ACTIVATION OF SUCCINATE DEHYDROGENASE BY ELECTRON FLUX FROM NADH AND ITS POSSIBLE REGULATORY FUNCTION

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SUMMARY. It is well known that succinate dehydrogenase (SD) is transformed from an inactive to an active state on combination with substrates or competitive inhibitors and that SD becomes deactivated on removing the activator. Activation has a high temperature coefficient and is thought to be due to a conformational change in the enzyme. In the present paper it is shown that in well washed membrane preparations, in which SD occurs largely in the deactivated form, NADH induces activation of SD. The activation progresses as long as NADH is present; once NADH is removed by oxidation, SD reverts rapidly to the deactivated form. NADH-induced activation has the same temperature coefficient as that produced by succinate and the kinetic properties of SD activated by either agent are the same. Activation is not due to a combination of NADH with SD, since rhein and piericidin, inhibitors of NADH oxidation, interfere with the activation of SD by NADH. Extraction of CoQ_{10} with pentane prevents the activation by NADH nearly completely, while readdition of CoQ_{10} restores activability by NADH. It is suggested that the agent responsible for this type of activation is reduced CoQ and that NADH serves only to maintain CoQ_{10} in the reduced state. Since thenoyltrifluoroacetone prevents succinate oxidation but not activation of SD by NADH, it appears that $CoQH_2$ can interact with and induce changes in SD in the absence of electron transport. The possibility that this type of rapid activation-deactivation may be of regulatory significance in the Krebs cycle is discussed.

INTRODUCTION

Succinate dehydrogenase (SD) from mammalian and other aerobic cells has a unique property: on combination with substrate or any competitive inhibitor it is converted to an activated state and it remains in this form until the substrate or inhibitor is removed, whereupon it reverts to an inactive (or slightly active) form (1,2). The activation is characterized by a high energy of activation (36 Kcal/mole) and appears to be a reversible conformation change in the enzyme. In fact, this was one of the earliest instances of the possibility of enzyme regulation by conformation change (1). The SD or succinate oxidase activity of a given preparation, whether whole cells, membranes, or

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soluble purified preparations, depends on the manipulations during isolation (washing by centrifugation, gel exclusion or dialysis), which determine the extent to which bound substrate has been removed, unless provisions are made to activate the enzyme prior to assay (2,3).

The importance of the activation-deactivation process has been emphasized (3) and the possibility pointed out that this may be a control mechanism in the Krebs cycle (4). Until now, however, the mechanism of this type of control has been centered around the prevailing concentrations of succinate, fumarate, and oxalacetate. In the present paper another, possibly much more important mechanism for regulating SD activity is pointed out, one which involves the prevailing concentrations of the substrates of other respiratory chain-linked dehydrogenases (such as NADH) and, therefore, the redox state of coenzyme Q in the respiratory chain. The fundamental observation reported in this note is that not only substrates and inhibitors of SD itself but also NADH can activate SD in membrane preparations, that upon exhaustion of NADH, SD is rapidly deactivated, and that these changes in SD activity are sufficiently rapid at physiological temperatures to be of considerable importance in regulating SD activity and, therefore, the rate of ATP synthesis.

MATERIALS AND METHODS

ETP and ETP_H were prepared as in previous work (5). Prior to this the particles were washed with 0.18 M sucrose-50 mM Tris-acetate-5mM $MgSO_4$, pH 7.4 (STM buffer) and resuspended in the same buffer. Activation of SD was carried out at 0.33 or 1 mg/ml protein concentration in STM buffer in the presence of 1 mμmole of antimycin A/mg of protein or 1 mM KCN. Activation was started by adding 0.25 M NADH and samples were removed periodically for assay of SD activity. SD was assayed spectrophotometrically as in previous work (2) except that a fixed (0.33 mg/ml) phenazine methosulfate concentration was used. The assay mixture contained, in addition, 40 mM P_i buffer, pH 7.4, 20 mM succinate, 1 mM KCN, and 0.0016 mg/ml of 2,6 dichlorophenolindophenol. The assay temperature was varied, as indicated in the paper. Activation during assay was avoided by measurement of initial velocity. The fumarate-NADH reaction was measured at 340 mμ in ETP_H preparations at 30° in the presence of 0.25 M NADH, STM buffer, 1.7 mμmoles of antimycin A/mg of protein and 20 mM fumarate to start the reaction. Suitable correction was made for NADH oxidation via the complete respiratory chain due to incomplete antimycin inhibition.

