EPR STUDIES ON THE MECHANISM OF ACTION OF SUCCINATE DEHYDROGENASE IN ACTIVATED PREPARATIONS

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SUMMARY

Reductive titrations and rapid kinetic studies are reported on extensively or completely activated, particulate and solubilized succinate dehydrogenase (SD) preparations. There is one iron-sulfur (Fe-S) center of the ferredoxin type present per flavin which is reduced by succinate, but even in activated preparations at most 60% of these centers were reduced within the turnover time of the enzymes. Flavin semiquinone formation does not precede or significantly lag behind the reduction of the Fe-S centers. On reduction of the soluble enzymes an accumulation of semiquinone is observed. No qualitative difference between the behavior of preparations containing 4 Fe or 8 Fe per flavin was found. Succinate-ubiquinone reductase (Complex II) contains an Fe-S component with properties of high-potential Fe-S proteins (See Ruzicka and Beinert, Biochem. Biophys. Res. Communs., this issue). It occurs at a concentration close to that of the bound flavin and has been observed to be reduced by succinate at approximately the same rate as the ferredoxin type ($g = 1.94$) component. With dithionite, reduction of additional Fe-S groups (0.2 to 0.5 per flavin) is observed but the significance of this is uncertain.

Although it has been known for years that succinate dehydrogenase (SD) contains, besides covalently bound flavin, iron-sulfur (Fe-S) groups which give characteristic EPR signals on reduction, the catalytic function of these components and the mechanism of intramolecular electron transfer in SD have remained poorly defined (1). The reason for this has been that definitive studies on the oxidation-reduction behavior of the SD components by rapid reaction techniques require the use of fully activated preparations. Until recently SD preparations, both particulate and soluble, were mixtures of activated and deactivated forms and activation required the use of succinate or malonate which interfered with subsequent rapid kinetic experiments. Thus, it was observed (2) that of the Fe-S components of SD which appear in the reduced state after treatment with dithionite or after several minutes exposure to succinate, only a small fraction is rapidly reduced by

succinate. It was suggested that the reason for the slow reduction may have been the presence of deactivated enzyme in the preparations.

The discovery (3,4) of a class of activators (anions) which fully activate SD, but do not reduce, oxidize, or inhibit SD, prompted us to reinvestigate the catalytic function of the enzyme with a method capable of providing information on its flavin and Fe-S components.

MATERIALS AND METHODS

Complex II was prepared according to Baginsky and Hatefi (5). Soluble SD was prepared by the method of Davis and Hatefi (6), but was extracted without succinate ("perchlorate extracted or Type 3 SD"), and by the procedure of Coles et al. (Type 1 SD) (6). SD activity was measured with PMS-DCIP at V_{max} and SD content by bound flavin analysis (7). ETP was prepared according to Ringler et al. (8).

SD preparations (except Type 3, which is fully activated as prepared) were activated with 500 mM $NaNO_3$ + 100 mM semicarbazide in 50 mM Hepes, pH 7.0, at 20°, at 5 mg/ml protein. The oxalacetate released during activation (9) was removed from soluble SD by passage through Sephadex G-50 and $(NH_4)_2SO_4$ precipitation and from particles by repeated washing with the activation mixture. EPR spectroscopy, anaerobic titrations and rapid kinetic experiments were carried out and the results evaluated, as described (10).

RESULTS AND DISCUSSION

EPR signals of SD. Complex II, in the oxidized form, has a dominant signal centered at g = 2.01 with 28 gauss width. This signal is only detectable at $\leq$ 25° K. It is present in intact heart tissue and presumably originates from a high-potential (Hipip) type of Fe-S structure, as discussed in the accompanying report (11). In addition there are signals from the cytochromes at g = 3.3 to 3.4 (12) and a high spin iron signal at g = 4.3. The quantities represented in these additional signals are variable and since these components are catalytically inert, they will not be considered here. Ideally, soluble SD preparations show no g = 1.94 or radical signal in their oxidized forms; however, in most preparations minor signals of this type, along with some Hipip or g = 4.3 component (2 to 10% of the flavin), were present. On reduction with succinate signals of the reduced ferredoxin (Fd) type Fe-S center (g = 2.027, 1.93, 1.91) and of flavin semiquinone (g = 2.00) appear, and with an excess of dithionite the signal of the Fd-type changes in shape and becomes more intense. This is true for all preparations.

