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Stabilized Ubisemiquinone in Reconstituted Succinate Ubiquinone Reductase

Xu, Y.[1], Salerno, J. C.[2], Wei, Y. H.[1,*], and King, Tsoo E.[1]

[1]Department of Chemistry and Laboratory of Bioenergetics,
State University of New York at Albany, Albany, N. Y. 12222
[2] Biology Department and Biophysics Group,
Rensselaer Polytechnic Institute, Troy, N. Y. 12180

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QP-S, a ubiquinone (Q) protein, accepts electrons from succinate through succinate dehydrogenase (SDH). A new method has produced a preparation of QP-S which has a different amino acid composition and SDS gel electrophoretic pattern from that of the old preparation (Biochemistry 19, 3579-3585 (1980)). The new preparation contains less than 1 nmol heme/mg protein; the activity of the preparation was not proportional to its heme content. A thenoyltrifluoroacetone sensitive free radical signal was detected by EPR spectroscopy in succinate-Q reductase reconstituted from this QP-S and SDH; the characteristics of this species identify it as ubisemiquinone. At pH 7.4, the Em of the two electron step was about 70 mV with E_1 = 5 mV and E_2 = 125 mV. The properties of the radical differed slightly from those of "Qs" radical in more intact preparations (e.g. submitochondrial particles). The present is the simplest system in which such a succinate reducible ubisemiquinone free radical has been demonstrated.

While succinate dehydrogenase (SDH)* does not react directly with ubiquinone (Q), succinate → Q electron transfer can be catalyzed by the combination of SDH and another component, QP-S (1, 2). Improvement of the original procedure for solubilization and purification of QP-S has been reported (3, 4). Of the two preparations recently described (4), one has been claimed to possess the highest purity (4). However, we have found that by

*Present address: Biochemistry Department, National Yang Ming Medical College, Shih-Pai, Taipei, Taiwan

Abbreviations: DCIP, 2,4 dichlorophenolindophenol; DMSO, dimethylsulfoxide; ETP, electron transfer particles; PMS, phenazine methosulfate; Q, ubiquinone; QCR, ubiquinone-cytochrome c reductase; QH_2, ubiquinol; QP-C, a Q binding protein in the cytochrome b-c_1 complex; QP-S, a Q binding protein accepting electrons directly from SDH; SCR, succinate-cytochrome c reductase; SDH, succinate dehydrogenase; SQR, succinate-quinone reductase; SDS, sodium dodecylsulfate; TTFA, thenoyltrifluoroacetone.

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amino acid sequencing, this preparation (4) is a mixture containing QP-C (results to be published).

Several other laboratories have reported functionally similar preparations (5-7). All QP-S preparations have two SDS-PAGE bands with molecular weights of about 15 kD and contain variable amounts of b cytochrome, typically 8-16 nmol/mg. An essential role for this cytochrome b_{560} in SDH-Q electron transfer has thus been proposed (7).

Stabilized ubisemiquinone radicals have been observed in whole tissue, mitochondria, ETP, SCR and "Complex II." The EPR signals take two forms; a split signal arising from dipolar interactions between two bound ubisemiquinones (8-10) and a rapidly relaxing free radical (11-13). These two signals are both thenoyltrifluoroacetone (TTFA) sensitive, both have stability constants near unity between pH 7 and 8, and are of maximum intensity near 100 mV at pH 7.

Ubisemiquinone radical has not been previously demonstrated in a system consisting only of SDH and QP-S. We have recently succeeded in demonstrating the formation of the radical in such a system using a new preparation of reconstitutively active QP-S.

Materials and Methods--QP-S was prepared from SQR, which was prepared from SCR (14, 15) as previously described with modifications. Triton X-100 at a final concentration of 1% was used to cleave SCR into SQR and QCR. SCR was made from the Keilin-Hartree heart muscle preparation (HMP). The SQR was separated from SCR using hydroxyapatite chromatography in the presence of 0.2% Emasol.