RESULTS

Fig. 1 illustrates the basic experiment on which the findings of this paper are based. In a standard ETP_H preparation (6) SD is about 80 to 90% in the deactivated state. If NADH is added in the aerobic state and its rate of oxidation is slowed to a fraction of the uninhibited rate with a suitable inhibitor (in the present experiment antimycin to yield > 98% inhibition) so as to permit the maintenance of NADH for a sufficiently long period to prevent activation of SD at the low temperature (22 to 24°) prevailing, SD becomes

gradually activated. When NADH is exhausted by slow aerobic oxidation, further activation of SD stops and thereafter SD activity declines to the initial, largely deactivated state. A second addition of NADH once again initiates the conversion of deactivated to activated SD (Fig. 1).

If the oxidation of NADH is completely prevented by cyanide, activation of SD proceeds to a maximum and the enzyme remains in the activated state for the entire period of observation. The extent of activation, estimated by SD activity at V_{max} with respect to the electron acceptor, and K_m value for phenazine methosulfate characteristic of the activated form of SD are the same whether NADH or succinate is used to initiate the activation.

In tracing the components involved in the NADH-induced activation of SD, it was first ascertained that NADH itself cannot be the direct activating agent, acting by direct combination with SD, since rhein, an efficient competitive inhibitor of the initial reaction between NADH and NADH dehydrogenase (7), prevented the activation almost completely (Fig. 2). Piericidin A (630 μμmoles/mg of protein) also inhibits the activation by NADH, although not as much as rhein, as may be seen from comparison of Fig. 2 with Fig. 1, which serves as the uninhibited control sample. It seems therefore that both the

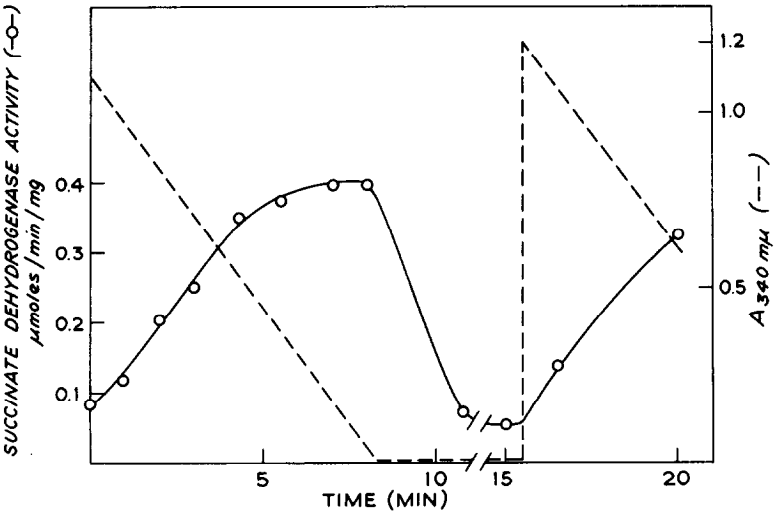

Fig. 1. Activation of SD by NADH. An ETP_H preparation (succinoxidase activity = 1.18 μmoles succinate/min/mg at 30°) was washed by centrifugation in STM buffer and resuspended in the same buffer to 1 mg of protein/ml. Antimycin A (1 mμmole/mg of protein) was added to slow the rate of aerobic NADH oxidation, followed by 0.25 mM NADH. Oxidation of the latter at 23° was monitored spectrophotometrically (dashed line). Samples were removed periodically and assayed immediately for succinate dehydrogenase activity in the presence of 0.33 mg phenazine methosulfate/ml (solid line). At 16 min a second aliquot of 0.25 mM NADH was added.

reduction of NADH dehydrogenase by its substrate and its reoxidation by the CoQ pool are necessary for activation of SD by NADH. On the other hand, 0.23 M thenoyltrifluoroacetone (TTF), which inhibits succinoxidase activity or the reduction of fumarate by NADH in the presence of antimycin over 99%, does not block the activation to any great extent (cf. open circles in Fig. 2 with control in Fig. 1 or triangles and solid circles in Fig. 2). Hence normal electron flux between SD and the CoQ pool does not seem essential for the activation.

Notwithstanding the results with TTF, the simplest visualization of the activation by NADH is that NADH, by way of its dehydrogenase, reduces CoQ and that reduced CoQ is the agent responsible for the activation. This is in line with the usual concept of the CoQ pool as the point of junction of the two dehydrogenases. It was decided, therefore, to examine the role of CoQ in the NADH-induced activation of SD.

