ON THE EXISTENCE OF DIFFERENT FORMS OF SUCCINIC DEHYDROGENASE IN PURIFIED PREPARATIONS FROM HEART*

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Some 6 years ago no significant differences were known to exist in the properties of succinic dehydrogenase (SD) in different particulate preparations from heart muscle or in the various soluble preparations then available. Although some differences in the behavior of the enzyme between soluble and particulate preparations had been noted by then, the purified enzyme was not known to be modified in essential respects (Singer et al., 1957; Giuditta and Singer, 1959). With the advent of improved methods of assay and of the determination of the SD content of preparations, however, it could be shown that, as judged by low turnover number in the phenazine assay as compared with intact mitochondria and increased reactivity with $FMNH_2$, all soluble preparations from heart and even some respiratory chain preparations are to some extent modified during isolation (Singer et al., 1962a; Singer and Kearney, 1963).

King (1961) has reported that while the purified SD preparation of Wang et al. (1956) (isolated without CN^-) restored the succinoxidase activity of alkali-treated Keilin-Hartree preparations (ATKH), that of Singer et al. (1956) did not, although the two showed identical composition and catalytic properties. On this basis he concluded that the former preparation was more native or physiological than the latter. King (1962) later found, however, that the difference above was not due to different methods of extraction or purification but to the presence of succinate during extraction in the Wang et al. preparation which turned out to be essential for SD preparations active in reconstitution. When included

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in the extraction of the enzyme by the method of Singer et al., it also became active in reconstitution tests. These interesting observations required further exploration, all the more since the reconstitution phenomenon has turned out to be considerably more complex (Kimura et al., (1963)) than the simple replacement of SD postulated to have been lost in the preparation of ATKH.

It has been reported (King, 1961) that on ageing soluble SD the ability to reconstitute succinoxidase on combination with ATKH declined faster than phenazine activity and from this it was concluded that "reconstitutive" activity is a more reliable indication of the native state of a preparation than are catalytic criteria. As shown in Fig. 1, although the ability to restore succinoxidase activity to ATKH is indeed lost more rapidly than catalytic activity in the phenazine assay (at V_{max}), modification of the dehydrogenase is readily detected by a change in the K_M for phenazine methosulfate, long before catalytic activity is lost. An increase in K_M for a dye is usually taken as an indication of protein modification. It is further shown that the ability of SD to combine with ATKH (flavin binding) is lost much more slowly on storage than the capacity to restore oxidase activity.

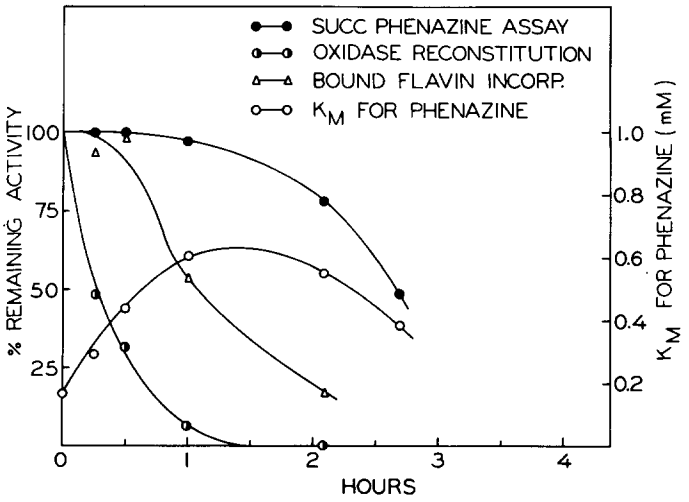

Fig. 1. Differential inactivation of soluble SD. A strictly fresh preparation of SD (Wang et al., calcium phosphate gel eluate, 3.9 mg./ml. (biuret basis), act. = 9.5 µmoles succ./min./mg. in phenazine assay at 38°) was incubated in air at 22° in 75 mM PO_4 - 5 mM succinate, pH 7.8. Aliquots were assayed for dehydrogenase activity (spectrophotometric phenazine assay), for oxidase reconstitution by direct addition of 0.14 mg. SD preparation/mg. ATKH protein directly to Warburg vessels, for flavin peptide (bound flavin) incorporation by adding 0.71 mg. SD preparation/mg. ATKH and reisolation, washing, and fluorometric analysis of the bound flavin content in the resulting complex (Singer et al. (1962b)). Methods and preparations as per Kimura et al. (1963).

