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KINETIC STUDIES OF SUCCINATE DEHYDROGENASE BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

D. V. DERVARTANIAN*, C. VEEGER**, W. H. ORME-JOHNSON AND H. BEINERT

Institute for Enzyme Research, University of Wisconsin, 1710 University Avenue, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

The electron paramagnetic resonance signals of succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) were studied during reductive titrations, and the kinetics of their appearance and disappearance were followed on rapid reduction of the enzyme with succinate and after treatment of the reduced enzyme with ferricyanide, fumarate or O_2. At most 1 equiv of unpaired electrons per mole of enzyme-bound flavin is accounted for in the EPR signal at $g = 1.94$. Thus, according to present information on the structure showing this signal[14] 2 iron atoms out of the total 6–8 present per enzyme molecule are involved in the oxidoreductive process observed by EPR. Only a fraction (about 16%) of this iron, however, responds rapidly to reduction by succinate, whereas after complete reduction with succinate, reoxidation of a larger portion of the iron with ferricyanide and fumarate is rapid. After reduction with NADH and phenazine methosulfate, reoxidation by fumarate is slow as is reoxidation by O_2 under any conditions. During reduction the amount of iron which rapidly reacts does not depend on substrate concentration, whereas the extent of free radical formation does, rising with increasing substrate concentration. The maximal radical concentration observed accounted for 20–30% of the bound flavin present.

INTRODUCTION

SINGER AND KEARNEY[1,2] and independently WANG *et al.*[3] reported in 1955 that preparations of succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) contain iron which is not bound to porphyrin. Subsequently, different types of succinate dehydrogenase preparations were shown by chemical analysis to contain amounts of iron varying from 2 to 8 iron atoms per mole of flavin[3–7]. In 1960 BEINERT AND SANDS[8] found a new type of electron paramagnetic resonance (EPR) signal in reduced succinate dehydrogenase preparations, which they tentatively attributed to

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Abbreviation: PMS, phenazine methosulfate.

* Present address: Department of Biochemistry, University of Georgia, Athens, Ga. 30601, U.S.A.

** Permanent address: Agricultural University, Department of Biochemistry, Wageningen, The Netherlands.

iron. Since then this signal has been seen by other workers[9–11], and it has been inferred from data on simpler iron–sulfur proteins that it is due to an iron complex of unknown structure, presumably containing sulfur[12–17].

Kinetic studies by ZEYLEMAKER *et al.*[19] indicated that all enzyme molecules in a preparation as obtained[20] have the same turnover number (50–65 $\sec^{-1}$ at 25°). Incubation of such a preparation with succinate will then convert all enzyme molecules into a more active form (110–130 $\sec^{-1}$ at 25°)[19]. Thus far, except for a preliminary statement[18], no kinetic studies on succinate dehydrogenase preparations have been published which cover reaction rates of the EPR-detectable components in the time range of the turnover of either the activated or not activated form of the enzyme.

The main reason for the fact that thus far no such attempts have been made to obtain quantitative information on the reaction rates of the components of this otherwise thoroughly studied enzyme must be found in the difficulties introduced by this "activation" phenomenon. These difficulties have been discussed and summarized by SINGER[21]. Briefly stated, efficient activators are either substrates or inhibitors of the enzyme, and their effect is said to be at least partly reversed after their removal[22]. Because of the requirements of the EPR technique, it is not feasible to dilute these substances out, as is usually done in routine activity assays. Also for the studies intended here, the enzyme should obviously not be fully or even partly reduced, which again is irrelevant for routine assays. It also appears at this time that certain aspects of the activation phenomenon are not completely understood and remain controversial.

We were not able to overcome these difficulties in a satisfactory manner in the present work. Fortunately, however, only the results on the rates of reduction of the various enzyme components can be affected. The enzyme used for reoxidation experiments must certainly have been activated by exposure to succinate, and the results on the titrations should also be free of interference by the activation phenomenon. We, nevertheless, present our results on rates of reduction, although their interpretation may be ambiguous because of the unknown activation state of the enzyme. However, much work has been done in the past few years with this and related enzyme preparations at similarly poorly defined states of activation, and we feel therefore that the data obtained contain useful information on the behavior of succinate dehydrogenase under such conditions.

With these reservations in mind, in this paper we report quantitative data on rates and extent of participation of the EPR-detectable components, *viz.* the iron complex and a free radical, presumably flavin semiquinone, in the turnover of succinate dehydrogenase.

MATERIALS AND METHODS

The enzyme used in this work was the preparation described by WANG *et al.*[3] as modified by DERVARTANIAN AND VEEGER[20]. This type of enzyme preparation at the purity used contains 4–5 μmoles of flavin and 35–40 μgatoms of iron per g of protein[7]. The protein was stored in small lots under liquid N_2 and only thawed once, immediately before use. All solutions were 0.1 M with respect to potassium phosphate buffer (pH 7.4) and were equilibrated with "high purity" N_2 unless otherwise specified. Enzyme solutions contained 1 mM EDTA. All manipulations involving the purified enzyme were carried out with the exclusion of O_2 as far as was practical without resorting to elaborate techniques. In the kinetic experiments, however, previously described anaerobic techniques[23] were used. The N_2 gas used in these experiments, as well as in the titrations with NADH and phenazine methosulfate (PMS)[24], was passed through alkaline pyrogallol before use. In the titration the level of PMS used was quite critical. In a total reaction mixture of 0.275 ml, 5 μl of a 0.0002% solution of PMS were optimal.

The titration procedure using dithionite has been briefly described elsewhere[14,25] and will be published in the near future[62]. Helium ($\leq$ 1 ppm of O_2) was used in this case. Iron was determined by the method of VAN DE BOGART AND BEINERT[26].

