RECONSTITUTION OF SUCCINIC-COENZYME Q REDUCTASE AND SUCCINIC OXIDASE ACTIVITIES BY A HIGHLY PURIFIED, REACTIVATED SUCCINIC DEHYDROGENASE[1]

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Succinic-coenzyme Q reductase particles (SQ) have been resolved into a soluble succinic dehydrogenase (SD) and a cyt. b-rich fraction (referred to hereafter as cyt. b)[3]. The SD fraction, purified with ammonium sulfate, contains per mg of protein 6-7 nmoles of acid-nonextractable flavin and about 50 nmoles each of iron and acid-labile sulfide. It catalyzes the oxidation of succinate in the PMS-DCIP system at a rate of 26 - 32 μmoles/min x mg protein ($V_{max}^{PMS} \simeq 37$). The succinate-reduced enzyme exhibits a free radical signal at g=2.0 and a strong iron-sulfide signal at g=1.94 by EPR spectroscopy at 90°K. The enzyme can be stored indefinitely under nitrogen at -20° without loss of dehydrogenase activity. Although considerably more active and purer than all the preparations of succinic dehydrogenase yet reported, SD is unable to reconstitute succinoxidase activity when combined with alkali-inactivated ETP. King (1) has shown such a reconstitution "activity" in preparations of succinic dehydrogenase isolated from mitochondrial particles by alkaline extraction in the presence of succinate. As compared to our preparation of SD, the King dehydrogenase contains per mg of protein only about 3.2 nmoles of flavin, 26 nanoatoms of iron and 24 nmoles of labile sulfide (2, 3). The absence of succinate during alkaline extraction resulted in an enzyme with similar composition, spectral properties and PMS reductase

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[3] Abbreviations: PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenol indophenol; TTFA, 2-thenoyltrifluoroacetone; cyt., cytochrome; Q and QH_2 oxidized and reduced coenzyme Q (ubiquinone); ETP, electron transport particle preparation; SD_R, succinic dehydrogenase reactivated for reconstitution.

activity (2), but with complete loss of reconstitution "activity".

Studies directed in this laboratory toward understanding the difference between the two forms of SD have shown that treatment of the enzyme with ferrous ammonium sulfate, sodium sulfide and mercaptoethanol yields a product (SD_R) capable of reconstituting both succinic-Q reductase and succinoxidase activities when added back respectively to the cyt. b fraction of SQ and to alkali-treated ETP. Thus in the succinate → Q → DCIP reaction where SD alone, SD plus cyt. b, cyt. b alone and SD_R alone had an activity of 0.02 to 0.4, SD_R plus cyt. b showed an activity of about 11.5 (Table I). Addition of TTFA inhibited this reconstituted activity by 80%. Other experiments have shown that preparations of complex III (QH_2-cyt. c reductase) which contain large amounts of cyt. b[4] could also be used, instead of the cyt. b fraction of SQ, for reconstitution of the above activity.

The polarographic traces of Fig. 1 show reconstitution of succinoxidase activity with alkali-treated ETP and SD_R. Thus, alkali-treated ETP showed no succinoxidase activity and addition of 63 μg SD did not elicit oxygen uptake (Fig. 1A). However, addition of 66 μg SD_R (or 33 μg SD_R, but no SD as in Fig. 1B) resulted in rapid oxidation of

