ON THE POSSIBLE INTERRELATIONS OF THE REACTIVITY OF SOLUBLE SUCCINATE DEHYDROGENASE WITH FERRICYANIDE, RECONSTITUTION ACTIVITY, AND THE HIPIP IRON SULFUR CENTER.

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Summary: It was recently reported (Vinogradov et al.,Biochem. Biophys. Res. Commun. 65, 1264-1269, (1975)) that fresh preparations of succinate dehydrogenase, extracted anaerobically in presence of succinate, contain a reaction site for ferricyanide which had not been previously recognized. This site has a low K_m for ferricyanide ($\sim$200μM); it is very unstable to air and is not seen either in preparations extracted without succinate or in membrane-bound forms of the enzyme, presumably because in the latter the site is inaccessible to ferricyanide. This type of ferricyanide reduction is thus distinct from that conventionally measured using high concentrations of ferricyanide ($K_m \sim$3mM).

The lability of the "low K_m site" for ferricyanide is reminiscent of the lability of reconstitution ability and the Hipip iron sulfur center of the soluble enzyme. This note presents evidence that the labile ferricyanide site and the reconstitution activity may both hinge on the integrity of the same component. It is shown that both activities decay at identical rates at three pH values on exposure of the enzyme to O_2 at 0°. The possibility is considered that the site involves the Hipip center. Concurrently with the disappearance of these activities, some 50-55% of the phenazine methosulfate reductase activity also disappears. The question whether this loss suggests different reaction sites for this dye in fresh and O_2 modified preparations is discussed in terms of current knowledge of the rate-limiting step in catalysis by succinate dehydrogenase.

The catalytic activity of mammalian succinate dehydrogenase is usually determined either with phenazine methosulfate (PMS) or ferricyanide as electron acceptor (1). As used in the past ferricyanide reductase activity is based either on an assay conducted at a single high ferricyanide concentration ($\sim$5mM (2)) or on a series of high ferricyanide concentrations (1.7 - 10mM) and extrapolation to V_{max} (1). Under either set of conditions the activity with ferricyanide is only a fraction of

that measured with phenazine methosulfate, at least at temperatures above 25° (1, 3).

Recently one of us (3) described a variant of the ferricyanide assay in which activity is determined at a series of low ferricyanide concentrations (50-250μM). This activity is characterized by a low K_m for ferricyanide (∿200μM), the fact that in freshly prepared soluble preparations it measures the same maximal activity as does PMS, and that this activity is very labile and decays rapidly on contact with O_2. Moreover, the activity measured at these low ferricyanide concentrations is not seen in membrane-bound enzyme preparations, presumably because the component which donates electrons to ferricyanide is not exposed to the outside of the vesicles and ferricyanide cannot penetrate the inner mitochondrial membrane (4). In this and all other respects mentioned the activity measured at high ferricyanide concentrations in conventional procedures differs from the new activity, so that it appears likely that different redox groups in the enzyme (i.e., different "sites") are involved in the two types of ferricyanide assay.

Our interest in this "low K_m ferricyanide site" stems from the suggestion (3) that because of its lability, which is reminiscent of the lability of "reconstitution activity", both of these activities may hinge on the integrity of a particular group in the enzyme, possibly the Hipip Fe-S center, which is similarly labile. This Hipip center, along with a ferredoxin-type of Fe-S center ("center 1") and histidyl 8α-FAD are present in stoichiometric amounts in the enzyme in Complex II and a substantial fraction of each is reduced at rates compatible with the turnover number in the PMS assay (5, 6). Of these three recognized redox components of the enzyme, the Hipip center is most labile: most of it is lost on extraction of the enzyme in the absence of succinate, although full catalytic activity of Complex II survives under these conditions (5, 6). If the enzyme is extracted anaerobically with succinate

present, much of the Hipip EPR signal is conserved, although with a modified signal shape (7). The catalytic competence of the Hipip center in the soluble enzyme has not yet been demonstrated.