The apparatus and techniques used in EPR spectroscopy and rapid freezing have been described in various recent papers[23,25,27–31]. The cavity used for the measurements was a standard rectangular V-4531 cavity of Varian Associates with a loaded Q of 4500–5000 under the conditions of our experiments. The concentrations of reactants given are final concentrations, *i.e.*, after mixing with various additions, by hand or in the rapid mixing apparatus. As in previous work using the rapid freezing technique the reaction times given refer to the times calculated from the dimensions of the apparatus and the speed of the syringe drive but neglect the unknown freezing time[31], which appears to be less than 10 msec.

The spectra obtained represent the first derivative of the EPR absorption curve (as for instance shown in Fig. 8). In the evaluation of the data obtained in the various experiments, we have normalized all signal heights that were observed on different days, on the basis of a standard of solid copper sulfate and to an enzyme concentration, after mixing, of 12.5 mg/ml, which was representative of the majority of experiments. The presence of isopentane in the frozen mixtures was also taken into account in this normalization. In agreement with previous work[30] 43% of the volume was found to be occupied by the frozen aqueous phase. The conditions of EPR spectroscopy chosen for normalization were: 23 mW microwave power; 12 gauss modulation amplitude; scanning rate of 100 gauss/min; time constant 0.5 sec; and temperature 98°K. The data from the titration experiment of Fig. 3 were treated separately as the observation temperature was 82°K in this case.

Since we did not observe any significant changes in signal shape, the observed "heights" of the derivative signals can be taken to be proportional to signal intensities and therewith to concentrations of the components represented. The proportionality factors are given by double integrations of derivative signals of the dehydrogenase and comparison with suitable standards. Thus in the experiment of Fig. 4 the iron signal obtained with dithionite corresponds to 30 μM in the sample after mixing, and the radical signal obtained at 6 min with the higher succinate concentration corresponds to 5.6 μM flavin, 11% of the enzyme-bound flavin present in this experiment. For the iron signals at $g = 1.94$ and $g = 4.3$, a copper–EDTA standard was used; for the radical signals, the flavoprotein from Azotobacter[32,33], which is quantitatively converted to the semiquinone by dithionite, was used. The semiquinone concentration for this protein can thus be based on a flavin determination. For integration, the radical signals were measured at 10 μW, where saturation appears to be $<$ 20% for the enzyme samples as well as for the standard. Because of the relatively low intensities of the signals and the signal-to-noise ratio of the spectrometer, operation at lower power did not result in an advantage in overall accuracy. With the iron signals, corrections were applied for transition probabilities[34] and population of states as far as presently known[35]. With several substances we have examined (iron–conalbumin, iron–EDTA), the signal at $g = 4.3$ only accounted for 30–50% of the iron present even after the mentioned corrections were applied (*cf.* refs. 35–37). We have therefore assumed that this same situation holds for the signal of succinate dehydrogenase and have made the additional correction of doubling the values in order to approximately account for this. The values given in this paper for the signal at $g = 4.3$ are therefore subject to an appreciable uncertainty. The temperature at which reactions were allowed to proceed was 22°. A relative measure of the spin relaxation rates was obtained by comparing signal heights over several orders of magnitude of incident microwave power. The logarithm of the signal height in arbitrary units divided by the square root of the power, $\log(S/\sqrt{P})$ (abscissa), was plotted *versus* the logarithm of the power. Details are found in recent publications[25,38]. Light absorption of the liquid samples contained in the round quartz tubes, as used for EPR spectroscopy, was measured with the aid of a specially constructed attachment, which will be described in the near future[63].

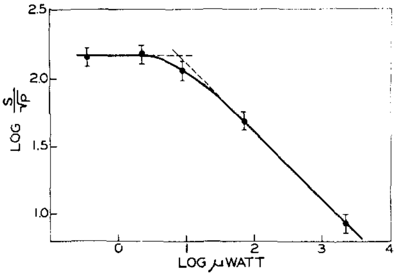
RESULTS

EPR signals of succinate dehydrogenase

The EPR signals of succinate dehydrogenase have been previously described[8–10,13,39–41]. The only EPR signal in the enzyme, as isolated, was that at $g = 4.3$, which is typical of Fe^{3+} in the high spin state[42]. This signal represents an amount of iron of the order of the flavin content of the enzyme. This iron is partially and slowly reduced by succinate and, when previously reduced, is rapidly reoxidized by ferricyanide or fumarate. The significance of this signal is not clear (see DISCUSSION).

On reduction by substrate or by a chemical reducing agent, a signal at $g = 2.00$, typical of a free radical, can be observed at room[39] and at low temperature[8–11], and concomitantly a signal appears at $g = 1.94$ (*cf.* Fig. 8), which is only seen at low temperature. It is reasonable to attribute the radical signal to a semiquinoid form of the bound flavin of the enzyme. According to the results obtained on simpler iron proteins, exhibiting a signal at $g = 1.94$ on reduction, the corresponding signal of succinate dehydrogenase is due to an iron complex[12,14]. For the sake of simplicity, it will be called the iron signal, and appearance of this signal will be referred to as indicating "iron reduction", although details of the electron distribution in the iron complex are not known. The radical signal is rather readily saturated with microwave power at the temperature necessary for observation of the iron signal. Saturation is observed at powers of 3–10 μW (110°K), the lowest at which the signals obtained in this work could still be measured with acceptable precision. Saturation varies to some extent with the conditions under which the radical is generated, as will be discussed below. The iron signal is not saturated at 77°K up to powers of 250 mW but is saturated at 4°K at powers as low as 3 μW (Fig. 1). The power of half saturation (*cf.* ref. 38) is approx. 10 μW at 4°K.