Stoichiometry of SD components. Table I summarizes the results of anaerobic reduction experiments on SD preparations. The quantities given are all based on double integrations of spectra and determinations of bound flavin, not on comparison of signal sizes. The following points stand out: the quantity of the Hipip component in Complex II as well as that of the succinate-reducible Fd-type Fe-S centers in all types of preparations is close to that of the bound flavin. Fe-S centers are evident which are reducible only by

dithionite; these amount to 50% of the succinate reducible centers at the most and, on the average, to between 20 and 30%. Contrary to a report in the literature (13) both Fd-type centers are readily detectable at 100° K (cf. Table I). From the stoichiometry it is obvious that the type of Fe-S center only reducible by dithionite cannot be present in every molecule of SD. In view of this and its inertness toward succinate the significance of this component is questionable. In view of the model of the Fe-S center of Fds put forth recently (14) the occurrence of a Hipip structure raises the interesting question as to whether the same Fe-S group that produces the Hipip type of signal, after uptake of $2e^-$ per center, reappears as a ferredoxin-type structure with a signal at g ~ 1.94. Our kinetic observations, namely that in a number of preparations there is little rapid reduction by succinate of the Hipip component, whereas about 50% of the g ~ 1.94 signal appears rapidly, would seem to rule out the possibility that these two signals arise from the same structure, just differing by $2e^-$. It seems possible, however, that the g ~ 1.94 signal elicited only by dithionite could arise from a Hipip type center after reduction with $2e^-$.

The semiquinone concentration, after addition of succinate, amounts to approximately 10% of the bound flavin in Complex II and to 70-80% in soluble SD. In the presence of TTFA the semiquinone concentration in Complex II is approximately doubled. It appears (cf. Table I) that in soluble SD succinate is not capable of reducing the flavin beyond the semiquinone state, as up to 80% of the flavin is accumulated in this form. This may reflect an important difference between catalytic mechanisms of soluble and more integrated preparations.

Anaerobic titrations with dithionite are summarized in Fig. 1. The three types of enzyme preparations studied show a very similar pattern of reduction. As expected, more equivalents (eq.) are required for complete reduction of Complex II, as the Hipip Fe-S component (g = 2.01) and in addition cytochrome(s) $\underline{b}$ and $\underline{c}_1$ are reduced. Only the Hipip component is quantitatively of significance during the early phase of the titration (< 4 eq.). The component which is apparently only reducible by dithionite is not reduced until all of the flavin and the ferredoxin-type Fe-S center has been reduced. This is evident from the signal shape, which changes significantly with the onset of reduction of the center reduced only by dithionite. The flavin is reduced in Complex II only after most of the Hipip and ferredoxin type Fe-S centers have been reduced. In this behavior, as well as in the quantity of semiquinone formed, Complex II differs from soluble SD. Because of variable quantities of ubiquinone and cytochromes present in Complex II it is probably not useful to consider the overall capacity for e^- uptake of this preparation in

TABLE I

Stoichiometry of EPR Detectable Components in SD Preparations

Preparation	Activation %	Temperature of Observation °K	Electron equivalents per mole of bound flavin (eq./mole) recovered in			
			g = 2.01[#]	g = 1.94 (succ.)	g = 1.94($Na_2S_2O_4$)	g = 2.00[†]
Complex II, (1)	96	13	1.04		1.41	
(2)	100	6	1.18	0.95	1.19	
		13	1.14	1.03	1.26	
		13			1.35	
		97		1.03	1.22	
		97			1.25	0.08
(3)	82	13			1.18	
(4)	86	13	0.55	0.85	1.03	0.13
		13		0.55*		0.22*
Type 3 (1)	88	13	0.167	1.09	1.35	
(2)	100	13	0	0.79	1.37	
		97		0.91	1.35	0.78
Type 1	100	13	0	0.87	1.46	
		13			1.49	
		97		0.84	1.29	0.72

[†]Semiquinone. [#]Hipip. *In presence of 1.5 mM TTFA. Turnover numbers of preparations at 38°, 100% activated: Complex II (2) = 12,380, (3) = 12,524, (4) = 9,640; Type 3 (2) = 10,120; Type 1 = 8,580.