The experiments to be described bear on the question of the possible interrelations of "low K_m ferricyanide activity", reconstitution activity and a functional Hipip Fe-S center.

METHODS

Keilin-Hartree preparations, soluble succinate dehydrogenase and alkali-inactivated Keilin-Hartree preparations were prepared essentially as described by King (8). The soluble enzyme was prepared anaerobically in the presence of succinate and stored in liquid N_2. Covalently-bound flavin was determined according to Singer et al.[2] (9) and protein by the biuret method (10). Succinate-PMS reductase was measured according to Singer (1) and succinate ferricyanide reductase measured spectrophotometrically at 420nm using low concentrations of ferricyanide (50-250μM(3)). Reconstitution was measured in the presence of 1% bovine serum albumin in an oxygen electrode using substoichiometric amounts of soluble enzyme (7μg soluble enzyme/mg inactive particles). All activities were measured at 30° in 10mM TRIS-H_2SO_4 buffer, pH 7.4, containing 20mM succinate and 1mM EDTA and are expressed as μmoles succinate oxidized/min/mg soluble enzyme.

RESULTS AND DISCUSSION

Table I illustrates the following: 1) The turnover number of soluble preparations of the enzyme, immediately after extraction in the presence of succinate from Keilin-Hartree preparations, is the same as in the parent particles in the PMS assay. 2) As expected, the activity of freshly prepared soluble enzyme at low ferricyanide concentrations equals that measured in the PMS assay. 3) Some 70% of this catalytic activity of the soluble enzyme may be measured as succinoxidase in the reconstituted system. Although this figure is relatively high, it is probably not maximal because in the succinoxidase assay the rate-limiting step is in the cytochrome b-c_1 region of the respiratory chain, and not at the level of the dehydrogenase. This limitation is minimized in the present experiments by adding to alkali-treated particles soluble enzyme in about one tenth the amount originally present. Thus the

Table I

Comparison of various activities of reconstitutively active succinate dehydrogenase

	Preparation	Histidyl flavin content (nmol/mg protein)	Activity (30°) PMS reductase	Ferricyanide reductase*	Reconstitution test
1.	Keilin-Hartree particulate preparation	0.14	0.87 (6,200)**	-	-
2.	Soluble succinate dehydrogenase	2.33	16.0 (6,900)	14.7 (6,300)	11.0 (4,700)

*Ferricyanide reductase activity refers specifically to that measured with low ferricyanide concentrations as described in METHODS.

**Numbers in parentheses are moles succinate oxidized/min/mole histidyl flavin.

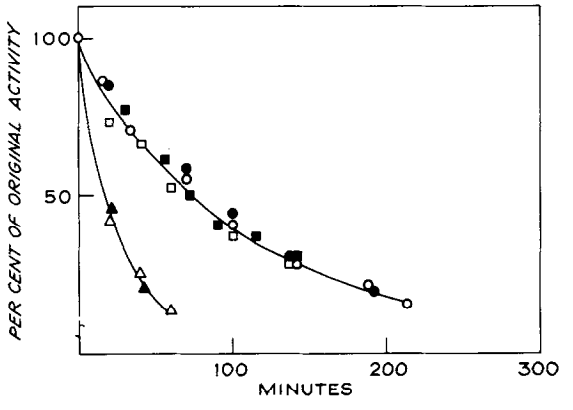

Figure 1. Effect of pH on aerobic decay of reconstitution and ferricyanide reductase activities of soluble succinate dehydrogenase. Aerobic decay was induced by exposing 2 ml of argon saturated enzyme to air in a 25 ml beaker in ice at pH 8.5 (triangles), pH 7.4 (circles) and pH 6.5 (squares); reconstitution activity (closed symbols), ferricyanide reductase activity measured at 150μM ferricyanide (open symbols).

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respiratory chain as electron acceptor was in excess.