On quantitative determination, maximally 30% of the bound flavin and, after reduction with dithionite, maximally 16% of the total iron were accounted for in the respective signals. Low recoveries in the signal at $g = 1.94$ with respect to the total iron present have rather generally been observed, particularly with the more complex nonheme iron proteins[12,25,44–46]. Possible interpretations of this finding will be offered below. In the case of succinate dehydrogenase the low recovery is not due to the



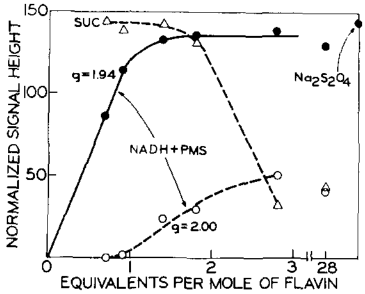


Fig. 1. Saturation of $g = 1.94$ signal ($g_\perp$) of succinate dehydrogenase at 4°K. The enzyme was prepared according to ref. 43. Enzyme prepared according to ref. 6 showed the same behavior within the limits of error of the method. The logarithm of the power incident on the cavity (abscissa) is plotted against the logarithm of the ratio of signal height, in arbitrary units, to the square root of the power (ordinate) (*cf.* refs. 25 and 38). The conditions of EPR spectroscopy were microwave power as indicated; modulation amplitude, 3.5 gauss; scanning rate approx. 200 gauss/min; time constant 0.3 sec; and temperature 4° K.

Fig. 2. Anaerobic titration of succinate dehydrogenase with NADH as reductant and phenazine methosulfate as mediator (*cf.* ref. 24). After manual mixing of the reactants in the dark, the samples were incubated in the dark for 1 h at room temperature, then frozen and their EPR spectrum was recorded. The sample tubes were subsequently evacuated, filled with N_2 and an excess of solid sodium succinate was added under N_2 to each tube, the samples were refrozen and their EPR spectrum was recorded. Signal (first derivative of absorption) heights measured in mm were normalized to routine settings of the EPR apparatus. Unless otherwise specified, these values refer to an enzyme concentration of 12.5 mg protein per ml after mixing, at a microwave power of 23 mW, modulation amplitude of 12 gauss, scanning rate of 200 gauss/min and time constant of 0.5 sec, at 98°K. In this experiment as well as all others, 0.1 M potassium phosphate buffer (pH 7.4), containing 1 mM EDTA, was used for the enzyme preparations and for the reactants the same buffer without EDTA. All reactant concentrations reported are final, and the temperature at mixing was approx. 22°. ○— — —○, radical signal; ●—●, iron signal; △- - -△, radical signal after addition of solid succinate (SUC).

TABLE I

CONCENTRATION OF UNPAIRED ELECTRONS IN SUCCINATE DEHYDROGENASE AFTER REDUCTION WITH DITHIONITE

Concentrations of iron and flavin are derived from protein determination by the biuret method on the assumption of a molecular weight of 240 000 and 1 mole of flavin per mole of enzyme (*cf.* ref. 7).

Iron (mM)	*Flavin (mM)*	*Temp. (°K)*	*Unpaired electrons in EPR signal at $g \approx 1.94$ and $g \approx 2.01$* (mM)*
Not determined	0.123	32	0.099
Not determined	0.123	81	0.10
Not determined	0.123	103	0.09
0.394	0.061	81	0.065

* On extensive reduction with dithionite radical signals at $g = 2$ are negligible.

temperature dependence of the signal, as shown in Table I. At least at temperatures higher than 30°K the signal merely broadens, probably through increased spin relaxation, but the integrated intensity remains unchanged up to temperatures (about 100°K) at which broadening sets a practical limit (*cf.* ref. 40).

Anaerobic reduction

Titration with NADH and phenazine methosulfate. It was shown by VAN GELDER[24] and VAN GELDER AND SLATER[47] that cytochrome *c* or cytochrome *c* oxidase can be titrated with NADH, when PMS is used as a mediator. Attempts to apply[18] this technique to succinate dehydrogenase showed that the EPR-detectable iron and flavin components can indeed be reduced and titrated with these reagents, when sufficient time is allowed for equilibration. Since in approx. 30 min maximal EPR signals were obtained, we routinely let the samples stand in the dark for 1 h at 22°. A titration curve is shown in Fig. 2. It can be seen that with 1 electron equiv of titrant the maximal iron signal was produced and that radical signals were only seen when more reductant was added. The amount of radical at its maximal development corresponded to 2% of the bound flavin. This must mean that out of the approximately eight iron atoms that are present per molecule, the equivalent of at most one is reduced, although according to findings with simpler iron proteins[14,48] at least two iron atoms may be involved in the one electron process giving rise to the $g = 1.94$ signal. Flavin is apparently only reduced after the iron complex has taken up an electron*. Since appearance of radical signals does not *per se* mean reduction, but could equally well indicate oxidation, we applied an independent test to the state of the flavin in the enzyme at the various stages of the titration. After addition of NADH *plus* PMS, incubation, freezing and measurement, the sample tubes were kept frozen with solid CO_2 and were again evacuated, filled with N_2 and thawed. The tops were removed and, while N_2 was blown down into the tubes, a small amount of solid sodium succinate was added, and the contents were frozen immediately after mixing (approx. 1 min). The radical signals observed after this treatment are shown by the open triangles in Fig. 2. Radical signals, amounting to 8% of the bound flavin, appeared in the samples with up to 2 reducing equiv of NADH, whereas with 3 and more reducing equiv of NADH present, succinate had less influence on radical concentration. Obviously succinate elicits strong radical signals as long as no radical has been formed by NADH *plus* PMS, whereas succinate in fact diminished the radical concentration, after NADH *plus* PMS has produced maximal radical signals. We take this to indicate that flavin was indeed left in its oxidized form with 1 electron equiv of NADH per mole of flavin but was reduced with larger quantities of NADH. Under these conditions, succinate served only to reduce flavin even further, thus diminishing the signal.

