BBA 65637

STUDIES ON SUCCINATE DEHYDROGENASE

III. ELECTRON-SPIN RESONANCE MEASUREMENTS AT LIQUID-NITROGEN TEMPERATURE

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(Received March 20th, 1967)

SUMMARY

1. When measured at 77 °K and under anaerobic conditions, purified succinate dehydrogenase reduced with succinate shows two ESR signals, belonging to the flavin semiquinone and reduced non-heme iron. Upon standing at 20° for 5 min the signal amplitudes (measured at 77 °K) increase. On prolonged standing (2 h) the free-radical signal declined somewhat while the signal due to reduced non-heme iron increased.

2. *p*-Chloromercuribenzoate does not affect the ESR spectrum of the enzyme when added after the succinate. No signals are observed when *p*-chloromercuribenzoate is added to the enzyme before addition of succinate.

3. Irradiation of the enzyme at 77 °K with light from a mercury-arc lamp leads to the formation of ESR signals belonging to the flavin semiquinone and reduced non-heme iron.

4. D-Malate, L-malate, L-chlorosuccinate and DL-methylsuccinate also bring about the formation of the signals belonging to the flavin semiquinone and reduced non-heme iron. The ESR spectrum of the enzyme after reduction with L-malate disappears within 10 min standing at room temperature. During reduction with L-chlorosuccinate and DL-methylsuccinate the transient formation and disappearance of an additional free-radical signal can be observed.

5. The introduction of oxygen into the ESR tube causes a decline of the flavin semiquinone signal of the enzyme, while concomitantly the reduced non-heme iron signal increases.

6. The different effects observed are explained in terms of the existence of several equilibria in succinate dehydrogenase reduced by succinate.

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INTRODUCTION

The preceding papers of this series[1,2] have dealt with the effects of substrates, competitive inhibitors and other compounds on the absorption spectrum of succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1). In this paper, these effects are correlated with ESR measurements made at liquid-nitrogen temperature. Preliminary accounts as well as some discussions of these results have appeared elsewhere[3–8].

MATERIALS AND METHODS

Descriptions of the method of preparation of succinate dehydrogenase, the determination of enzyme activity, the materials used and other experimental procedures have been published in the first two papers of this series[1,2]. ESR measurements were performed with a Varian 4500-10A X-Band spectrometer. For cooling to liquid-nitrogen temperatures, the Varian V-4546 liquid-nitrogen accessory, combined with the 100-kcycle cavity with irradiation slots, was utilized. Irradiation studies were carried out with a Philips (SP 500) 500-W high-pressure mercury arc.

Samples to be measured at liquid-nitrogen temperature were placed either in quartz tubes of 4 mm diameter and 20 cm length or in a similar quartz tube, jointed to an upper chamber, in which repeated evacuations (5–6 times) by high vacuum were followed by flushing with O_2-free nitrogen (alkaline pyrogallol) to ensure anaerobic conditions. This upper chamber contained 2 or 3 side-arms by which additions of components could be made. Measurements at room temperature were made with the Varian aqueous sample cell.

Experiments with succinate dehydrogenase were always done anaerobically and the cells with enzyme and reactants in separate side-arms were kept under nitrogen at 0° before and after measurements at room or liquid-nitrogen temperature. With very labile systems, the cells immediately after mixing of reactants were maintained in liquid nitrogen until ready for measurements. Further experimental details are described in the legends of the figures.

RESULTS

Effect of succinate in the absence of O_2

Addition of succinate to succinate dehydrogenase under anaerobic conditions leads to the appearance at 20 °C of one signal, at $g = 2.00$, representing the flavin-semiquinone free radical, as suggested by BEINERT AND SANDS[9].

Measurements at 77 °K under the same conditions show two sets of signals (Fig. 1, upper records): the flavin semiquinone signal at $g = 2.00$ and an asymmetric signal at $g_{\perp} = 1.94$, $g_{//} = 2.01$, attributed to a reduced iron species[9]. The intensities of the signals at 20 °C or at 77 °K increased during standing for 5 min at 20 °C under anaerobic conditions. This increase may be due to activation of the enzyme as described by KEARNEY[10] and re-emphasized by KIMURA, HAUBER AND SINGER[11], but other explanations are possible (see DISCUSSION). The increase was found with all substrates, except L- and DL-malate, and was particularly striking in the case of