Fig. 1 compares the kinetics of the loss of activity measured at

low ferricyanide concentrations and of reconstitution activity when a freshly prepared, soluble enzyme is exposed to O_2 at three pH values. Both activities are evidently extremely labile and decay at identical rates. The first order rate constants were $8x10^{-2}min^{-1}$ at pH 6.5 and 7.4 and about 5 times higher at pH 8.5. The coincidence of the loss of the two activities compared here may implicate a common component.

Fig. 2 compares the decay of reactivity with low ferricyanide concentrations and with PMS. It is interesting to note that, although activity in the latter assay is often regarded as quite stable, as compared with reconstitution activity, actually, by the time 80-90% of the latter decays, some 50% of the activity in the PMS assay also disappears. The remainder, however, is moderately stable, although on continued exposure to O_2 all catalytic activity is eventually lost. The kinetics of the loss of activity in the PMS assay, accompanied by parallel increase in the K_m for the dye, suggests that PMS may accept electrons from different sites in the fresh and in the O_2-inactivated forms of the enzyme. It would not be judicious to conclude, however, that the same labile group responsible for reconstitution activity in the fresh enzyme is also required for full activity in the PMS assay, because preparations extracted from Complex II with and without succinate have equal turnover numbers in the PMS assay (10,000 at 38° (6,11)).

The hypothesis that the loss of PMS activity on incubation with O_2 may be viewed as a shift in reaction site of the dye implies that, at least after O_2 inactivation, reoxidation of the enzyme by PMS is rate-limiting. This would require verification by EPR methods, in view of recent experience with Complex II preparations (12). Experiments in which the reduced enzyme in Complex II was rapidly mixed with PMS at 0° showed that both the ferredoxin-type and the Hipip Fe-S centers are completely reoxidized within 6 msec, well within the turnover time of the enzyme, with reoxidation of the ferredoxin-type Fe-S being, if

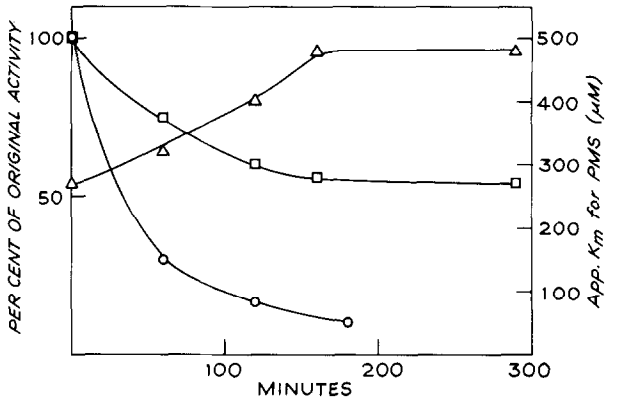

Figure 2. Changes in the activities of reconstitutively active succinate dehydrogenase under aerobic conditions at pH 7.4: (□), PMS reductase activity; (Δ) apparent K_m value for PMS; (O) ferricyanide reductase activity measured at low ferricyanide concentrations. Aerobic decay was induced as in figure 1. Measured activities were obtained by extrapolation to infinite dye concentration at all time points.

anything, faster. Thus, definitive interpretation of the reason for the rapid, partial loss of activity in the PMS assay on exposure of the enzyme to O_2 must await the identification of the rate-limiting step in catalysis in both fresh and O_2-inactivated samples of the soluble enzyme.

The results in Table I and Fig. 1 are compatible with the idea that activity in both the reconstitution test and in reaction with low concentrations of ferricyanide depends on the integrity of the same group, and the Hipip Fe-S center is indeed a logical candidate because of its lability and its presumed position in the intramolecular sequence of electron transport. Evidence for this hypothesis must eventually rest on the demonstration that a catalytically competent Hipip component, as measured by EPR, accompanies reconstitution and "low K_m" ferricyanide activity in a variety of preparations and that the three parameters are lost at the same rate under a variety of conditions. Should such evidence eventually be produced, the ferricyanide assay of Vinogradov et al.(3), would become a simple and

convenient means of ascertaining the degree of intactness of the Hipip center and of reconstitution activity.

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