Titration with sodium dithionite. Fig. 3 shows the results of an anaerobic titration of succinate dehydrogenase with solid sodium dithionite, diluted to weighable quantities by KCl. The left-hand side of Fig. 3 refers to the EPR measurements on the frozen samples, while the right-hand side refers to the light absorption measurements on the

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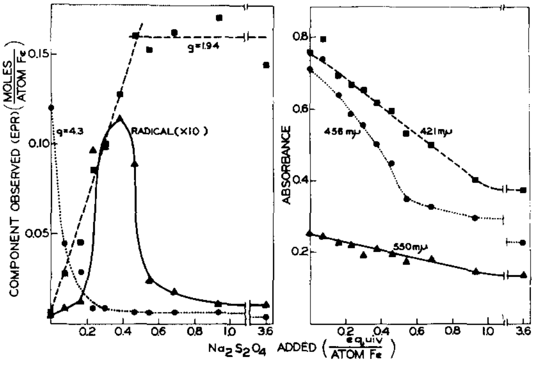
[*] Changes in the signal at $g = 4.3$ were not measured during this titration. Since it was observed in the titration with dithionite (see below) that flavin radical is not formed unless the iron represented in the signal at $g = 4.3$ is reduced, it is possible that the delay of flavin radical formation, presumably indicating flavin reduction, is related to the behavior of the signal at $g = 4.3$ rather than to that at $g = 1.94$.



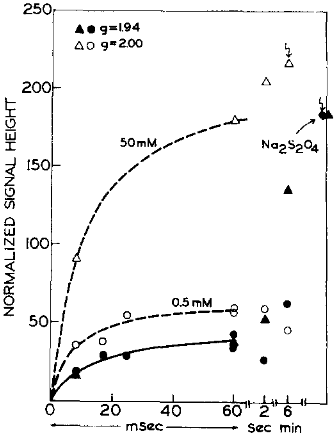


Fig. 3. EPR signal heights observed during titration of succinate dehydrogenase with dithionite. Enzyme, 0.4 mM with respect to iron, was titrated with solid dithionite, which was diluted to weighable quantities by solid KCl (refs. 14 and 25). Before freezing and observation of the EPR spectra, light absorption was measured in the EPR sample tubes. This was done by an accessory to the Cary spectrophotometer, Model 14, to be described elsewhere (ref. 49). The effective light path under these conditions was 2.8 mm. The left side of the figure refers to the EPR measurements and the right side to light absorption. The ordinate referring to the optical measurements gives the actual absorbance values measured under the conditions just described. The ordinate referring to the EPR measurements gives the concentrations of the three EPR-detectable components, as calculated from double integrations of the observed signals divided by the total iron concentration of the preparation, as determined chemically. Note that the values on the ordinate should be divided by 10, when applied to the concentration of flavin semiquinone. The conditions of EPR spectroscopy were: microwave power, 27 mW; modulation amplitude, 6 gauss; scanning rate, 200 gauss/min; time constant, 0.5 sec; and temperature 82°K for the signal at $g = 1.94$; the same conditions, except 12-gauss modulation for the signal at $g = 4.3$; and 0.2 mW, 6-gauss modulation, the same scanning rate, and a temperature of 101°K for the radical. The symbols used in the right half of the figure refer to absorbance at the wavelengths indicated and those in the left half to EPR signals as follows: ●·····●, $g = 4.3$; ■— — —■, $g = 1.94$; ▲—▲, radical at $g = 2.0$.

Fig. 4. Signal heights during anaerobic reduction of succinate dehydrogenase at two levels of succinate. Samples were frozen at the times indicated. The conditions of EPR spectroscopy were those of Fig. 2. ○ — —○, 0.5 mM succinate, radical signal; ●—●, 0.5 mM succinate, iron signal; △— — —△, 50 mM succinate, radical signal; ▲—▲, 50 mM succinate, iron signal.

same samples, immediately before freezing. The EPR results show that a portion of the first equivalents to enter the enzyme is used for reduction of the high spin Fe^{3+} represented in the signal at $g = 4.3$ and another portion is accomodated in the iron complex, which after electron uptake, exhibits the signal at $g = 1.94$.

According to double integrations of the signals obtained, the maximal signal at $g = 1.94$ (at 0.92 equiv of reductant) accounted for 1.1 equiv of unpaired electrons per mole of flavin or 16.5% of the total iron found by chemical analysis, *i.e.*, 1/6 of the iron. The signal at $g = 4.3$ accounted for 0.3–1 gatom of iron per mole of flavin and the maximal radical signal (at 0.375 equiv of reductant) for 7% of the flavin in this particular experiment, whereas in other experiments as much as 20–30% of the flavin was accounted for in the radical signal after dithionite reduction. Since the two iron species are present in roughly equal amounts (within the limits of error of the determination of the high spin ferric species) the question may be raised whether, when ferric, the iron represented in the $g = 4.3$ species may be the same that on reduction produces the signal at $g = 1.94$. However, neither the titration curves shown in