Table I presents two types of evidence for the role of $CoQH_2$ as a mediator of the activation. In exp. 1 it is shown that nearly the same activation is reached with 175 µM

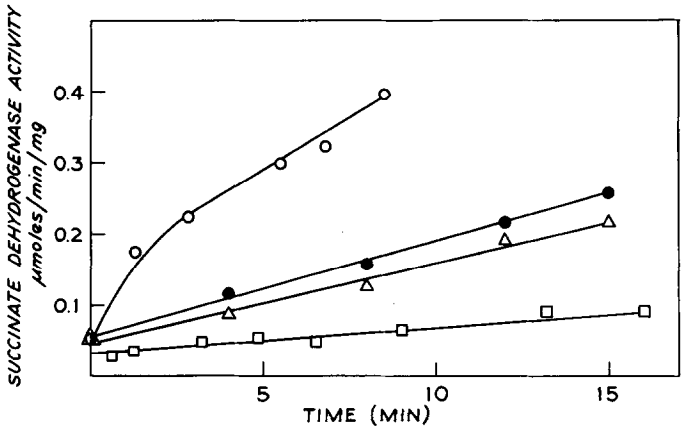

Fig. 2. Effect of various inhibitors on the NADH-induced activation of SD. Experimental conditions were as in Fig. 1. Open circles, 1 mµmole of antimycin/mg plus 0.233 µM TTF; solid circles, 630 µµmoles of piericidin A/mg of protein preincubated with the particles for 30 min at 0° prior to adding NADH; triangles, piericidin as above plus 0.233 mM TTF; squares, the ETP_H was diluted to 0.33 mg of protein/ml and 0.66 mM rhein was added before NADH.

$CoQ_{10}H_2$ (under N_2) as with succinate or NADH. Oxidized CoQ_{10} does not activate SD. Exp. 2 shows that after pentane extraction of the endogenous CoQ_{10}, activation of SD by NADH is very slight but on restoration of the CoQ_{10} by the technique of Szarkowska (8) activability by NADH returns. The restoration of activability by NADH is not quite complete, but neither is reconstitution of NADH oxidase activity under these con-

Table I. Evidence for the Role of CoQ_{10} in the NADH-Induced Activation

Exp.	Particles	Activator	% of maximal activity reached at 0	1 min	2 min	4 min	8 min	20 min
1	ETP_H	NADH	20	50	54	63	78	100
	"	$CoQ_{10}H_2$	10	25	36	52	75	93
	"	CoQ_{10}	15			15		15
2	Lyophilized ETP_H	NADH	35	49	59	68	73	73
	Same after pentane extr.	"	33	36	40	46	52	54
	Same + 175 μM CoQ_{10}	"	29	37	46	59	68	69

ETP_H particles as in Fig. 1, suspended in 0.18 M STM buffer with 1 mμmole of antimycin A plus 1 mM KCN present, were activated under N_2 with the agents shown at 30°. Aliquots removed at the times indicated were rapidly chilled and assayed for SD activity at 17° (where no activation occurs during assay) at fixed phenazine methosulfate concentration. NADH was 0.25 mM; CoQ_{10} or $CoQ_{10}H_2$ 175 μM. In exp. 2 the lyophilized particles were extracted 5 times with pentane (9), resulting in the lowering of the chemically measurable CoQ_{10} content below the limit of detection and of the NADH oxidase activity by 99%. Restoration of CoQ content was with 175 μM CoQ_{10} by Szarkowska's method. Results are expressed as % of the maximal SD activity obtained on activation with 20 mM succinate at 30° in 15 min.

ditions (9). It should be noted that extraction or readdition of CoQ_{10} have no effect whatsoever on activation by succinate. The data are expressed as % of maximal activity reached on activation with 20 mM succinate. In normal ETP_H activation by succinate or NADH yield the same specific activity but in lyophilized ETP_H activation by NADH is less extensive (exp. 2), perhaps because lyophilization damages part of the NADH dehydrogenase present (9).

Fig. 3 is an Arrhenius plot of the activation by NADH. The energy of activation for the control sample (33 Kcal/mole) is close to that previously reported (1) for activation by substrates, showing again the probable identity of the activated form, regardless of the agent used for activation. It may also be seen that the rate of activation is much slower in CoQ-depleted samples but is restored on adding back CoQ_{10}.

Activation of SD not only increases phenazine methosulfate activity but also overall succinoxidase and succinate-cytochrome reductase activities (2). Fig. 4 illustrates the influence of the state of activation of SD on electron flux in ETP_H. Aliquots of an ETP_H

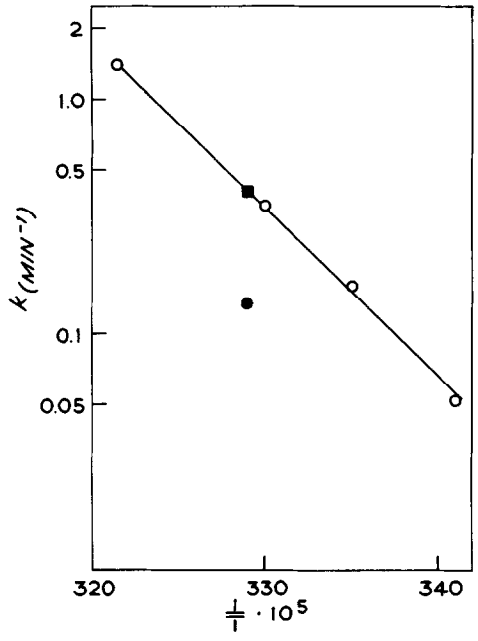

Fig. 3. Arrhenius plot for the NADH-induced activation of SD. Experimental conditions were as in Fig. 1 except that both antimycin A and 1 mM KCN were present and that the experiment was repeated at several temperatures. Open circles, normal ETP_H; shaded circle, CoQ-depleted particles, prepared by 5 pentane extractions; shaded square, depleted particles plus 150 µM CoQ_{10} added in alcoholic solution (8) to the activation medium.