After dialysis, the SQR was treated with 1% Tween 20 and 2M urea, frozen and thawed. The QP-S was separated using hydroxyapatite chromatography. The crude QP-S was precipitated with ammonium sulfate at 35% saturation; the floating precipitate was collected and dispersed in 50 mM Tris-acetate buffer, pH 7.8, 0.5 M sucrose and dialyzed against the same buffer overnight. QP-S was stored at -40°C or used immediately.

EPR experiments were carried out using an IBM Bruker ER-200 spectrometer with an Air Products liquid helium cryostat. Samples were quickly frozen in a 1:5 cyclohexane/isopentane precooled with liquid nitrogen. Relative spin concentrations were estimated by simulation using a computer program with appropriate parameters (12).

Potentiometric titrations were performed as described by Dutton (16, 17) using a mediator system selected to avoid free radicals derived from mediators. The mediators were indigocarmine disulphonate, duroquinone, 1,4 napthoquinone, 1,2 napthoquinone and 2-hydroxy-1,4 napthoquinone. TTFA was obtained from Sigma; mediators were obtained from Sigma, Eastman and Aldrich. All other chemicals were the purest commercially available.

Results and Discussion--The QP-S prepared by the new method was rather stable and could be stored for 24 hrs at 0-4°C or for at least 14 days at -40°C

without appreciable change in reconstitutive activity or physical properties. It showed two bands on SDS gel according to the method of Laemmli (18), with apparent molecular weights of approximately 13 and 15 kD. These properties were different from previously reported preparations (4). The preparation contained less than 1 nmol heme per mg protein (apparently as a b cytochrome). Reconstitutive activity in the previously reported assay system (4, 7) was 16 μmol succinate oxidized per min per mg total protein present at room temperature, which is comparable to very active SQR preparations. The amino acid composition using the method of Houston (19), was clearly different from either QP-C or the previously reported QP-S preparation (20, 4).

In order to observe the EPR signal of the ubisemiquinone radical, several methods were used to obtain the desired redox state of the complex. When succinate and fumarate in a 1/100 ratio were added to a mixture of reconstitutively active SDH and QP-S, a radical was generated which could be observed at 100 K. The signal had a g value of about 2.003 and a linewidth of 10-10.5 Gauss, and could be almost completely abolished by the addition of 3 mM TTFA.

The TTFA sensitive signal could also be observed during potentiometric titration of the reconstituted system as shown in Fig. 1. Figure 2A shows the results of such a titration in the presence of added Q_2 (210 μM) in the absence of TTFA. The plot of radical signal vs. Eh does not have a symmetrical

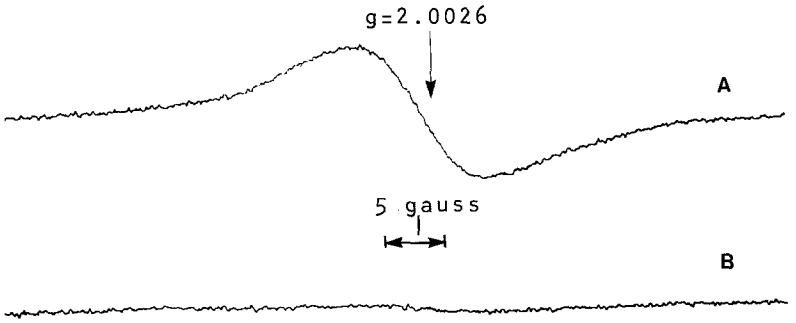

Fig. 1 (A). EPR signal of QP-S in reconstituted SQR. The sample was prepared by potentiometrically poising QP-S at +85 mV, anaerobically transferring 0.3 ml of sample to an EPR tube and freezing immediately. The EPR conditions were: microwave frequency 9.47 GHz; modulation frequency, 100 KHz; modulation amplitude, 6.3 Gauss; microwave power 8.2 mW; time constant, 0.2 s; scan rate 30 Gauss/min; sample temperature 100 K; gain 1.25×10^3. (B) The sample was made the same as that described in (A) but the system also contained 0.3 mM TTFA in (DMSO). The final concentration of DMSO is 0.3% (vol.) and the potential was poised at +88 mV.