Fig. 3 nor the kinetic course of the reduction and reoxidation of these two species are compatible with the idea that the $g = 4.3$ species gives rise to the $g = 1.94$ species on reduction. They must be separate entities. It does, however, appear that the high spin ferric species ($g = 4.3$) may be able to accept electrons *via* flavin, by intra- or intermolecular oxidoreduction since flavin reduction only begins when the high spin species is reduced. Similar observations were previously made on NADH–cytochrome *c* reductase[50]. Flavin semiquinone formation is maximal while the $g = 1.94$ species is partly developed. Further reduction of flavin sets in as soon as the iron complex ($g = 1.94$) has been saturated with reducing equivalents. With 0.55 equiv added per iron all EPR-detectable components are reduced. If we assume that the enzyme contained eight atoms of iron per flavin, 0.55 equiv per iron would mean 4.4 equiv consumed per flavin. It appears then that of these 4–5 equiv per flavin, 2 are consumed in complete reduction of the flavin and approx. 1 each for production of the $g = 1.94$ species and the reduction of the high spin Fe^{3+} ($g = 4.3$). The light absorption data show, however, that additional electron acceptors are present which are most likely additional nonheme iron components. Similar observations have been reported for the titration of aldehyde oxidase from rabbit liver[25].

These observations indicate that those additional nonheme iron components, which are not detected by EPR in our experiments, have an absorptivity of the same order of magnitude as the EPR-detectable ones and can therefore not readily be distinguished from the latter. It appears from the plots of the light absorption data at the different wavelengths that the curve describing primarily flavin absorption (456 mμ) shows a break at the position where flavin reduction is complete, according to EPR, in contrast to the curves more related to iron reduction.

Kinetics of reduction with succinate. Fig. 4 shows the time-course of anaerobic reduction of iron and flavin in succinate dehydrogenase at final succinate concentrations of 50 and 0.5 mM. It can be seen that both signals appear at similar rates but that only a fraction of the maximal iron signal, which can be obtained with dithionite, is readily produced initially with succinate. A slow reduction of iron then follows. The half-time for the rapid phase of the reduction is of the order of 10 msec, in agreement with the results of measurements by the stopped-flow technique[19]. The number of observations points is insufficient to make a closer distinction. It is apparent that the intensity of the iron signal at early reaction times does not significantly depend on the succinate concentration, whereas the radical concentration is 3 times higher at the higher succinate level. In the time range of minutes, the higher succinate concentration also leads to stronger iron signals. The points indicated by the arrows in the figure correspond to 30 μM iron and 5.6 μM flavin.

The concentration of enzyme-bound flavin was 52 μM. Enzyme preparations of the type used here have a turnover number of approx. 65 moles of succinate per mole of flavin and per sec at 25° with ferricyanide or PMS as acceptor[51]. Depending on the electron accepting and transfer arrangement in the enzyme (*cf.* footnote 2 of refs. 52 and 46), one might then expect a cycling of oxidation–reduction catalysts in the enzyme every 8–16 msec. The rate of reduction of the components observed in the experiment of Fig. 4 at 22° is compatible with these numbers derived from overall activity assays.

An experiment similar to that illustrated in Fig. 4 was carried out with succinate–coenzyme *Q* reductase*. With 20 mM succinate and 50 μM enzyme the rapid phase

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* These experiments were done in collaboration with Dr. D. M. ZIEGLER.

of iron reduction was 60% completed in 6 msec and complete in 60 msec. Semiquinone formation followed a similar course and was maximal at 60 msec. The intensity of the rapidly produced $g = 1.94$ signal was only 10% of that elicited by dithionite.

During the anaerobic reduction of succinate dehydrogenase with D-malate, which has been shown to be a substrate of the enzyme[51], only small signals were observed. The iron and flavin signals developed simultaneously as with succinate. At 16 msec less than 1% of the maximally observed (with succinate) radical or $g = 1.94$ signal is seen and at 2 sec 14% of the radical and 7% of the iron signal. The turnover number of D-malate with succinate dehydrogenase is approx. 1.5 mmoles of D-malate per mole of flavin and per sec at 25° (*cf.* ref. 20). It is only possible to deduce approximate rates from the available EPR data, but the EPR observations are in general agreement with the conclusions drawn from overall rate studies and support the relationship to enzyme activity of the components observed by EPR spectroscopy.

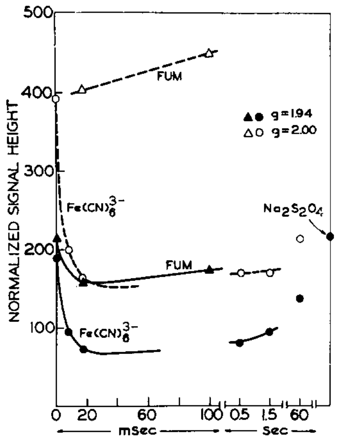
Reoxidation of reduced enzyme

Kinetics with ferricyanide. The enzyme was reduced anaerobically, either with a small quantity of succinate or with NADH and PMS and reoxidation of the enzyme was attempted by ferricyanide, fumarate or O_2.

Fig. 5 shows an experiment with ferricyanide or fumarate as oxidant. Since the enzyme is reoxidized very rapidly and is inactivated with large amounts of ferricyanide, we chose a ferricyanide concentration of 0.5 mM, one tenth of the original succinate concentration. At the concentration used (52 μM), the enzyme will turn over approx. 5 times. From the turnover number at an infinite ferricyanide concentration, it can be concluded that the activated enzyme turns over every 8–10 msec. From the dependency of the reaction rate on both donor and acceptor concentrations for this enzyme[19], it can be calculated that under the conditions of the experiment it takes approx. 250–300 msec for one turnover. Fig. 5 shows that oxidation of the reduced enzyme by ferricyanide to a steady-state level is approx. 85% complete in 8 msec, the first point of measurement. In the steady state observed after 16 msec, the iron is about 35% reduced as compared to the initial reduced enzyme. As expected, after 500 msec very little change in steady-state level is observed; however after 1.5 sec the reduction level starts to increase. In the reduced enzyme 90% of the $g = 1.94$ signal that can be produced by dithionite was observable. This experiment indicates that the reduction of the enzyme is a slower reaction than reoxidation by ferricyanide. Similar observations were made with NADH dehydrogenase[45].

