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On the prosthetic group of succinic dehydrogenase*

The problem of the prosthetic group of succinic dehydrogenase has been the subject of much conjecture, but a definitive experimental approach to its study had to await the isolation of the pure enzyme. Since the suggestion of AXELROD *et al.*[1], based on nutritional experiments that flavin may be the prosthetic group of the enzyme, the presence of a flavin moiety in the dehydrogenase has been widely speculated upon. Other investigators favored the view that the primary dehydrogenase is a hemoprotein, possibly identical with cytochrome B. The availability of essentially homogeneous preparations of the enzyme[2] has opened the way to a critical re-examination of the problem.

The authors have reported that the prosthetic group of the dehydrogenase contains iron but not in the form of hemin[2,3]. The total iron content equals the inorganic iron which is liberated as Fe^{++} by boiling or acidification. Ultracentrifugally homogeneous preparations of the dehydrogenase, prepared from *fresh* mitochondrial acetone powders contain 1 g atom of Fe^{++} per 60,000 to 65,000 g protein; similar preparations isolated from stored acetone powders (several months at -10°) contain 1 g atom Fe^{++} per 110,000 to 130,000 g. From the preliminary value of the molecular weight (about 140,000, based on sedimentation rate and diffusion[3]), it may be concluded that the two types of preparations contain 2 and 1 g atom of Fe^{++} per mole, respectively. Since by physico-chemical criteria the two preparations appear identical and since the specific activity varies with the iron content[2], it seems that both iron atoms are needed for full activity and that one of the two becomes readily labilized and lost on storage or purification. In contrast, the iron in the one-iron type of preparation is so strongly held that neither dialysis against buffer or Fe^{++}-chelators nor passage through cation exchangers liberates it, although it is set free on denaturation. Iron complexors (*o*-phenanthroline, 8-oxyquinoline, thiocyanate, α,α'-bipyridyl, versene, iron-specific versene) do not inhibit the enzyme but the apo-protein of crystalline β_1-globulin from plasma strongly inhibits the dehydrogenase; this can be prevented and partly reversed by ferrous iron. While *o*-phenanthroline does not inhibit the enzyme, it forms a red complex with it and the resulting spectrum is similar to but not identical with that of ferrous *o*-phenanthrolinate. The *o*-phenanthroline compound of succinic dehydrogenase has full activity; it is bleached by succinate in the same way as the free enzyme, and it does not dissociate on dialysis.

The absorption spectrum of succinic dehydrogenase (Fig. 1) is atypical for a flavoprotein: there is no definite maximum in the region of 460 or 375 mμ; there is a gradually increasing absorption below 400 mμ as well as a measurable absorption in the entire visible range. The enzyme is partly bleached by hydrosulfite and the resulting difference spectrum (Fig. 1, insert) exhibits a maximum at 460 mμ. Succinate bleaches the enzyme less completely than hydrosulfite and this reduction is inhibited by malonate. The preparation shown in Fig. 1 is the one-iron type; the two-iron enzyme shows almost 50% more color at and below 450 mμ, whereas above 520 mμ, where the iron moieties of other ferroflavoproteins[4] are thought to absorb light, the two-iron enzymes shows twice as much color.

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Although the difference spectrum (Fig. 1) suggested a long while ago that the dehydrogenase might be a flavoprotein, doubt was cast on this possibility by the observations[5] that neither heat-denaturation nor cold acid liberated more than a minute fraction of the color of the enzyme (assayed as total flavin or as FAD). Green and his colleagues[6] confirmed these findings and further found that digestion of the *particulate* enzyme[7] or of cholate-solubilized derivatives thereof with trypsin set free considerably more flavin (personal communication from Dr. GREEN). In this Laboratory, experiments with highly purified preparations of the soluble enzyme have confirmed that prolonged digestion with proteolytic enzymes greatly increases the yield of flavin-like material in deproteinized filtrates. After incubation with trypsin plus chymotrypsin, followed by boiling, the filtrate showed an absorption spectrum and fluorescence which were qualitatively similar to flavins, but, as judged by the color at 450 mμ, using the extinction coefficient of FAD, somewhat less than 50% of the amount of flavin required for 1 mole per 140,000 g protein could be recovered in the filtrate, although the coagulated protein was colorless after this extraction. On the basis of fluorescence after acid hydrolysis the flavin content of the extract was even lower (1 mole per 600,000 to 1 million g protein), and in terms of coenzyme activity in the D-amino acid oxidase test, the yield of FAD was about 1 mole per 1 million g protein.

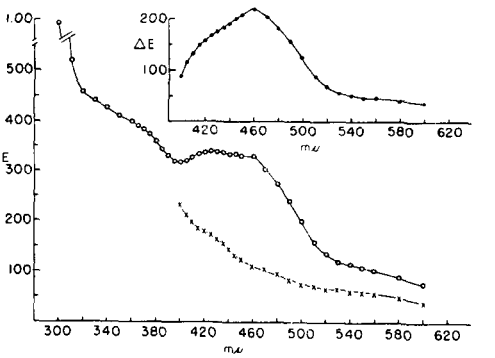

Fig. 1. Absorption spectrum of succinic dehydrogenase. One-iron type preparation at highest purity; 4.55 mg protein per ml in tris(hydroxymethyl)aminomethane buffer at pH 7.8. Open circles, untreated enzyme; crosses, after hydrosulfite; the inset is the difference spectrum after hydrosulfite.

A detailed study of the optimal conditions for liberation of the colored material gave the following results. (1) Maximal color liberation occurs in about 4 hours at 38°, pH 7, with 0.5 mg each of crystalline trypsin and chymotrypsin per mg dehydrogenase. (2) In the course of digestion the color of the enzyme *diminishes* throughout the visible spectrum. (3) During the same period the slight initial fluorescence of the enzyme *increases* over 10-fold and reaches a maximum. (4) On boiling the proteolytic digest there is no further increase in fluorescence but the visible color further decreases and it disappears entirely in the red region of the spectrum, where iron-protein linkages are thought to absorb. (5) Digestion for 4 hours at 100° in 1 *N* H_2SO_4 liberates about the same amount of 450 mμ color as proteolytic enzymes.

The experiments cited lend a clue to the nature of the prosthetic group of succinic dehydrogenase. It appears likely that both iron and some type of flavin are present in the dehydrogenase. The flavin and at least one of the iron atoms are very tightly bound to the apoenzyme. Heat denaturation or cold acids release the iron but not the flavin; proteolysis liberates the flavin but subsequent boiling or acidification are required to liberate all of the iron as inorganic Fe^{++}. The color of the native enzyme appears to be due in part to its flavin content, in part to intact protein-flavin and protein-Fe linkages. Thus neither the color of the native enzyme at 450 mμ nor the color bleached by hydrosulfite are a reliable measure of the flavin content. The mechanism of electron transport by the enzyme may then involve the events: succinate→flavin→Fe→acceptor.

As to the nature of the flavin group, the authors have been unable to obtain satisfactory evidence of its identity with either of the known flavin nucleotides. After chromatographic purification its color, fluorescence, and activity in the D-amino acid oxidase test differ appreciably from authentic FAD. The chemical nature of this compound is under active investigation.

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