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Regulation of Succinate Dehydrogenase Activity by Reduced Coenzyme Q_{10}*

M. Gutman,† Edna B. Kearney, and Thomas P. Singer‡

ABSTRACT: It is known that, upon combination with substrates or substrate competitors, succinate dehydrogenase is converted from an inactive to active form and that on removal of the activator the enzyme reverts to an inactive form. The activation is characterized by a high energy of activation (36 kcal/mole).

It has been found that during the oxidation of NADH by inner membrane preparations a similar activation of succinate dehydrogenase occurs. The maximal extent of activation reached, the energy of activation of the process, and the kinetic properties of the activated form of the enzyme are the same as when activation is induced by the substrates or substrate analogs. When NADH is exhausted succinate dehydrogenase is rapidly deactivated. The activation–deactivation processes are sufficiently rapid at 37° to be of significance in metabolic regulation. Extraction of endogenous coenzyme Q (CoQ) from the membrane results in loss of activation by NADH but on reconstitution of the particles with respect to CoQ, activation by NADH is restored. These observations and studies with inhibitors indicate that NADH itself is not the activating agent but merely serves to reduce endogenous CoQ and that $CoQH_2$ is the activating agent. It is suggested that $CoQH_2$ is a positive modifier of succinate dehydrogenase. The site at which $CoQH_2$ acts may not be the same as the one involved in electron transport from succinate dehydrogenase to CoQ since thenoyltrifluoracetone abolishes electron flux from the dehydrogenase to CoQ without affecting activation by $CoQH_2$. The role of the activation–deactivation processes in electron flux between the dehydrogenase and the respiratory chain and in the control of the Krebs cycle are discussed.

It has been known since 1955 that succinate dehydrogenase is activated by substrates and substrate analogs which act as competitive inhibitors (Kearney *et al.*, 1955; Kearney, 1957). Thus succinate dehydrogenase was one of the first examples of homotropic regulation discovered, since the succinate serves not only as a substrate but as a positive modifier of the enzyme. The activation has been observed in intact mitochondria, membrane preparations, and the soluble, purified enzyme from a variety of mammalian sources and aerobic yeast cells (Kearney, 1957; Thorn, 1962; Singer *et al.*, 1966) and was found to be reversible upon removal of the activator (Kimura *et al.*, 1967). Two characteristics of the activation process are noteworthy; the high energy of activation (36 kcal/mole), suggestive of a protein modification (Kearney, 1957), and the fact that activation affects only certain activities of the enzyme

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(the reduction of PMS,[1] ferricyanide, and of the respiratory chain by succinate, which are thought to involve nonheme iron), but does not alter the fumarate reductase activity (which is thought to involve only the FAD moiety of the enzyme) (Kearney, 1957; Kimura *et al.*, 1967).

It has been recently observed that activation of succinate dehydrogenase occurs in inner membrane preparations during NADH oxidation in the absence of added succinate or substrate analogs (Gutman *et al.*, 1971b). The activation is reversible and in this and all other respects studied appears to be identical with that induced by succinate. NADH itself is not an activator but acts by reducing endogenous CoQ_{10} which appears to be the direct activating agent. Since $CoQ_{10}H_2$ does not combine at the substrate site, its apparent modulating effect on succinate dehydrogenase may be an example of a second site or allosteric regulation. Thus succinate dehydrogenase appears to be regulated by at least two types of effectors. Another type of regulatory effect of CoQ on succinate dehydrogenase has been reported by Rossi *et al.* (1970). This phenomenon is different from the one reported here, as it does not deal with changes in activity in response to the redox state of CoQ nor with activation of the enzyme.

Materials and Methods

ETP_H, CoQ_{10}, rhein, and TTF were prepared or obtained as in previous work (Gutman *et al.*, 1971a; Kean *et al.*, 1971). Succinate dehydrogenase activity was measured by the PMS–DCIP assay (Arrigoni and Singer, 1962), but at a fixed (0.1 mM) PMS concentration except where otherwise noted, at the temperature specified in the text. During activation by NADH or $CoQ_{10}H_2$ samples of ETP or of ETP_H at a protein concentration of 1 mg/ml in 0.18 M sucrose–50 mM Tris-acetate–5 mM $MgSO_4$ buffer (pH 7.4) (STM buffer) were incubated with 250 μM NADH or the specified concentration of $CoQ_{10}H_2$. Samples were withdrawn at intervals, and assayed immediately for succinate dehydrogenase activity. Special care was taken in each experiment to minimize activation during assay either by carrying out assays at or below 20° (where activation is quite slow) or by a combination of close timing and rapid initial rate measurements (recorder speed of 12 or 24 in. per min). Control assays without succinate showed that only negligible amounts of reducing equivalents were carried over from the activation mixture, and that these were instantaneously oxidized by PMS. Thus, no correction for this was needed.