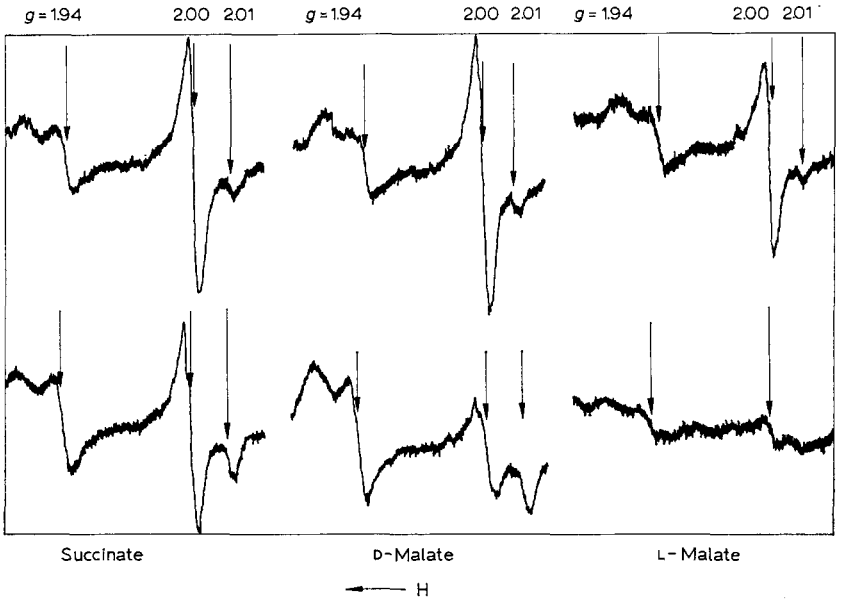


Fig. 1. ESR spectra at 77 °K of succinate dehydrogenase containing 24.8 mg protein/ml and 163 units[1]/mg protein in 0.1 M phosphate buffer (pH 7.6) and 1 mM EDTA. The solution was evacuated at least 8 times under high vacuum followed by purified nitrogen (alkaline pyrogallol) with the latter ending the cycle. The spectra were recorded with the same instrumental settings. The upper records, made directly after mixing, give the spectra obtained with succinate (24 mM), D-malate (110 mM) and L malate (110 mM) showing the simultaneous appearance of two sets of signals at $g = 2.00$ and at $g_{\perp} = 1.94$, $g_{||} = 2.01$. The lower records show the effect of standing for 2 h at room temperature. In the case of succinate and D-malate, the $g = 2.00$ signals have decreased while the $g_{\perp} = 1.94$, $g_{||} = 2.01$ signals have increased. With L-malate the signals almost completely disappeared within 10 min.

L-chlorosuccinate and DL-methylsuccinate. With L- or DL-malate the signals declined rapidly (see below).

The signals found with enzyme to which succinate was added were similar to those reported by BEINERT AND SANDS[9] as well as KING, HOWARD AND MASON[12]. The distance between the points of maximum slope (ΔH_{ms}) for the $g = 2.00$ signal was found to be about 12 gauss, which is to be expected for a free radical of a conjugated aromatic system such as a flavin semiquinone. It is in agreement, for example, with the width of the signal of an aromatic mononegative ion[13] in a rigid glass and is considerably less than the value obtained with succinic acid crystals irradiated with X-rays[14].

Titration of a constant amount of succinate dehydrogenase (about 0.1 mM flavin) with varying amounts of succinate under anaerobic conditions showed that almost maximal ESR signals were observed with 0.8 mM succinate, and slight signals were detected with as little as 40 μM.

In agreement with BEINERT AND SANDS[9] the signal with $g_{\perp} = 1.94$, $g_{||} = 2.01$, but not that at $g = 2.00$, disappears completely at temperatures above −100 °C. The two sets of signals also showed totally different saturation characteristics when the microwave power was steadily increased. The $g = 2.00$ signal reached maximal

intensity at low power, typical of an organic free radical, whereas the asymmetric signal could not be saturated even at higher power (*cf.* ref. 15).

In the absence of succinate, the enzyme (5 h after the solubilization with butanol) showed no ESR signals either at room or liquid-nitrogen temperature. This is in contrast to the results of KING, HOWARD AND MASON[12] who found both sets of signals with a similar preparation under the same conditions.

Irradiation of the oxidized enzyme with ultraviolet light at 77 °K for 10 min under anaerobic conditions led to the same, although rather weak, signals as found with succinate except that the peak distance for the $g = 2.00$ signal was somewhat broader (Fig. 2).