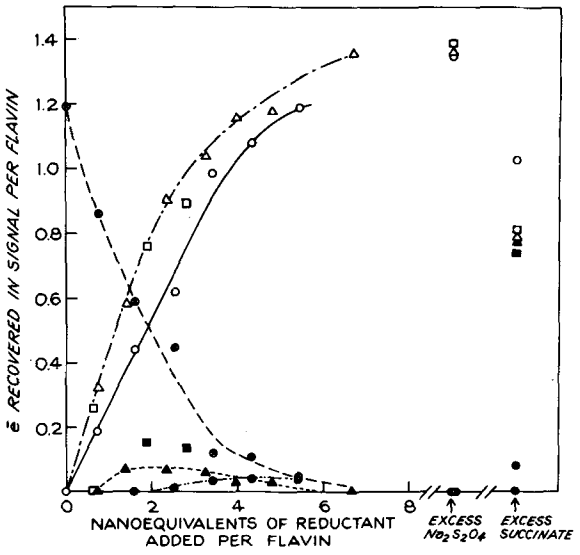

Fig. 1. Titration of SD preparation with dithionite. Equivalents of dithionite added are expressed per mole of flavin on abscissa. The preparations used were those of Table I. Complex II (2): ○, g ~ 1.94; ⊗ Hipip; ●, radical. Type 3 (2): △, g ~ 1.94, ▲, radical. Type 1: □, g ~ 1.94, ■, radical.

detail. However, with soluble SD it appears from the pattern of semiquinone formation, as well as from the onset of shape change in the signal of the Fd-type Fe-S center, that 4 and not the expected 3e⁻ are required to reduce the flavin completely and the succinate reducible Fe-S center. Since serious interference by oxygen seems excluded according to the initial slopes of the curves, the question of an additional e⁻ acceptor is raised.

Kinetics of reduction of the EPR detectable components of succinate dehydrogenase

Hipip component. In most preparations a partial rapid reduction of this component was observed concomitant with the formation of the signal at g ~ 1.94. However, the amount reduced varied from preparation to preparation between 10 and 50% of the total present. In ETP, anaerobically and in the presence of TTFA, ~ 50% of the Hipip signal disappeared rapidly ($t_{1/2}$ at 16° ~ 100 msec), concomitant with formation of the succinate-produced signal at g ~ 1.94. Without TTFA, reduction of all carriers by succinate was relatively slow. Our tentative conclusion is that an Fe-S center with the general features of the high potential Fe-S proteins of photosynthetic bacteria (15) is associated with the succinate-ubiquinone reductase complex and that this Fe-S center may well have a catalytic function. It would appear, however, that the Hipip component is very labile and is one of the first components to lose activity. This agrees with observations on the purified material (11).

TABLE II

Typical Rates of Reduction of EPR Detectable Components in SD Preparations

Preparation	Activation* %	Turnover time (msec), 16°	Temperature of reaction °C	Signal	Electron equivalents recovered in signal per mole of bound flavin at time t								
					← msec →								← min →
					0	6	13	17	25	40	60	100	10
Complex				g≈1.94	0.09	0.61		0.60		0.68		0.62	1.14
II (4)+	86	34	13.5	Radical	0	0.07		0.05		0.09		0.05	0.02
Same + 1.5													
mM TTFA	86	34	14	g≈1.94	0.02	0.46		0.50		0.49		0.53	0.71
				Radical	0	0.022		0.026		0.026		0.026	0.22
Type 3,(2)+	100	23	14	g≈1.94	0.20				0.80		0.79	1.22	1.40
				Radical	0.0535				0.355		0.60	0.595	0.33
Same + 1.5													
mM TTFA	100	23	13	g≈1.94					0.33			0.47	1.01
				Radical					0.19			0.38	0.13
Type 1,(1)+	100	42	13	g≈1.94		0.13	0.15		0.19		0.21	0.29	0.98
				Radical		0.083	0.10		0.11		0.14	0.19	0.16
(2)	89	33	16.5	g≈1.94	0.14		0.19		0.215		0.30	0.29	0.635
				Radical	0.051		0.11		0.15		0.235	0.23	0.092

