinate dehydrogenase which is very stable in particulate preparations but extremely labile once solubilized

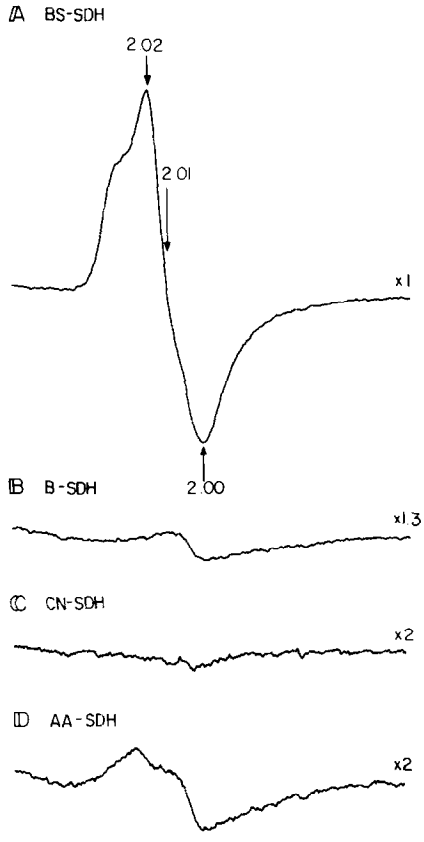

Fig. 3. EPR spectra of the iron-sulfur Center 3 of various succinate dehydrogenase preparations. Enzyme concentrations used were:
(A) BS-SDH -- 20 mg protein/ml of 3 nmoles flavin/mg protein,
(B) B-SDH -- 16.5 mg protein/ml of 1.8 nmoles flavin/mg protein,
(C) CN-SDH -- 9.7 mg protein/ml of 5.3 nmoles flavin/mg protein, and
(D) AA-SDH -- 30.2 mg protein/ml of 1.9 nmoles flavin/mg protein.
Other experimental conditions were the same as in Fig. 1.

(cf. 6 and references cited therein). Present findings clarify why Beinert et al. (3) could not detect any qualitative difference between the behaviors of the soluble succinate dehydrogenase preparations containing 4 iron or 8 iron per flavin. These investigators have used 8 iron-8 sulfide succinate dehydrogenase which is extracted by perchlorate (13) but without succinate preincubation, thus obtaining a reconstitutively inactive enzyme as in the case of B-SDH (Fig. 3, Spectrum B).

The midpoint potential of Center S-3 has been found to be +60 ± 15 mV with n = 1 determined potentiometrically in the particulate, antimycin A

sensitive succinate-cytochrome c reductase preparation at pH 7.4. This value is reasonable for an electron carrier functioning in the succinate dehydrogenase region of the respiratory chain. Center S-3 is the first example of a Hipip-type iron-sulfur center having a relatively low midpoint potential. Possibility also exists that another Hipip type iron-sulfur center occurs in mitochondria, since Hipip type center with a high midpoint potential (> +150 mV) was previously observed in the pigeon heart mitochondria (14). It may explain the observation of a Hipip signal in Complex III other than the structural contamination of the Complex as previously discussed (9, 12) as well as the isolation of the Hipip protein with a molecular weight of 100,000 daltons (5). As will be discussed in the accompanying paper (15), both Centers S-1 and S-2 are probably of the 2-iron-2-sulfide type as in the case of spinach ferredoxin or adrenodoxin (16). All succinate dehydrogenase preparations examined contain both Centers S-1 and S-2 in approximately equivalent concentration to that of the bound flavin (cf. Ref. 3). No active iron-sulfide centers other than Centers S-1, S-2 and S-3 have been detected so far in the dehydrogenase molecule which contains 8 Fe and 8 S per flavin. It should be emphasized that Center S-3 exhibits EPR absorbance in the oxidized form similar to bacterial Hipip-type iron-sulfur proteins which contain 4 Fe and 4 S per active center (17). Furthermore, Sweeny et al. (18) have recently demonstrated that "superoxidation" of 4 Fe-4 S ferredoxins give rise to EPR spectra centered at g = 2.01, similar to that of Center S-3. To our knowledge, this EPR absorbance has not been observed in any well characterized iron-sulfur proteins containing less than 4 iron and 4 sulfide. All these observations would suggest that Center S-3 possesses a 4 Fe-4 S structure in contrast to the 2 Fe-2 S for Centers 1 and 2. At any rate, it is reasonable to conclude that Center S-3 is an innate constituent of succinate dehydrogenase, and at least as a working hypothesis we are inclined to believe that Center S-3 plays a role as an electron carrier, most probably between iron-flavoprotein (Fp) and ubiquinone.

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