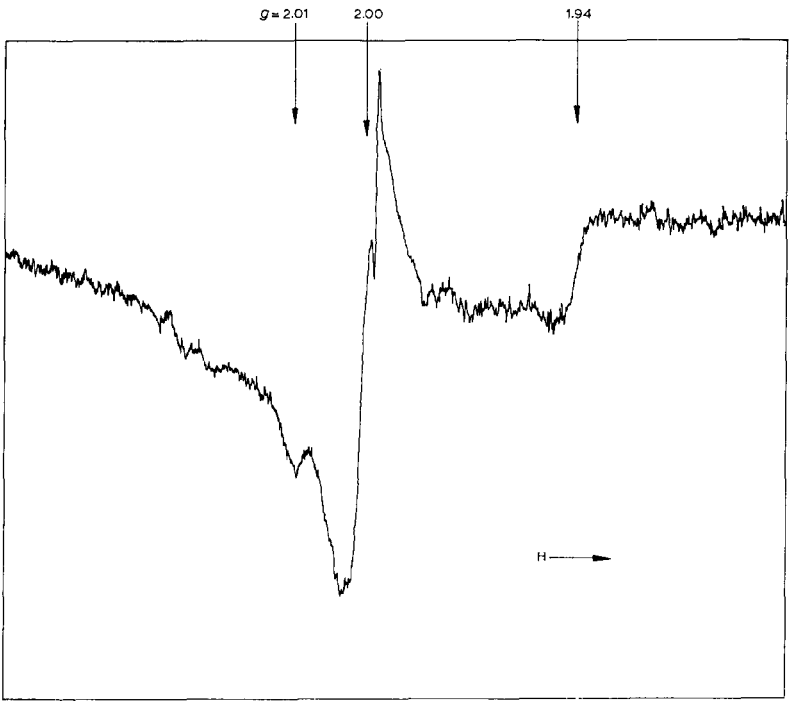


Fig. 2. ESR spectrum at 77 °K of succinate dehydrogenase after irradiation with ultraviolet light for 10 min under nitrogen (120 units/mg protein, 33 mg protein/ml).

On addition of excess $Na_2S_2O_4$ under N_2 to the enzyme in the absence of succinate, only the asymmetric signal at $g_\perp = 1.94$, $g_{||} = 2.01$ could be found (*cf.* ref. 9). After irradiation of this system with ultraviolet light for 10 min at 77 °K the signal of the flavin semiquinone wsa also present.

When succinate was added under N_2 to an active enzyme, followed by *p*-chloromercuribenzoate, normal ESR signals characteristic of the succinate-reduced enzyme were obtained. On the other hand, when *p*-chloromercuribenzoate was first added to the enzyme, followed by succinate, no signals could be detected. This is in agreement with BEINERT[15] who found further that, on the addition of $Na_2S_2O_4$ to the *p*-chloromercurisulphonate-treated enzyme, the ESR signal at $g_\perp = 1.94, g_{||} = 2.01$

appeared with normal intensity. These observations are consistent with the finding[16,17] that succinate protects the enzyme against *p*-chloromercuribenzoate inactivation. Pre-treatment of the enzyme with reduced glutathione had no effect on the ESR signals obtained after the subsequent addition of succinate (*cf.* ref. 16).

The effect of competitive inhibitors

The addition of fumarate, malonate or oxaloacetate to the enzyme did not result in any detectable ESR signals even at very high concentrations of inhibitor. When fumarate at concentrations as low as 0.3 mM was added to the succinate-reduced enzyme under nitrogen, there was a decline in both ESR signals, indicating formation of an ESR-inactive enzyme–fumarate complex. D-Chlorosuccinate, which is a competitive inhibitor of succinate dehydrogenase, also produced no ESR signals. This is in contrast to the stereoisomer, which elicited both signals.

The effect of substrates other than succinate in the absence of O_2

The addition of other substrates, *viz.* L-chlorosuccinate, L-malate, D-malate, DL-malate, DL-chlorosuccinate or DL-methylsuccinate, to the enzyme under N_2 at 77 °K led to the same ESR signals as found with succinate.