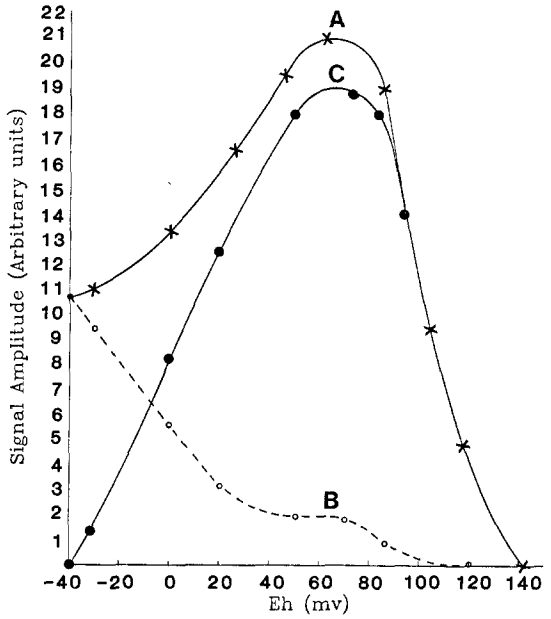

Fig. 2. Potentiometric titration of EPR signal from reconstituted SQR. The sample was made as described in context. The EPR conditions were microwave frequence, 9.47 GHz; modulation frequency, 100 KHz; modulation amplitude, 6.3 Gauss; microwave power, 0.82 mW; time constant, 0.2 s; scan rate 30 Gauss/min; sample temperature 100 K; gain 2 x 10^5. (A) titration as it is. (B) Addition of 3 mM TTFA, (C) (A) minus (B).

bell shape. The maximum spin concentration was about 5% that of center S-1 of SDH by simulation. The results of a similar titration in the presence of TTFA are shown in Fig. 2B. The difference between Fig. 2A and 2B clearly represents the titration of TTFA sensitive radical. The bell shaped curve is reasonably close to the theoretical line for the $E_1(Q/Q^{\cdot})$ = 5mV and $E_2(Q^{\cdot}/QH_2)$ = 125mV.

The power saturation of the TTFA sensitive species is shown in Fig. 3. The saturation of the broader TTFA insensitive radical is similar to that of the sensitive one. The $P_{1/2}$ value of 7.26 mW is lower than that of QP-S but considerably higher than that of the bound semiquinones of the cytochrome $\underline{b}$-$\underline{c}_1$ (12, 13, 21). The TTFA insensitive species may be flavin; its linewidth is 12-12.5 Gauss and the g value about 2.006, consistent with such an assignment (22-25).

Ubisemiquinone radical signals have been previously observed in a number of more complex systems containing SQR. No such radical has been reported in

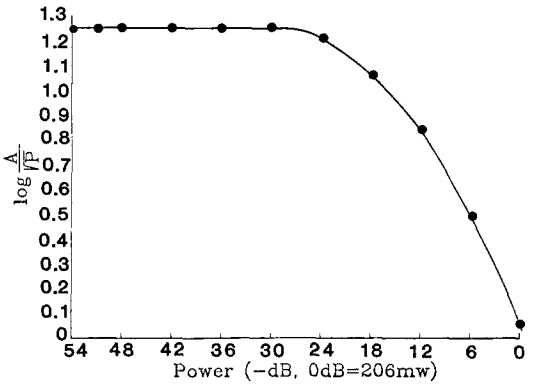

Fig. 3. Power saturation behavior of QP-S. EPR spectra of the sample used in Fig. 2A (without TTFA treatment) were measured at different microwave powers. Other EPR conditions were as in Fig. 2. A signifies signal amplitude; p, microwave power in milliwatts, P 1/2 is the power ratio level at which A/ p is half maximal.

either purified SDH or purified QP-S, and we have been unable to observe any such signals from either kind of preparation after addition of Q and QH_2 in various ratios which would have produced radical in SCR or SQR.