Endogenous CoQ_{10} was removed by pentane extraction and added back to the depleted particles by the technique of Szarkowska (1966), as in previous work (Gutman *et al.*, 1971a). Cytochrome *b* reduction was monitored at 563 − 575 mμ with an Aminco-Chance dual-wavelength spectrophotometer. The reduction of NAD^+ by succinate was measured as per Ernster and Lee (1967).

Results

Activation of Succinate Dehydrogenase during NADH Oxidation. The reversible activation of succinate dehydrogenase by NADH is shown in Figure 1. The activity showed a five-

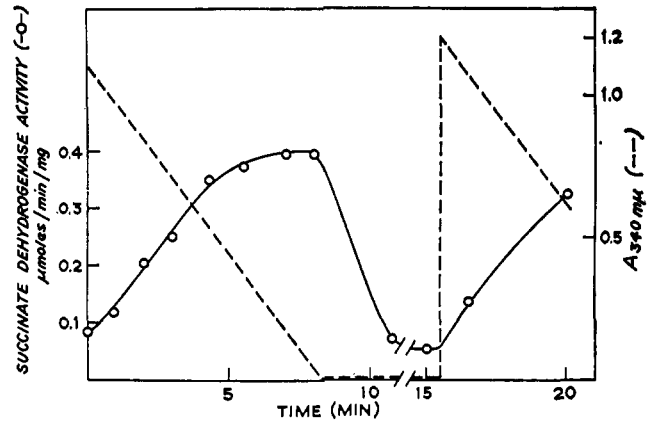


FIGURE 1: Activation of succinate dehydrogenase by NADH. An ETP_H preparation (succinoxidase activity = 1.18 μmoles of succinate/min per mg at 30°) was washed by centrifugation in STM buffer (pH 7.4) and resuspended in the same buffer to 1 mg of protein/ml. Antimycin A (1 mμmole/mg of protein) was added to slow the rate of aerobic oxidation of NADH, followed by 0.25 mM NADH. Oxidation of the latter at 23° was monitored spectrophotometrically at 340 mμ (dashed line). Samples were removed periodically and assayed immediately for succinate dehydrogenase activity in the presence of 0.33 mg of PMS/ml (solid line). At 16 min a second aliquot of 0.25 mM NADH was added.

fold increase by the time most of the added NADH was exhausted (dotted line), after which spontaneous deactivation occurred. If a second aliquot of NADH was added at this time, activation of succinate dehydrogenase was once again observed.

Under the conditions of this experiment activation of succinate dehydrogenase was not maximal since NADH was slowly oxidized despite the presence of antimycin A and was exhausted before activation reached completion. Inclusion of 1 mM KCN along with antimycin A, however, permitted maintenance of NADH for a sufficient period to allow maximal activation. Under these conditions the same degree of activation was reached with succinate or NADH in the majority of experiments. In typical samples the specific activity reached was 1.5 μmoles of succinate oxidized per min per mg of ETP_H protein at 30° in the PMS–DCIP assay at V_{max} and the apparent K_m for PMS was 0.51 mM, regardless of the activator used. In occasional experiments activation induced by NADH was somewhat less (80–95% of the value reached on activation with succinate).

Effect of Inhibitors on Activation Initiated by NADH. The first evidence that NADH itself is not the activating agent came from the demonstration that rhein prevents activation initiated by NADH. This inhibitor is known to interrupt NADH oxidation by inhibiting competitively the reaction of NADH with the FMN moiety of NADH dehydrogenase (Kean *et al.*, 1970, 1971). Rhein was added at a high enough concentration (0.66 mM) to ensure that NADH would not be exhausted within the time interval needed for full activation; under these conditions NADH oxidase was inhibited 95%. During the experimental period the activity of succinate dehydrogenase increased only from 0.05 to 0.095 μmole per min per mg despite the continuous presence of significant concentrations of NADH (Figure 2). The control curve is that shown in Figure 1. This very low degree of activation is not due to inactivation of succinate dehydrogenase by the high rhein concentration since the rhein-treated particles have full activity when activated by succinate. The fact that

[1] Abbreviations used are: CoQ_{10}, coenzyme Q_{10}; DCIP, 2,6-dichlorophenolindophenol; FAD, flavine-adenine dinucleotide; ETP_H, phosphorylating preparation of inner mitochondrial membrane; PMS, phenazine methosulfate; STM buffer, sucrose–Tris–Mg buffer; TTF, thenoyltrifluoroacetone.