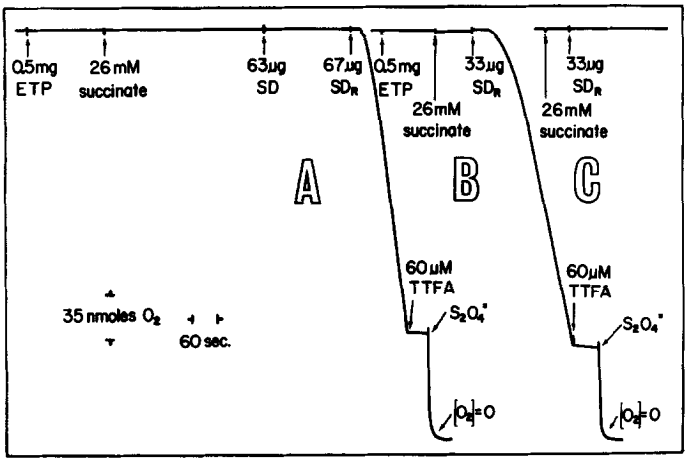

Figure 1. Reconstitution of succinoxidase activity

[4] Preparations of complex III contain about 8 nmoles of cyt. b and 4 nmoles of cyt. c_1 per mg of protein (4).

succinate. This reaction was completely sensitive to TTFA (addition of dithionite after TTFA shows that at the point of TTFA inhibition the system still contained a considerable amount of oxygen). In the absence of ETP, SD_R did not elicit oxygen uptake (Fig. 1C). Under optimal conditions, 96% of the succinoxidase activity of untreated ETP was restored when SD_R was added to alkali-inactivated ETP. Thus in an experiment where succinoxidase activity of ETP before alkali treatment corresponded to 500 nanoatoms oxygen uptake/min x mg protein, the activity of the reconstituted system corresponded to 480 nanoatoms oxygen/min x mg of SD_R plus alkali-treated ETP. In another experiment, premixing of alkali-treated ETP with SD_R before addition to the assay mixture increased the activity by 20% and presence of succinate during premixing by 40%. In both

TABLE I

Reconstitution of Succinate-Coenzyme Q Reductase Activity

Exp.	Additions	Specific Activity per mg SD or SD_R		per mg total protein	
		-TTFA	+TTFA	-TTFA	+TTFA
I	SD	0.02	0.02	0.02	0.02
I	SD_R	0.02	0.02	0.02	0.02
I	cyt. b	----	----	0.07	0.03
I	SD + cyt. b	0.40	0.14	0.09	0.03
II	SD_R + cyt. b	11.5	2.2	4.0	0.8

Exp. I. 6.3 μg SD or SD_R and 20 μg cyt. b per ml of the reaction mixture.
Exp. II. 1.57 μg SD_R and 3 μg cyt. b per ml of the reaction mixture.

The assay mixtures at 38° contained 50 mM potassium phosphate, pH 7.4, 20 mM sodium succinate, 0.1 mM EDTA, 50 μMQ_2 in a final volume of 2% ethanol, 21 μg/ml DCIP and 2 mM TTFA where indicated. The enzyme mixtures were preincubated at 200 times the concentrations given above for 3 min. at 38° in 50 mM potassium phosphate, pH 7.2, plus 20 mM succinate before addition to the reaction media. Rates were calculated from the DCIP mM A_{600}=21. Specific activity is expressed as μmoles succinate oxidized/min x mg of protein as indicated.

the succinic-Q and the succinoxidase reconstitution experiments, gradual

increase of either partner (SD_R with cyt. b; SD_R with alkali-treated ETP) in the presence of a constant amount of the other resulted in a typical saturation curve. When the mixture of soluble SD_R and particulate cyt. b was centrifuged at the speed necessary for sedimenting the particles, SD_R was also sedimented with cyt. b as evidenced from the acquired succinic-Q reductase activity and the flavin content of the cyt. b pellet. During conversion of SD to SD_R, omission of either ferrous ammonium sulfate or sodium sulfide prevented the formation of SD_R. Similar to the preparation of King, the reconstitution "activity" of SD_R also declined rapidly, but addition of dithiothreitol stabilized SD_R considerably. Iron and labile sulfide assays of SD_R showed a five-fold increase in the concentration of each. However, the easy conversion of SD_R to SD precluded rigorous repurification of SD_R, and the presence of colloidal iron-sulfide in SD_R, beyond that amount of iron-sulfide taken up by the enzyme for repairing SD, remains a possibility. That iron, sodium sulfide and mercaptoethanol can form a complex or react with proteins such as serum albumin to form an artificial iron-sulfur protein is known (11).