preparation were activated with malonate for various times, cooled to 0° to stop the activation, and after adding antimycin A (1 mµmole/mg) and cyanide (1 mM) assayed for the rate of reduction of cyt. b by succinate (ordinate) and for succinate dehydrogenase activity with phenazine methosulfate (abscissa). Over the range where the extent of activation was low, so that SD activity limited the rate of the succinate-cyt. b interaction, a linear relation was found. When over 50% of the enzyme was in the activated form, further activation increased the rate of cyt. b reduction only slightly. Extrapolation of the linear part of the curve further suggests that the unactivated (or deactivated) form of SD is completely inactive in catalyzing the reduction of endogenous cyt. b by succinate.

DISCUSSION

The results presented suggest that SD may be activated not only by agents combining at the substrate site but also by interactions on the O_2-side of the enzyme. That NADH itself is not the immediate activator but serves only to reduce, by way of its dehydrogenase, the component which acts as the direct activator is clear from the inhibitions observed with rhein and piericidin A. The experiments of Table I and Fig. 3 suggest that endo-

genous $CoQ_{10}H_2$ might be the actual activator. The observation that although the CoQ pool is depleted about 99% as judged by chemical analysis or by the loss of NADH oxidase activity after pentane extractions but activation by NADH is still measurable, may be

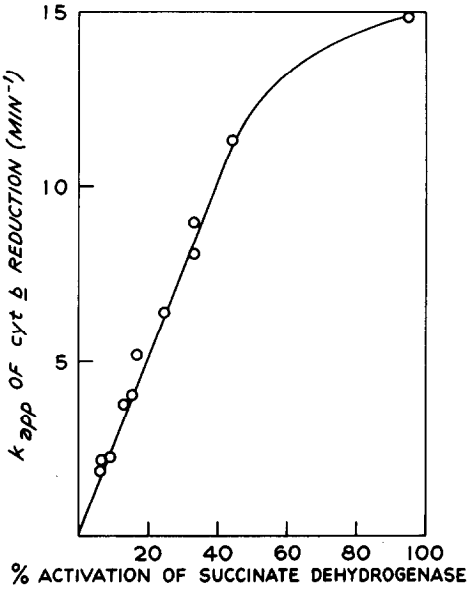

Fig. 4. Relation of the extent of activation of SD to the rate of the succinate-cytochrome b reaction in ETP_H. The particles were washed as in the experiment of Fig. 1, resuspended to 2 mg/ml, and activated for varying periods with 0.33 mM malonate at 24° to obtain different degrees of activation of SD. Samples were periodically removed, rapidly cooled to 0° to halt the activation, antimycin A (1 mμmole/mg) and KCN (1 mM) were added to prevent oxidase activity, and SD activity was determined as in Fig. 1. The results are plotted on the abscissa as % of the maximal activity reached. The ordinate represents the rate constant for the reduction of cytochrome b by 20 mM succinate at 11°, measured at 563-575 mμ.

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explained by the fact that the CoQ_{10} pool in inner membranes is functionally compartmentalized (9), so that only a fraction may participate in the activation. Further, the effective concentrations of $CoQH_2$ required for activation of SD may be much lower than have been required for succinate oxidation, just as the dissociation constants for succinate in activation is much lower than the K_m for succinate dehydrogenase activity (1).

The fact that TTF inhibits electron transport from SD completely but not the activation of SD by NADH has an important implication: if reduced CoQ_{10} is indeed the immediate activator, the combination of TTF with the nonheme irons of SD blocks redox reactions with CoQ but not the combination of $CoQ_{10}H_2$ with the enzyme. It is possible, of course, that the combining site of $CoQ_{10}H_2$ on the enzyme is not the same when acting as an activator and when acting as an electron donor. It is interesting to note that Rossi et al (10)

have recently reported that the removal of CoQ_{10} from membrane preparations changes the kinetic properties of SD.

Lastly, it should be emphasized that both the activation of SD by NADH and its deactivation on oxidation of NADH are rapid at 37°, so that a several-fold change in SD activity might occur in less than 1 min. This property makes SD a likely candidate as a regulatory enzyme in the Krebs cycle and, hence, in the overall rate of ATP synthesis.

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