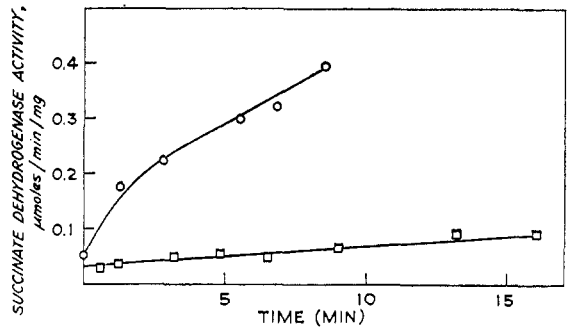


FIGURE 2: Effect of rhein and of thenoyltrifluoroacetone on the NADH-induced activation. Experimental conditions were as in Figure 1, which serves as the control. Open circles, 1 mμmole of antimycin A/mg plus 0.233 mM thenoyltrifluoroacetone. Squares, same as above but the ETP_H was diluted to 0.33 mg of protein/ml and 0.66 mM rhein was added without antimycin before the NADH.

rhein inhibits activation by NADH shows that NADH itself is not the activating agent but serves only to reduce some component on the substrate side of the antimycin block, since antimycin A does not interfere with the activation (Figure 1). These limitations suggest that either the reduced form of NADH dehydrogenase, reduced CoQ_{10}, or of cytochrome *b* is the actual activator.

Thenoyltrifluoroacetone, at a concentration sufficient to inhibit electron flux between succinate dehydrogenase and the respiratory chain completely in both directions (0.233 mM)

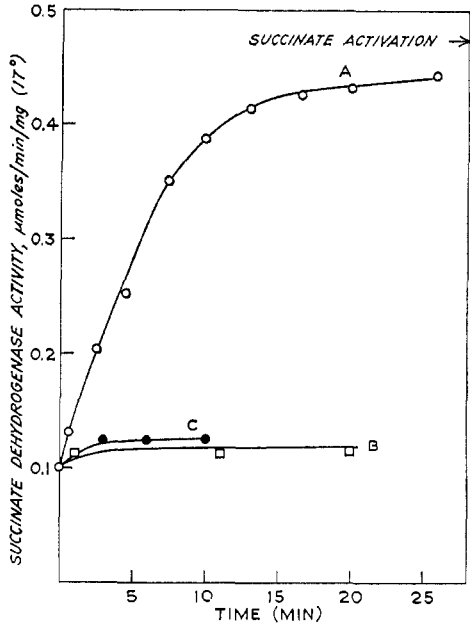


FIGURE 3: Activation of succinate dehydrogenase by reduced CoQ_{10}. ETP_H, washed with 0.25 M STM buffer (pH 7.4), was resuspended in 0.18 M STM buffer (pH 7.4) at 4 mg of protein/ml. Antimycin A (1 mμmole/mg) and cyanide (1 mM) were added and the sample placed under an atmosphere of N_2 to prevent autooxidation of $CoQ_{10}H_2$. CoQ_{10} was reduced with borohydride, neutralized with dilute acetic acid, and shaken till the first appearance of the yellow color of oxidized CoQ_{10} to ensure removal of unreacted borohydride, all at 0°. Activation of succinate dehydrogenase was started by adding 50 μl of either $CoQ_{10}H_2$ (curve A) or of CoQ_{10} (curve B) in absolute ethanol to 3 ml of enzyme, giving 175 mM final concentration of the quinone. Curve C, no addition. Samples were withdrawn at intervals and assayed immediately at 17°. The horizontal arrow indicates the maximal activation reached with succinate as activator.