Kinetics with fumarate. Reoxidation by 5 mM fumarate of the iron represented in the signal at $g = 1.94$ is a similarly rapid reaction. The radical signal is slightly increased with fumarate. In view of the ratio of the affinities of succinate and fumarate (K_D succinate/K_i fumarate $= 27$)[51] for the dehydrogenase it is not surprising that at an equimolar ratio of these substrates reoxidation of the iron does not exceed 25%.

In order to eliminate the competition between succinate and fumarate and to measure what might be the true reoxidation rate by fumarate, in another experiment, the enzyme was anaerobically reduced with 1.5 electron of equiv NADH (in the presence of PMS) per bound flavin and was reoxidized with 5 mM fumarate. This is shown in Fig. 6. It can be seen that reoxidation by fumarate is not a rapid reaction under these conditions. The half-time lies at approx. 1 sec. Unless the low velocity of reoxidation is due to the presence of PMS or NADH, this is an interesting observation



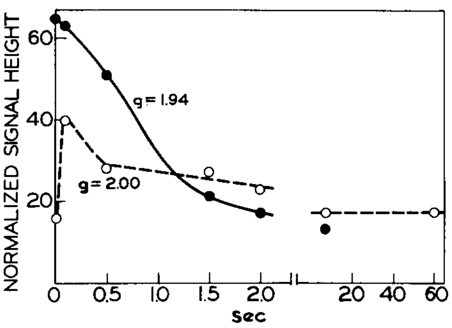


Fig. 5. Time-course of reoxidation by potassium ferricyanide or fumarate (FUM), of enzyme reduced by 10 mM succinate. The conditions of EPR spectroscopy were those of Fig. 2. Symbols of the curves referring to reoxidation by fumarate (5 mM) are: △— — —△, flavin radical; ▲—▲, iron signal. Symbols of the curves describing reoxidation by potassium ferricyanide (0.5 mM) are :○— — —○, flavin radical; ●—●, iron signal.

Fig. 6. Reoxidation by 5 mM fumarate of enzyme reduced by 0.5 equiv of NADH per mole of flavin as in the experiment of Fig. 2. ○— — —○, radical signal; ●—● iron signal. The conditions of EPR spectroscopy were those of Fig. 2.

insofar as it would imply that succinate conditions the enzyme for reoxidation.

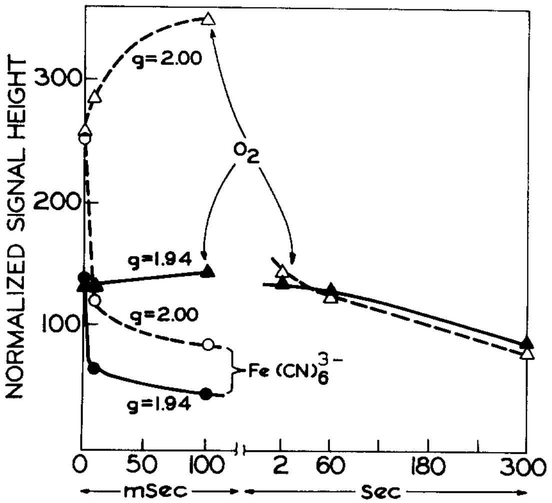
Kinetics with O_2. In a third type of experiment, the enzyme was first reduced anaerobically with an excess of succinate and was then mixed with O_2-saturated buffer. As shown in Fig. 7, O_2 causes first a rise and then a fall of the radical concentration and a decline of the iron signal. This behavior is most easily interpretated as a reoxidation, during which the radical passes through a maximum at the state of partial reduction and then vanishes as oxidation progresses. The decline of these EPR signals in the presence of O_2 has also been observed by KING *et al.*[9].

In the same series of experiments reoxidation by ferricyanide was repeated (Fig. 7) with results analogous to those obtained in Fig. 5.

Changes in signal shape and spin relaxation rate

It was observed that at the earliest times during reoxidation of succinate dehydrogenase, previously reduced with succinate, a change in the shape of the signal occurs in the $g = 2$ region. The effect is more pronounced with ferricyanide than with fumarate as oxidant. An example is shown in Fig. 8. The $g = 2.01$ peak of the iron signal is shifted 5–6 gauss downfield while the flavin signal is shifted by approximately half this value. Samples collected at 100 msec after initiation of reoxidation or later do not show this effect. No unique explanation can be given for this effect. It obviously belongs to the class of subtle changes, previously reported on[25,38,46,53] which include minor, but reproducible, changes in signal shape and electron spin relaxation rates, depending on the type of reactants present and on the time allowed for interaction.