While the literature on SD treats any given preparation as if it contained only one species of enzyme, the data in Fig. 1 show that, at least on ageing, molecular heterogeneity develops. Under these conditions the aged preparations contain species of SD which are catalytically active but do not combine with ATKH, those which do combine but do not yield an active oxidase, and those which combine and yield an active electron transport system. That a degree of molecular heterogeneity exists even in strictly fresh preparations of the Wang enzyme has already been shown in the previous paper (Kimura et al., 1963). It was pointed out that at the gel eluate stage only a small and variable fraction (usually 10 to 20%) of the SD molecules present, as determined chemically, is capable of combining with ATKH. Fractionation of unaged Wang et al. preparations by exclusion on Sephadex G-100 or G-200 columns at the eluate stage has now enabled us to separate the enzyme into two fractions (Fig. 2). One (type I) shows very low activity in reconstituting succinoxidase, even if used in excess; the other (type II) is highly active on a molar basis and imparts high oxidase activity to a limiting amount of ATKH (Fig. 3).

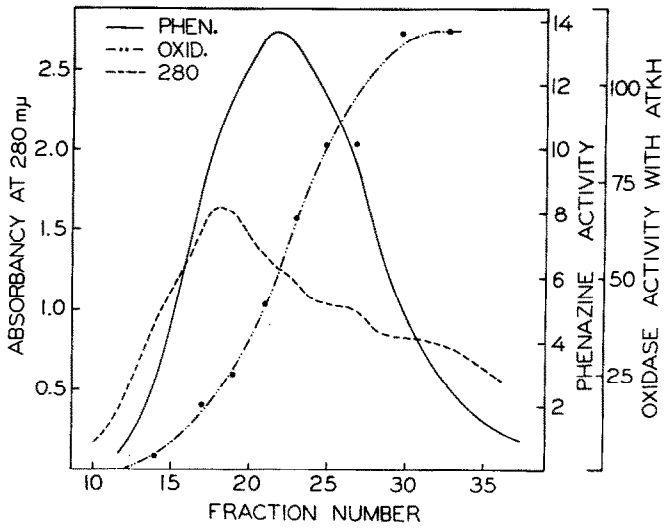

Fig. 2. Chromatography of SD on Sephadex G-100. To a column of Sephadex G-100 (V_o = 48 ml., equilibrated with 15 mM phosphate - 5 mM succinate, pH 7.6) 56 mg. of Wang et al. preparation (gel eluate), containing 326 μmoles/min. phenazine activity were applied at 0°. Fractions of 2.1 ml. were collected with the same buffer. Absorbancy refers to 1 cm. light path/ml. solution, phenazine activity to μmoles succinate/min./ml. as in Fig. 1; oxidase activity is expressed as μl. O_2/min./ml. at 38° resulting from the addition of 2.3 μmoles/min. of phenazine activity per mg. ATKH.

TABLE I. COMPARISON OF FRACTIONS SEPARATED ON SEPHADEX G-100 COLUMNS

Column separation as in Fig. 2. Type I denotes fractions (2 ml.each) 4 through 9 emerging after the holdback volume, sp. act. = 12 μmoles/min./mg. in phenazine assay. Type II is fractions 14 through 20, sp. act. = 11.7. [a] Turnover number is expressed per mole of newly incorporated bound flavin. The turnover numbers of the untreated Keilin Hartree preparations were 13,000 and 6,000 in the phenazine and oxidase assays. Conditions as in Figs. 1 and 2, except that all activities were measured after reisolation of the reconstituted particle.