Although the same type of signals appeared initially with D- and L-malate as with succinate, there were nevertheless striking differences in their behaviour during standing in the absence of O_2 (Fig. 1, lower records). The signals given by L-malate or DL-malate almost completely disappeared within 3–10 min standing at 20 °C, whereas after 2 h the signals given by succinate or D-malate showed a slight decrease (greater with D-malate) at $g = 2.00$ and a small increase in the asymmetric $g = 1.94$, $g = 2.01$ signal. In this manner, the signals produced by succinate and D-malate could be distinguished from those given by L-malate. DL-Malate behaved in the same way as L-malate. The addition of L-malate but not of D-malate was also found to

TABLE I

THE EFFECT OF SUCCINATE IN STABILIZING THE L-MALATE-INDUCED ESR SIGNALS OF SUCCINATE DEHYDROGENASE AT 77 °K UNDER ANAEROBIC CONDITIONS

Systems A, B, and C contain 18.5, 16.6 and 15.3 mg protein/ml, respectively, of enzyme containing 168 units/mg protein. Instrumental settings are the same in each system. Signal heights are given in arbitrary units.

System	*Sec*	$g = 2.00$	$g = 1.94$	$g = 2.01$
A. L-Malate (140 mM)	40	66	30	16
	216	35	23	8
	340	21	19	8
	660	None	None	None
B. L-Malate (120 mM) + succinate (1.7 mM)	40	66	32	11
	160	45	27	7
	340	31	24	6
	480	19	20	4
C. L-Malate (120 mM) + succinate (17 mM)	40	82	35	16
	160	97	41	17
	370	89	41	17
	1000	54	33	17

bring about a decrease in the signal intensity caused by succinate alone. Table I shows the effect of succinate on the signal intensity of L-malate-reduced enzyme. In the presence of 17 mM succinate and 120 mM L-malate (C), there was at first an increase of the signal (see above) followed by a decline. At the lower succinate concentration (B) only a slower rate of decline was seen.

The smaller initial intensities and the decline obtained with L-malate or DL-malate are due to the presence of small amounts of fumarate hydratase (EC 4.2.1.2) in the enzyme preparation, which convert L-malate into fumarate. This results in the formation of the succinate dehydrogenase–fumarate complex[1] which gives no ESR spectrum. Since D-malate is not acted upon by fumarate hydratase, the signals obtained with this substrate are equal in intensity to those obtained with succinate and do not decline on standing over a period of 10 min.

Although L-chlorosuccinate and DL-methylsuccinate produced the same sets of ESR signals as other substrates, a difference was observed with respect to the course of the development of the signals. Fig. 3 shows the ESR spectrum of L-chlorosuccinate-reduced enzyme after 1, 3 and 8 min standing under nitrogen at 20 °C and measured at 77 °K. An additional radical signal of low intensity, ΔH_{ms} approx.