Along these lines differences in electron spin relaxation of the radical signal were also observed with succinate dehydrogenase as they were with other flavo-



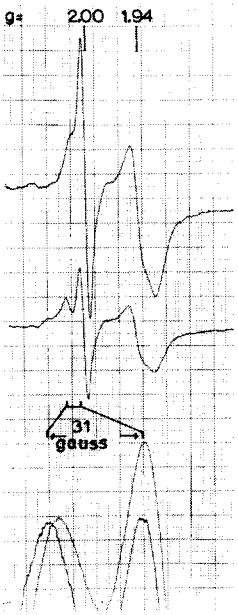


Fig. 7. Effect of O_2 on the EPR signal heights of enzyme reduced by 2.5 mM succinate. An equal volume of buffer saturated with O_2 was mixed with the anaerobically reduced enzyme. The conditions of EPR spectroscopy were those of Fig. 2. △— — —△, flavin radical; ▲—▲, iron signal. In the same experiment reoxidation of the same enzyme by 0.5 mM ferricyanide was repeated. The corresponding curves are: ○— — —○, radical signal; ●—●, iron signal.

Fig. 8. EPR spectra (first derivative) of reduced succinate dehydrogenase before and after partial reoxidation by ferricyanide. The conditions of EPR spectroscopy were: microwave power, 27 mW; modulation amplitude, 12 gauss; scanning rate, 200 gauss per min; and temperature 101° K for the two upper curves and scanning rate, 100 gauss per min; and temperature 82° K for the lowermost curves. The final concentrations of reactants were: enzyme, 8.65 mg/ml (approx. 0.036 mM); succinate, 6 mM; ferricyanide, 1 mM. Uppermost curve: reduced blank; center curve: 8 msec after mixing with ferricyanide; bottom curve: expanded scan (as indicated) of the samples used for the two upper curves at lower temperature, showing details of the shifts of the peaks (see text). The shifts as shown in the figure are subject to a number of inaccuracies of recording, whereas the gauss values given in the text and on the figure were derived from measurements with a proton probe. The latter values should therefore be used for quantitative evaluations.

proteins[25,38,46,54], depending on conditions. The differences were, however, not as pronounced as with aldehyde oxidase[25,52] or dihydroorotate dehydrogenase[46,53]. Particularly, the presence of fumarate or ferricyanide in addition to succinate had little effect, while effects of the time allowed for interaction with succinate were more pronounced.

DISCUSSION

The present work has shown that two of the EPR-detectable components of succinate dehydrogenase, a substance that can occur in the form of a stable free radical, and iron (g = 1.94) react at rates compatible with their participation in the catalytic turnover of the enzyme. These two components are thought to be the flavin prosthetic group and an iron–sulfur complex of the kind found in several other nonheme iron proteins[12,14,25,30,45,46,48]. A third EPR-detectable component, namely the high spin

Fe^{3+} represented by the signal at $g = 4.3$, is not considered a catalytically significant component, as will be discussed below.

It is at first sight disturbing that only a small fraction of the iron found in the enzyme by chemical analysis reacts rapidly. The following quantitative balance, derived from Fig. 4, illustrates this. In this experiment the concentration of bound flavin was approx. 0.05 mM so that 8×0.05 mM = 0.4 mM iron should have been present. On reduction with dithionite, 30 μM iron was accounted for in the signal at $g = 1.94$ and 15% of this, or 1% of the total iron present, had reacted at 17 msec. Although this overall balance may at first glance suggest that we are observing a negligible and possibly insignificant fraction of a component of the enzyme, the following considerations are pertinent: (1) The titration experiments of Figs. 2 and 3 show clearly that the total observable signal at $g = 1.94$ is produced by 1 equiv only per mole of enzyme. This information is independent of absolute quantitative evaluation of EPR signals which may involve some unknown errors but depends solely on the amount of reductant added and on the observation of maximal signal size by EPR, which cannot be significantly in error. When the $g = 1.94$ signal obtained with 1 reducing equiv per mole was quantitatively evaluated, between 0.4 and 1 equiv* were accounted for in different experiments. On the basis of this information from the titration experiment, at most the equivalent of one iron atom per flavin is represented in the $g = 1.94$ signal, which brings the fraction of rapidly reacting iron to 8% of that which we can actually follow by EPR spectroscopy. (2) Studies on a variety of proteins containing nonheme iron indicate that the minimum unit required for the appearance of the $g = 1.94$ iron signal on reduction consists of two iron atoms, although only one electron is taken up by this unit. A similar two-iron unit is very likely to be operative in succinate dehydrogenase. The fraction of iron involved in the oxidation–reduction process is therefore in fact twice that apparent from quantitative determination of the signal. (3) An additional point to be raised here concerns the behavior of the iron signal in the reduction experiment of Fig. 4 as compared to the reoxidation experiments of Figs. 5 and 7. It is evident from these experiments (Figs. 5 and 7) that on prolonged exposure (10 min) of the enzyme to succinate over 90% of the $g = 1.94$ signal, which can be produced by dithionite, does appear. On reoxidation with ferricyanide this signal disappears within a few msec (Figs. 5 and 7) and with fumarate a smaller fraction (25%) also disappears rapidly. While it could be argued that ferricyanide is a chemical oxidant, this argument does not apply to fumarate. An explanation for this unusual behavior of the iron signal may be found in the well-known requirement of succinate dehydrogenase for "activation" (*cf.* refs. 22 and 55). It is very difficult if not impossible to have preactivated enzyme under the conditions of concentration needed for EPR spectroscopy, without having either substrate present (in which case the enzyme would be reduced to start with), or an inhibitor (fumarate, malonate, *etc.*). It is easier to circumvent these problems in the usual catalytic assays for enzymes, where substrates or inhibitors can be effectively diluted out and the oxidation state of the enzyme at the outset of the experiment is not relevant, as only turnover is observed, *e.g.*, by product formation or reduction of an acceptor. It might thus be suggested that a fully activated enzyme should have a

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* The higher recoveries were obtained in more recent experiments, when an improved temperature and humidity control system allowed us to record spectra at approx. 80° K during all seasons.

larger fraction of rapidly reacting iron complex of the type producing the $g = 1.94$ signal, although we were not able to design experiments to verify this directly.