*At the time of EPR experiments. Initially all preparations were 100% activated. +As in Table 1.

Fd-component. In deactivated preparations no signal at g ~ 1.94 appeared with succinate even in minutes at 16°. However, even in preparations activated to the extent of 80-100% only about 50% of the Fe-S groups became detectable within the turnover time of the respective enzymes, after addition of succinate (Table II). Complex II was more effective in this respect than the soluble enzymes. With Complex II practically all the Fe-S groups that were rapidly reducible were reduced within 6 msec at 14° C, in agreement with the turnover time measured in the PMS-DCIP assay.

Flavin. Complex II again differed from the soluble enzymes in that considerably less flavin was found in the semiquinone form on reduction with succinate. The high level of semiquinone observed with the Type 3 enzyme (6) is particularly striking.

Activation state and reactivity of Fe-S groups. Comparison with previous work. Although in the previously reported EPR study on SD(2) a different type of soluble preparation (Type 2) was used, the results obtained with EPR spectroscopy at ~ 100° K are compatible with those reported here, if it is considered that the preparations used were only partially activated, as pointed out at that time (2). Nevertheless, the present results show that even in extensively activated preparations the quantity of Fe-S groups reacting within the turnover time of the respective enzyme preparation is, in our opinion, significantly less than expected. We are inclined to discount the possibility that inactivation or deactivation occurred in the time required until enzyme and substrate were mixed, since in the critical experiments this was only 5 to 8 minutes at 0 to 14° C after thawing the stored protein. It appears that in a certain percentage of molecules, which in some instances may be as high as 60%, the Fe-S centers react as required for a catalytically active enzyme component; in the rest of the molecules they will only react after prolonged exposure to substrate. We consider two possibilities, viz., either that the difference in reactivity of the Fe-S groups observed by EPR is not apparent in the catalytic assays, or that purified preparations in which each molecule is fully active have never been obtained. The latter case would be similar to that experienced with xanthine oxidase, where for many years all studies were done on preparations composed of species differing in the makeup of their active centers, but not in features of protein structure (16,17), which would be readily detected by the usual methods of protein chemistry.

In analogy to the experience with xanthine oxidase, difficulties arise in the interpretation of the meaning of EPR signals if several signals appear simultaneously but at concentrations less than stoichiometric to those of the active sites of the enzyme. For example, it is probably reasonable to attribute the rapidly appearing Fd-type signals, after addition of succinate, to the reactive centers in the preparation, but it might be

that the semiquinone signals arise from those centers whose Fe-S groups do not respond rapidly. Similar ambiguity exists for the Fd-type signal which only responds to dithionite.

CONCLUSIONS

There is one Fd-type Fe-S center in SD which responds to succinate, but even in activated preparations we have never seen more than ~ 60% of this to react within the turnover time of the enzyme. Flavin semiquinone formation is observed simultaneously and there is no indication that flavin reduction precedes reduction of the Fe-S center. In the soluble preparations flavin accumulates in the semiquinone form, indicating that reduction to the fully reduced form is impaired. No qualitative differences were seen between the behavior of preparations with 4 Fe and 8 Fe per flavin, respectively, although the latter showed a higher fraction of rapidly reacting Fe-S centers. The possibility must be considered that an Fe-S component of the Hipip type functions in more complex preparations in electron transfer from SD toward the acceptors on the oxygen side of the electron transfer system.

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