HMP prepared as previously described exhibits both the split signal and the rapidly relaxing QP-S radical signal (12), although the intensity of the split signal appears somewhat smaller than in some other preparations. SQR prepared from SCR and poised potentiometrically in the neighborhood of 90 mV did not exhibit the split signal, but at higher temperatures (50-100 K) a TTFA sensitive radical could be observed with a linewidth of about 15.6 Gauss (not shown). This is considerably broader than even the signal of the covalently bound flavin radical of SDH. The power saturation of this species indicated that its relaxation was slower than QP-S in more intact systems, but much more rapid than that of the bound ubisemiquinones in the cytochrome $\underline{b}$-$\underline{c}_1$ region (12, 21). No split signal was observed in reconstituted SQR.

Several lines of evidence demonstrate that the radical observed by EPR spectroscopy in reconstituted SQR is ubisemiquinone. It has a linewidth and g value consistent with ubisemiquinone; the line is narrower than flavin radical. The midpoint potential is close to that of ubiquinone, and is 150-200 mV more positive than the flavin of SDH. The radical is sensitive to TTFA, which acts at QP-S. Finally, it may be emphasized that the heme group in the preparation

was not reducible by succinate and was present in a molar ratio of less than 1:60. The heme content varied from 0.4 to 0.9 nmol/mg protein, and did not correlate with the reconstitutive activity of the preparation. It seems unlikely that the heme functions as an electron transfer intermediate from SDH to Q.

Some interesting differences remain between this radical and corresponding radicals previously reported in more intact systems (12, 13). The slower relaxation and loss of the split signal both suggest "modifications" of the quinone binding site, but this does not appear to be enough to significantly affect the activity. The unusually broad linewidth of the semiquinone observed in SQR may represent remnants of the interaction which produces the split signal in "more intact" preparations. Reduction of the observed linewidth to 10 Gauss in the reconstituted system suggests that only relatively isolated single quinone radicals are formed there. In both these systems, relaxation is slower than in more intact preparations with a higher level of complexity.

We have recently commented on the b cytochrome content of SCR (26, 27); in some active preparations cytochrome b_{560} must be greatly substoichiometric with cytochrome b-c_1 and SDH components. The similarity of Hatefi's cytochrome b_{560} to QP-S argues against contamination of Complex II with modified cytochrome b-c_1 components as an explanation for cytochrome b_{560}, however, and it is unlikely that free heme has migrated from cytochrome b-c_1 polypeptides.

Some connection may exist between the properties of the semiquinone signal and the content of cytochrome b. We have not yet proven any cause and effect relationship, but our results suggest that some of the rapid relaxation of QP-S radical may be due to magnetic interaction with the ferriheme.

While the split signal cannot be due to Q-ferriheme magnetic coupling, heme may be necessary for the binding or simultaneous stabilization of two semiquinones. Binding of the heme should internally crosslink two portions of the polypeptide or crosslink between polypeptides and stabilize the native conformation, since low spin heme requires two axial ligands from the polypeptide. Yu et al (28) suggested that heme plays a structural role in SQR. They report-

ed shifts in the EPR spectra of the ferriheme associated with reconstitution of SDH and QP-S which provide evidence that these two components structurally recombine.

In conclusion, it appears that the minimum system for effective Q reduction by succinate does not require any heme. Whether 'cytochrome b_{560}' and 'QP-S' polypeptides may be identical with the heme and Q binding sites sharing the same polypeptide remains to be shown. If so, then it would suggest that the native preparations should contain stoichiometric amounts of heme, although its function still remains unclear.

Acknowledgments

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