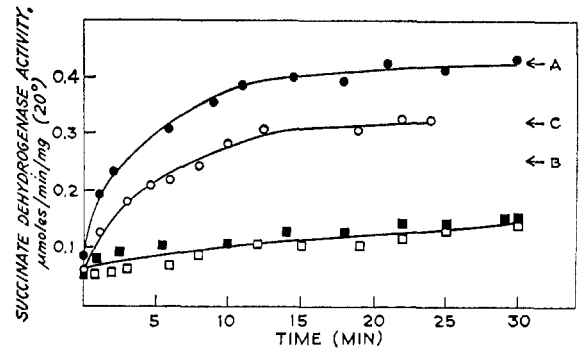


FIGURE 4: Effect of pentane extraction and of reincorporation of CoQ_{10} on activation of succinate dehydrogenase by NADH. ETP_H particles were washed, as described in Methods, lyophilized, and five-times extracted with cold anhydrous pentane as in Szarkowska's (1966) procedure. Pentane was removed in high vacuum. Samples were then activated at 30° with succinate or NADH, in the presence of 1 mM KCN and 1 mμmole of antimycin A. Solid circles, lyophilized ETP_H activated by NADH. The arrow at A represents maximal activation of the same sample by succinate. Solid squares, and open squares: activation of pentane-extracted particles by NADH in the presence and absence of cyanide and antimycin A, respectively. The arrow at B denotes maximal activation of these samples by succinate. Open circles: pentane-extracted ETP_H following readdition of 50 μM CoQ_{10} in ethanol activated by NADH. The arrow at C denotes maximal activation of this sample by succinate. Activity was determined at 20°.

is without effect on activation by NADH (*cf.* Figure 2 with the control curve in Figure 1).

Role of CoQ_{10} in the Activation. The following lines of evidence suggest that $CoQ_{10}H_2$, rather than NADH dehydrogenase or cytochrome *b*, is the component directly involved in the activation of succinate dehydrogenase initiated by NADH. Figure 3 demonstrates that under anaerobic conditions nearly the same maximal activation is reached with 175 μM added $CoQ_{10}H_2$ as with succinate, while oxidized CoQ_{10} does not activate the enzyme.

While this experiment shows that exogenous $CoQ_{10}H_2$ is capable of bringing about activation of the enzyme, it is quite possible that it does so by reducing endogenous CoQ_{10}. The effect of depletion and of reincorporation of endogenous CoQ_{10} was therefore investigated. The experiments illustrated in Figure 4 compare activation by NADH in lyophilized, unextracted ETP_H with the same preparation after removal of the CoQ_{10} by pentane extraction and after replenishing the CoQ_{10} content by the method of Szarkowska (1966). In these experiments activation by succinate is taken as the maximal activity which can be reached, noted in Figure 4 as A, B, and C. In the unextracted particles NADH and succinate give the same activation. After depletion of CoQ_{10} NADH cannot activate the enzyme significantly, whereas succinate still activates. After replenishing the CoQ_{10} content NADH can again activate as much as succinate. The levels of activity observed in the enzyme fully activated by succinate in normal and depleted particles concur with the observation of Rossi *et al.* (1970), who interpreted their findings in terms of the suggestion (Giuditta and Singer, 1959) that membrane-bound succinate dehydrogenase has two reaction sites for PMS, one of which is thought to require CoQ_{10} for operation (Rossi *et al.*, 1970). The difference in activation reached in unextracted (level A) and extracted, reincorporated (level C) samples may be in part due to incomplete reincorporation of CoQ_{10}, in part to a slight inactivation of the enzyme during pentane extraction.

These experiments strongly suggest that activation by

NADH requires the presence of CoQ_{10} and that, in fact, it is the reduced form of CoQ_{10} which is involved in the activation under these conditions.