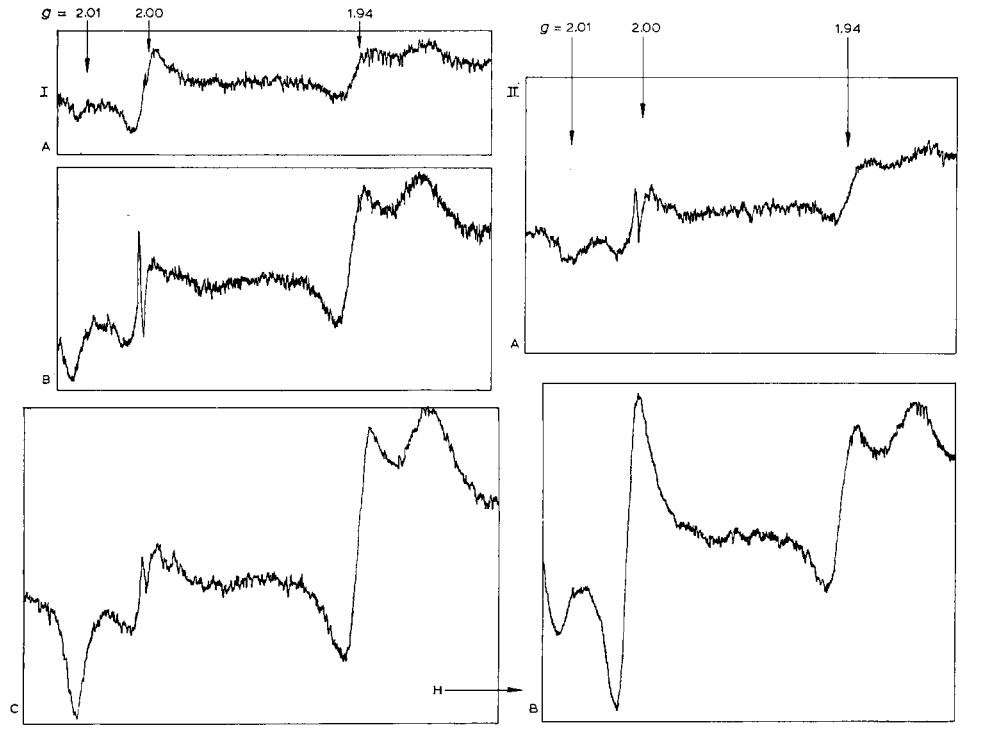


Fig. 3. Effect of L-chlorosuccinate and DL-methylsuccinate on ESR spectrum, measured at 77 °K under anaerobic conditions, of succinate dehydrogenase (44 mg protein/ml, 167 units/mg protein). Series I shows the enzyme in the presence of L-chlorosuccinate (74 mM) after standing for 1 min (A), 3 min (B), and 8 min (C) at 20 °C. Series II shows the enzyme in the presence of DL-methylsuccinate (78 mM) after standing for 1 min (A), and 6 min (B) at 20 °C. These experiments were performed with the same instrumental settings.

4 gauss, not observed with succinate or malate, developed strongly and then declined, while the flavin-free-radical and iron signals were reaching full intensity. The same sequence of signals was observed with DL-methylsuccinate. The fact that the changes in intensity of the small signal in the vicinity of $g = 2.00$ occurred while the other signals increased to full intensity shows that it was not a cavity signal. Furthermore, the ESR recordings were made under identical instrumental settings. Although the signal has not been observed with any other substrate tested, we have often observed with succinate and D-malate a small but reproducible shoulder on the major free-radical signal at $g = 2.00$.

The nature of the additional signal is unknown; since it is very narrow, it cannot be an aliphatic radical. Since these substrates are not oxidized as readily as succinate[18,2], the additional signal might represent radical formation and breakdown as a consequence of secondary reactions occurring during the incubation.

The effect of oxygen on the ESR spectrum of succinate- or D-*malate-reduced succinate dehydrogenase*

A characteristic of the ESR signals found with the fully activated succinate-reduced enzyme is that the ratio of the amplitudes of the $g = 1.94$ and $g = 2.01$ signals is constant under a variety of conditions. The ratio $g = 1.94$:$g = 2.01$ found in the studies presented here (2.2–2.5) was similar to that reported by BEINERT AND SANDS[9] and KING, HOWARD AND MASON[12]. Table II shows the results obtained with two preparations in which the signals were fully developed under anaerobic conditions

TABLE II

THE EFFECT OF OXYGEN ON THE ESR SIGNALS GIVEN BY SUCCINATE-REDUCED ENZYME UNDER STRICTLY ANAEROBIC CONDITIONS AT 77 °K

Signal heights are given in arbitrary units. The instrumental settings are identical in experiments with the same preparation. In I, enzyme (190 units/mg protein) at a concentration of 19.0 mg protein/ml was used, and in II enzyme (125 units/mg protein) at 27.5 mg protein/ml. Unless specified otherwise, enzyme was allowed to stand at 20 °C and then frozen in liquid nitrogen before measuring.

	$g = 2.00$	1.94	2.01
I. A. Enzyme and succinate (10 mM) mixed under N_2 (4 cm Hg pressure) and allowed to stand for 2.5 min at 20 °C	254	94	41
2.5 h later	254	110	49
After 2.5 min at 20 °C, air admitted and the contents thoroughly mixed	125	131	60
4 h later	135	135	62
B. Enzyme and succinate as above mixed in the presence of air, after 2.5 min at 20 °C	111	97	47
After 4.5 min	76	110	48
After 9.5 min	53	123	51
II. A. Enzyme and succinate (20 mM) mixed under N_2 and allowed to stand for 10 min at 20 °C	240	85	38
Air admitted and contents thoroughly mixed, then allowed to stand for 8 min at 20 °C	110	87	41
B. Enzyme and succinate as in II, A mixed under N_2 (4 cm Hg pressure)	168	52	21
Contents mixed with air and allowed to stand for 8 min at 20 °C	113	77	37

in the presence of succinate and then exposed to oxygen. In each case, there was a significant decrease in the flavin semiquinone signal, depending on whether oxygen was allowed to enter efficiently into the reaction cuvette (more rapid in a cuvette under a slight vacuum) and to react with succinate-reduced enzyme. Concomitantly there was an increase in the asymmetric iron signal at $g_{\perp} = 1.94$, $g_{||} = 2.01$.