The maximal free radical in the experiment of Fig. 4 amounted to 11% of the total bound flavin and to approx. 2% at 17 msec at 0.5 mM succinate and 6% at 50 mM succinate. Since it is a feature, to our knowledge only observed with one flavoprotein[33,33], that all the flavin should be present in the semiquinone form, these recoveries appear entirely reasonable.

Two interesting aspects of the reduction experiment are as follows: (1) the rate of formation of the radical and $g = 1.94$ signals is independent of the succinate concentration, in agreement with the catalytic mechanism of ZEYLEMAKER *et al.*[19] and (2) the quantity of radical produced is 3 times higher at the higher succinate concentration. The half-time of appearance of the radical is not affected by the succinate concentration and has within experimental error the same value as that of the appearance of the signal at $g = 1.94$. These observations have a number of implications and are not readily explained in a unique fashion. In work of the kind described here one must always be aware of the possibility that different molecular species may be present and that different signals, which are observed (*e.g.* the radical and $g = 1.94$ signals), do not necessarily originate from the same molecules. Such heterogeneity of species could explain the kinetic behavior observed on reduction (Fig. 4). On the other hand, such behavior can also be explained on the assumption of a homogeneous population of molecules, if a sufficient number of intermediates with a particularly chosen set of rate constants for their interconversion is postulated.

The observation that the quantity of radical formed increases with succinate concentration, whereas the intensity of the $g = 1.94$ signal is independent of succinate concentration, suggests the presence of at least one more intermediate in addition to the ones proposed from kinetic experiments by ZEYLEMAKER *et al.*[19] in the reaction of succinate with the oxidized enzyme. It also militates against the suggestion that the flavin and nonheme iron react in a concerted fashion, simply each one accomodating 1 reducing equiv from an electron pair (hydride ion) transferred from the substrate. An argument against such a suggestion may also be seen in the titration experiment of Fig. 2, which indicated that the iron complex, but not the flavin, retains the first electrons which enter the enzyme. It should be kept in mind, however, that the titration experiments are concerned with an equilibrium situation and not with initial events and that the mechanism of reduction by NADH and PMS does not have to be identical with that by succinate.

The significance of the third EPR-detectable component, namely the high spin Fe^{3+}, with an EPR signal at $g = 4.3$ remains doubtful, although it is present in an amount approximately equivalent to the flavin and the nonheme iron complex that shows the signal at $g = 1.94$ in its reduced state. The reasons for questioning the significance of the high spin ferric component are these: (a) Signals of this kind are rather generally found in inorganic and organic matter and have been seen in many proteins, even in proteins which are not known as iron proteins. (b) The presence of these signals depends on the treatment of proteins during purification and is particularly observed when iron chelators such as EDTA are added or have been added at some stage during purification. Succinate dehydrogenase as used here was prepared in the presence of EDTA and EDTA was also added to all reaction mixtures. We have not attempted in this series of experiments to prepare the enzyme in the absence of

EDTA, but it has been found in previous work that in succinate and NADH dehydrogenase prepared without EDTA by other methods the signals at $g = 4.3$ were very weak (*cf.* ref. 56). (c) The signal at $g = 4.3$ does not respond to reduction by succinate to an extent or at a rate which would clearly indicate that it is a catalytically active component.

It is not certain whether the iron represented in this signal is adventitious iron picked up by the enzyme during manipulations or whether it is a constituent of the enzyme. In either case it is likely that we are dealing here with a ternary protein iron–EDTA complex, as has been suggested previously[56] and has also been postulated by other workers[57].

The experiments of Figs. 5 and 6 show that fumarate does effectively reoxidize the carriers in the enzyme and, at least in the presence of succinate, rapidly reoxidizes them. Fig. 7 relates to the question of whether the electron carriers in succinate dehydrogenase are oxidized by O_2. To our knowledge all flavoproteins are oxidized by O_2 although at low rates in some instances. MASSEY AND SINGER[58] established that soluble succinate dehydrogenase reacted with O_2 very slowly, *viz.*, at 0.02% of the rate shown with PMS. This has been confirmed by HÜFNER *et al.*[59]. On the other hand, a number of apparently contradictory effects have been observed with succinate dehydrogenase in concentrated solutions as they are required in EPR spectroscopy. HOLLOCHER AND COMMONER[39] reported that in the presence of O_2 the maximal radical concentration depended on the ratio of succinate to fumarate, while DERVARTANIAN *et al.*[10] found that under anaerobic conditions, the addition of fumarate of any concentration to a succinate-reduced enzyme caused a decline in both the radical and iron signals, which they ascribed to the formation of a complex between oxidized enzyme and fumarate. VAN VOORST *et al.*[60] observed that when the succinate-reduced enzyme was exposed to O_2, the radical signal declined and the iron signal increased, an effect which was not reversed when the system was again made anaerobic. However, when an enzyme reduced with D-malate was exposed to O_2, there was a complete loss of radical signal with no effect on the iron signal. On the other hand, in the presence of equimolar amounts of succinate and fumarate, exposure to O_2 caused an increase in both the radical and iron signals. GRIFFIN AND HOLLOCHER[61] have confirmed the observations that the EPR signals in the absence of O_2 are more intense and that O_2 causes a decline in the radical signal when succinate is present.

The results of the experiment of Fig. 7, namely an initial increase of the radical signal followed by a decline of both iron and radical signals, are most readily explained by assuming that a slow oxidation takes place, in disagreement with GRIFFIN AND HOLLOCHER[61] who concluded that oxidation was not involved in the signal changes of the kind described above.

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