DISCUSSION

Several important conclusions emerge from these studies:

1. Iron and labile sulfide in succinic dehydrogenase appear to be essential for electronic communication between this enzyme and the respiratory chain. Since the ratio of flavin:iron:sulfide in both the reconstitutively active preparation of King and SD is approximately 1:8:8, it seems that a slight damage to the iron-sulfide system is sufficient to render the enzyme completely inactive for reconstitution. The high PMS-DCIP reductase activity of SD as compared to activity of the King enzyme also indicates that this assay is incapable of detecting such significant defects in isolated succinic dehydrogenase.

2. In classical preparations of purified succinic dehydrogenase, the ratio of iron:sulfide:flavin is 4:4:1, but in SQ this ratio is 8:8:1. Therefore, it was tacitly assumed that, similar to DPNH-Q reductase particles (5, 6), the excess iron-sulfide in SQ is due to the presence of an iron-sulfur protein in addition to the iron-sulfur components of succinic dehydrogenase. The inactivity of the 4-iron dehydrogenases for reconsti-

tution combined with the reconstitutive ability of the 8-iron extract of King further strengthened this possibility. Our demonstration that the 8:1 iron-flavin ratio of SQ is recovered in a highly purified succinic dehydrogenase, which is easily converted to a reconstitutively active form without addition of a separate protein, strongly favors the absence of a separate iron-sulfur protein in succinic-Q reductase[5].

3. In experiments not reported here we have shown that, contrary to the report of Ziegler and Doeg (7), the cyt. b of SQ is slowly reduced by succinate and that this reaction is inhibited by TTFA. Other studies have suggested that, as suspected earlier (8), the presence of cyt. b in SQ preparations might be a consequence of cleavage of complex III by the conditions used (cholate, heat, ammonium sulfate) by Ziegler and Doeg (7) for isolation of succinic-Q reductase (see ref. 9). The fact that SD_R and complex III can reconstitute succinic-Q reductase activity is in agreement with this possibility. Nevertheless, the requirement for both succinic dehydrogenase and cyt. b as an operational unit in catalyzing electron transfer from succinic dehydrogenase to coenzyme Q is supported by the above studies as well as by those of Bruni and Racker (10).

4. The requirement of iron-sulfide for electron transfer from succinic dehydrogenase to the respiratory chain, the likely absence of a separate iron-sulfur protein in SQ, and the TTFA inhibition of cyt. b reduction in succinic-Q reductase suggest that the site of TTFA inhibition is between succinic dehydrogenase and Q-cyt. b.

EXPERIMENTAL

SQ was prepared by a modification of the procedure of Ziegler and Doeg (7). SD was extracted from SQ with 50 mM Tris-Cl, pH 8.0, and purified by ammonium sulfate fractionation. Fractions precipitating at 41.2% and 43.6% saturation were removed and the fraction precipitating at 54.6% saturation was collected. This fraction is referred to as SD. SD_R was prepared from SD at pH $\simeq$ 7.8 under conditions that iron and sulfide are taken up by serum albumin to form an iron-sulfur protein (11). The protein was then passed through Sephadex G-25 before using. Alkali-

[5] This argument assumes after other workers in the field that a succinic dehydrogenase of molecular weight $\sim$150,000 is a single protein and not a binary complex of a flavoprotein and an iron-sulfur protein.

inactivated (pH 9.4) ETP was prepared according to King (1) and the cyt. b fraction of SQ was obtained by various procedures including by removal of SD at alkaline pH. Succinic dehydrogenase assay at 38° was basically according to Arrigoni and Singer (12) and succinoxidase at 30° according to King (1). Complex III was prepared according to Hatefi et al (4).

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