Fig. 4 shows a similar effect when an anaerobic cell containing D-malate-

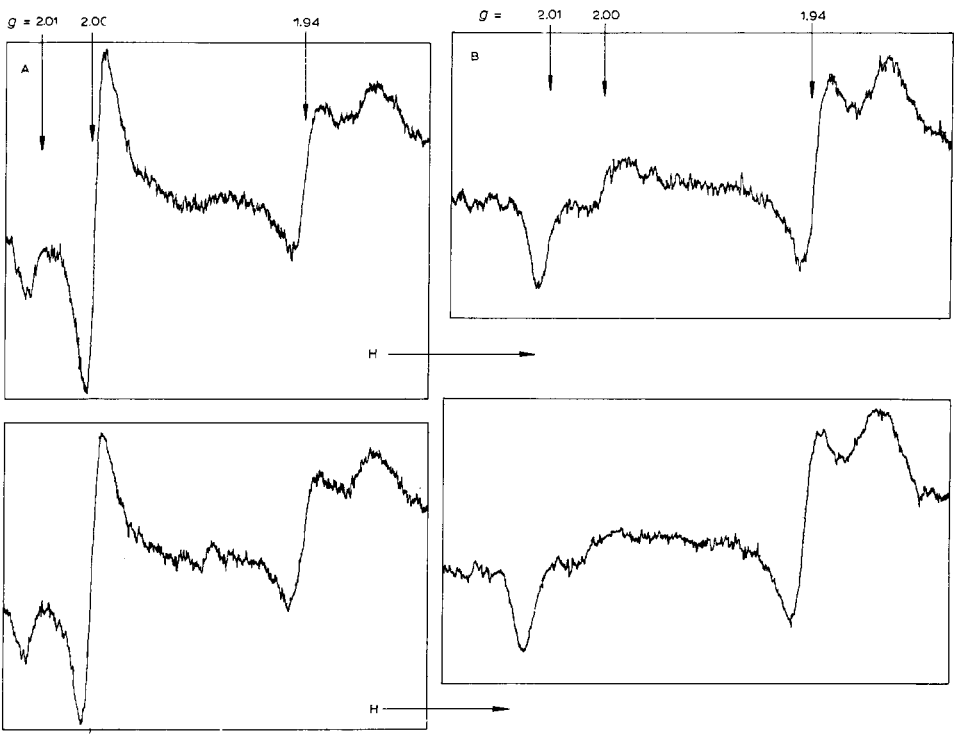


Fig. 4. The effect of oxygen on the ESR signals of succinate dehydrogenase (168 units/mg protein, 18.5 mg protein/ml) reduced by D-malate (170 mM). System A contained D-malate and enzyme under nitrogen recorded after 40 sec (above) and 160 sec (below) standing at 20 °C. System B is identical to A except that the contents were mixed in the presence of air and recorded after 40 sec (above) and 160 sec (below) at 20 °C. The slight radical signal seen at $g = 2.00$ after 40 sec has almost completely disappeared within 160 sec at 20 °C.

reduced enzyme was exposed to oxygen. The effect of O_2 was much more pronounced than with succinate as electron donor. Immediately after mixing in the presence of O_2 a slight signal at $g = 2.00$ developed, and this almost completely disappeared within 160 sec at 20 °C. The signals at $g = 1.94$ and $g = 2.01$, which did not change upon standing at 20 °C, were larger than the signals observed under anaerobic conditions.

DISCUSSION

The addition of substrates such as succinate, L-chlorosuccinate, D- or L-malate

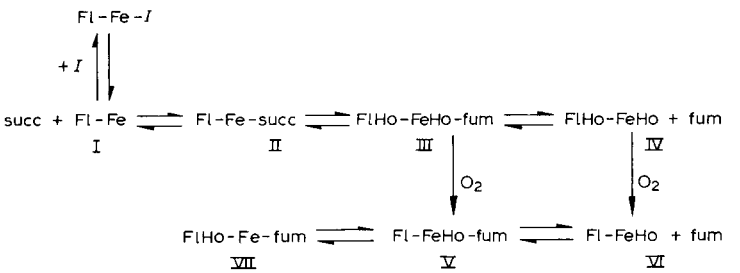
causes a significant decrease of optical absorption in the flavin region, the maximum decrease at 460 mμ with succinate amounting to 27% (refs. 1 and 2). In the ESR spectrum the $g = 2.00$ free-radical signal, derived from the flavin semiquinone, and the asymmetric iron signal at $g_{\perp} = 1.94$ and $g_{||} = 2.01$ appear simultaneously. These signals are the result of a one-electron reduction of the flavin moiety to the semiquinone level[9] and a, presumably also one-electron, reduction of the iron species[19–22]. The presence of some ESR-inactive reduced enzyme, with both reducing equivalents on the flavin, cannot be excluded. As is also the case with other flavoproteins such as D-amino-acid oxidase (EC 1.4.3.3) and L-amino-acid oxidase (EC 1.4.3.2)[23], competitive inhibitors of succinate dehydrogenase do not give any ESR signals but cause only spectral changes, interpreted as being due to the formation of enzyme–inhibitor complexes.