SD Sample	SD added (mμmoles flavin/mg. ATKH)	Bound flavin incorporated (mμmoles/mg.)	Turnover No.[a] (thousands) Phenaz. assay	Turnover No.[a] (thousands) Oxidase	% Incorporation of bound flavin
Before column Separation	0.22	0.020	11	4	9
	0.44	0.031	12	5	7
Type I	0.15	0.021	5	1	14
	0.30	0.029	7	1	10
Type II	0.24	0.027	12	4	11
	0.48	0.048	13	4	10

As shown in Table I, the % incorporation of SD flavin is not materially different with type I and II fractions, but while the former yiel not only a low turnover number in oxidase assay but also in phenazine assays after reisolation of the reconstituted particle, the latter yield about the same turnover number in both assays as untreated Keilin Hartre samples. It appears that gel exclusion affords partial separation of th SD molecules which on combination with ATKH give high phenazine and oxidase activity from those which bestow low activity on combination. The simplest interpretation is that the type yielding low activity is more modified in extraction than the other.

These findings suggest the existence of at least three forms of SD in the Wang et al. preparation at the gel eluate stage: besides the two just mentioned, there is a third one, catalytically active but incapable of combination with ATKH. The possible presence of still a fourth type is suggested by the following. On closer fractionation on Sephadex columns fractions yielding high phenazine activity but low oxidase activity on combination with ATKH and removal of uncombined SD have been noted. Also, when succinate is omitted during the extraction of SD., the resulting preparations will combine well with ATKH and increase considerably its phenazine activity but fail to yield active succinoxidase.

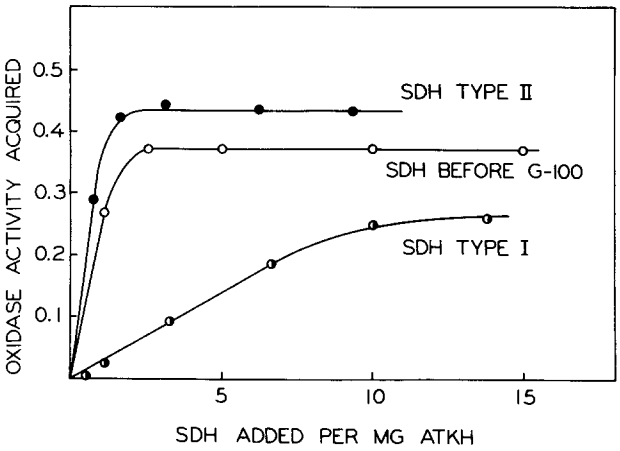

Fig. 3. Reconstitution of succinoxidase with SD fractions separated on Sephadex G-100. The units of SD added/mg. ATKH on the abscissa are expressed as μmoles succinate oxidized/min. in the spectrophotometric phenazine assay. On the ordinate oxidase activity is expressed in μmoles/min./mg. recombined preparation. Conditions as in Figs. 1 and 2. The phenazine activity of the SD preparations used (in μmoles succinate/min./ml.) was: unfractionated calcium phosphate gel eluate: 26.5 μmoles/min./ml.; Type I (Fig. 2, fractions 4 + 5), 8.87 μmoles/min./ml.; Type II (Fig. 2, fractions 14 + 15), 8.21 μmoles/min./ml.

Under all conditions tested there is a competition between these forms for the combining site of ATKH. Pretreatment of ATKH with SD extracted without succinate or with an aged SD preparation, either of which combine with ATKH but do not restore its oxidase activity, prevents subsequent binding of an SD sample which would otherwise yield an active oxidase. That configuration of SD which can restore oxidase activity is preferentially bound, however. This explains why in previous studies (Kimura et al., 1963) titration of ATKH with an unfractionated sample of SD (containing all species referred to) yielded the same turnover number in both phenazine and oxidase assays per mole of bound flavin taken up as given by untreated preparations.

These findings neither negate nor explain our earlier report (Kimura et al., 1963) that under the conditions of the alkaline exposure used in the reconstitution test SD is not significantly dissociated from the respiratory chain and that on combination of soluble SD with ATKH a double-headed respiratory chain is formed. In this respect the same results are obtained whether an unfractionated Wang et al. preparation or type I or type II fractions are used.

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