A prior incubation for about 5 min is essential for full development of the ESR signals observed with various substrates of succinate dehydrogenase.

The small decline of the $g = 2.00$ signal and the concomitant increase of the $g = 1.94$, 2.01 signal observed after prolonged incubation at 20 °C is probably due to the presence of traces of oxygen in the cuvette. Account must be taken of the presence of fumarate hydratase in the enzyme preparation. Although its level may vary from one preparation to another, sufficient fumarate will be made available by its action on L- or DL-malate to occupy all available active sites on succinate dehydrogenase, resulting in a loss of the ESR signals characteristic of the reduced enzyme. ESR measurements should be made as rapidly as possible in order to minimize this effect of fumarate. GRIFFIN AND HOLLOCHER[24], who confirmed the ESR signals with D- or L-malate at 77 °K, did not find any signal at room temperature. This is probably because the free radical and reduced iron obtained on addition of malate to the enzyme were stabilized when the solution was cooled to 77 °K for ESR measurements at this temperature, but rapidly disappeared when the samples were not frozen. We found a rapid disappearance of the signals obtained with L-malate on standing under anaerobic conditions (Fig. 1) and also a rapid loss of the free-radical signal (the only signal that can be observed at room temperature) with D-malate in air. In disagreement with our results[2], no oxaloacetate was detected by GRIFFIN AND HOLLOCHER[24]. Since they also did not observe significant spectral changes upon the addition of substrates to their preparation, it is likely that their preparation is of significantly lower activity than ours. The relative change in absorbance is related to the specific activity of a succinate dehydrogenase preparation[2].

The development and decay of the small signal at $g = 2.00$, observed when the enzyme is reduced with L-chlorosuccinate or DL-methylsuccinate, is, in contrast to the flavin-radical signal at that g value, not kinetically important. This can be concluded from the observation[18,2] that the rate of oxidation of L-chlorosuccinate by acceptors, catalysed by succinate dehydrogenase, is about 50% that of the oxidation of succinate. It follows that the time needed for the reduction of the enzyme by L-chlorosuccinate and appearance of the flavin-radical signal will be of the same order of magnitude as that for succinate, which is milliseconds[21] rather than minutes. The formation and decay of the extra signal must then reflect a secondary process. The nature of these effects is not clear; it is, however, possible that they are connected with the slow dissociation of chlorofumarate or oxaloacetate from the reduced enzyme.

It has been shown[8,25] that in the overall reaction catalysed by succinate dehydrogenase, *e.g.* the oxidation of succinate by $K_3Fe(CN)_6$, a fumarate-reduced enzyme complex is the catalytically active intermediate and it is oxidized by the acceptor before the product (fumarate) dissociates from the oxidized enzyme. This does not exclude the possibility, however, of a catalytically unimportant side reaction in which fumarate dissociates from the reduced enzyme. Such a reaction might be observed in the absence of a rapidly reacting electron acceptor, as is the case in the ESR studies. Furthermore the kinetic evidence[8,25] led to the postulation of a succinate-oxidized enzyme complex as the first step in the reduction of the enzyme by this substrate. Stopped-flow experiments[25] show that the reduction proceeds in two phases: a fast reaction with a half time of the order of 10 msec at 25°, followed by a much slower reaction with a half time of the order of minutes. Since the fast phase of the reaction accounts for the turnover of the enzyme in the presence of acceptor, the second phase of the reaction is presumably a side reaction.



Scheme 1. Different forms of succinate-reduced succinate dehydrogenase in the presence and absence of O_2. In this scheme no formal valency has been given to the redox-active iron in the non-heme-iron complex, because no information is available about its ligand field. A reducing equivalent has been given the symbol Ho regardless of whether a hydrogen atom or an electron is taken up. Fl is flavin. Two-equivalent-reduced forms of the enzyme with both equivalents on the flavin are left out of consideration. *I* = competitive inhibitors, such as fumarate, malonate, oxaloacetate.

Scheme 1 provides a reasonable explanation for some of the effects observed here.

The slow increase in intensity of the $g = 2.00$ and $g = 1.94$, $g = 2.01$ signals may be due to a slow shift in the equilibrium between the ESR-inactive intermediate II and the ESR-active intermediate III, caused by dissociation of fumarate from the reduced enzyme. If the amount of ESR-active intermediates III and IV increases, there would be an increase of signal intensity.

The effect of O_2 on the ESR signals can be explained if it may be assumed that oxygen oxidizes the flavin semiquinone but affects much less the reduced-iron species. The presence of oxygen would thus lead to the formation of intermediates V and VI, having the flavin in the oxidized form but the iron species still reduced. The oxidation of III and IV to V and VI, respectively, would lead to a complete conversion of II into V and VI (*via* III and IV). The net result is an increase in the signal at $g = 1.94$, 2.01.

It has been observed[21] that a maximally developed $g = 1.94$, 2.01 signal can be produced by selective reduction with one reducing equivalent while no radical signal is present. The intensity of the $g = 1.94$, 2.01 signal is as high as that obtained

with excess of $Na_2S_2O_4$ which gives a higher signal than that obtained by reduction with succinate under anaerobic conditions. Upon the addition of fumarate the $g = 1.94$, 2.01 signal decreases in intensity, while the $g = 2.00$ signal develops to some extent. Taking these observations into account it is probable that V is in equilibrium with VII which shows the $g = 2.00$ signal, whereas VI shows the maximum $g = 1.94$, 2.01 signal. The same explanation may hold for the effect of O_2 in case of reduction by D-malate, oxaloacetate taking over the role of fumarate.

The succinate dehydrogenase preparation used in these studies contains EDTA, which is probably relevant to the photoreduction observed, since MASSEY AND PALMER[26] have recently shown that photoreduction of flavoproteins to the semiquinoid state is strongly promoted by EDTA. It is interesting that on irradiation of succinate dehydrogenase, even in the frozen state, the $g = 1.94$, $g = 2.01$ signal develops to some extent.

The effect of *p*-chloromercuribenzoate and reduced glutathione on the optical[2] and ESR spectrum of succinate dehydrogenase indicates clearly that a functional sulphur group is involved in the mechanism of action of the enzyme. Although pretreatment of enzyme with *p*-chloromercuribenzoate prevented the induction of the signals by substrate, the results of BEINERT[15] indicate that $Na_2S_2O_4$ is still able to elicit the $g = 1.94$, 2.01 signal. Thus, although the substrate site has been altered in such a way as to prevent electron flow from substrate to flavin or iron, the latter moiety has not been destroyed. Indeed, the inactivation of the enzyme can be reversed by subsequent treatment under anaerobic conditions with cyanide or reduced glutathione[16,17], provided that exposure to *p*-chloromercuribenzoate inactivation has not been sufficient to cause irreversible denaturation.

It has been found[27–35] that non-heme-iron proteins contain acid-labile sulphur in amounts more than, equivalent to or less than the amount of iron present, and that there are iron–sulphur interactions[36,37]. DERVARTANIAN *et al.*[37] concluded from experiments on the incorporation of ^{33}S in purified iron proteins of two bacterial species that the splitting between individual lines of ^{33}S was 12 gauss on the basis of comparison with calculated spectra. However, since flavoproteins and other proteins containing non-heme iron differ in function, ESR behaviour, optical spectrum and other chemical and physical properties, they may not all contain equivalent amounts of iron and acid-labile sulphur.

The nature of the $g = 1.94$ signal and of the iron species responsible for it in biological materials is as yet unknown. BEINERT and co-workers[15,38–40] have discussed this in considerable detail. A multitude of non-heme-iron containing materials, including bacterial and plant ferredoxins[41,42], show the signal under reducing conditions. Its functional rate as a redox entity has been shown to be perfectly compatible with its rate of reduction by substrate and reoxidation by acceptor[21,43,44]. From studies with model compounds, several proposals for the ligand field of this redox-active iron in non-heme iron-containing proteins have been made[19,22,42,45] but none of them has proved to be identical with that of the biological species.

ACKNOWLEDGEMENTS

We are indebted to Professor E. C. SLATER and Professor G. J. HOIJTINK for

their valuable advice and interest. We wish to thank Dr. H. BEINERT and Dr. P. HEMMERICH for useful discussions and criticism. We also gratefully acknowledge the technical assistance of Miss A. SEARLE and Mr. R. SITTERS, and extend our thanks to Mr. H. DE HAAN for preparation of the anaerobic quartz tubes.

This investigation was supported in part by Research Grant RG 6569 from the U.S. Public Health Service and by the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).

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