Effect of Piericidin on the Activation. The probability that $CoQ_{10}H_2$ is the direct activator opens the way toward the study of the effects of piericidin on the NADH-induced activation, since this inhibitor is known to block both the reduction of the CoQ_{10} pool by NADH and interferes with redox reactions within the pool (Singer and Gutman, 1971). In analyzing the complex effects of piericidin A on the NADH-induced activation of succinate dehydrogenase, it is useful to summarize certain characteristics of the reaction of this inhibitor with the respiratory chain. There are two binding sites for piericidin A per NADH dehydrogenase molecule (Gutman *et al.*, 1970), which are responsible for most of the inhibition exerted by piericidin on the NADH oxidase system. These have been called specific binding sites and the noncovalent binding at these loci is so strong that bovine serum albumin does not dissociate the inhibitor from these sites. In addition there are numerous unspecific binding sites for piericidin present in inner membrane preparations. Binding at these sites is weaker, so that serum albumin releases the inhibitor from these loci, with resulting reversal of any inhibition which is due to unspecific binding (Gutman *et al.*, 1970). While the inhibition of NADH oxidase due to specifically bound piericidin is restricted to the region between the nonheme irons of NADH dehydrogenase and the CoQ_{10} pool, unspecifically bound piericidin may inhibit at several points in the respiratory chain, including the compartmentalized CoQ_{10} pool (Gutman *et al.*, 1970, 1971a), wherein it interferes with equilibration of the CoQ_{10} molecules. Piericidin has no effect on succinate oxidation at the low concentrations used in the present study (Horgan *et al.*, 1968), despite the involvement of CoQ_{10} in the process.

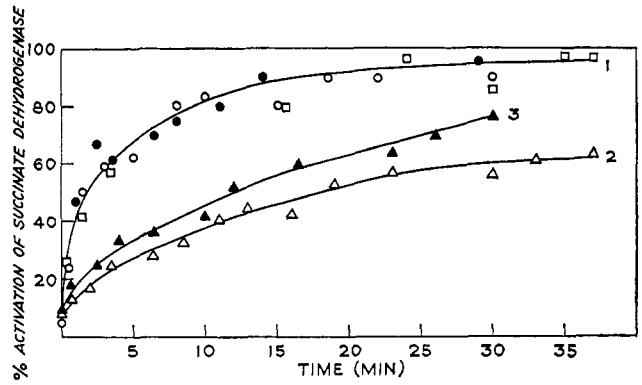


FIGURE 5: Effect of piericidin A on the NADH-induced activation. ETP_H, washed as in previous experiments, was resuspended in 0.18 M STM buffer (pH 7.4) at 16.4 mg of protein/ml and treated with 3.3 mμmoles of [^{14}C]piericidin A/mg of protein at 0° for 30 min. An aliquot was removed and the rest washed twice with 2% (w/v) bovine serum albumin in 0.18 M STM buffer and once with 0.18 M STM buffer. After removal of an aliquot the particles were washed once again with 2% bovine serum albumin in STM buffer and once with STM buffer. The piericidin content of the particles at each stage is given in the text. Activation by NADH was as in Figure 1, except that both antimycin A and cyanide were present and the temperature was 30°. Succinate dehydrogenase activity was assayed at 20°. Control samples were subjected to the same manipulations as piericidin-treated ones. Activation is expressed on the ordinate as % of the maximal activation reached with succinate as activator. Open circles, untreated control; solid circles, same after two washes with bovine serum albumin and one STM wash; open triangles, piericidin-treated sample before washings; solid triangles, same after two washes with albumin and one with STM buffer; open squares, same after an additional wash with albumin in STM and one with STM buffer alone.

The action of specifically and unspecifically bound piericidin on the activation triggered by NADH is examined in Figure 5. The experiments were carried out in the presence of 1 mM KCN and 1 mμmole of antimycin A per mg to assure complete inhibition of electron transport from reduced CoQ_{10} to O_2. Under these conditions maximal activation by NADH is obtained, without a secondary decay (curve 1, open and filled circles). The inclusion of piericidin (3.3 mμmoles/mg of protein, sufficient to cause 97% inhibition of NADH oxidation in the absence of antimycin and cyanide) greatly decreases both the rate and extent of activation by NADH (Figure 5, curve 2). This is as expected, if $CoQ_{10}H_2$ is the direct activator, since piericidin inhibits the reduction of the CoQ_{10} pool by NADH. The fact that some activation still occurs in the presence of piericidin is readily explained by the incomplete inhibition. Curve 3 shows that two washes with bovine serum albumin and one with 0.18 M STM buffer, by removing most of the unspecifically bound piericidin, partially restores activation by NADH. An additional wash with bovine serum albumin removes any remaining unspecifically bound inhibitor, lowering the piericidin content to the level expected for specific binding exclusively (36 μμmoles of NADH dehydrogenase/mg of protein, corresponding to 72 μμmoles of specifically bound piericidin/mg; experimentally found, 79 μμmoles of piericidin/mg). As seen in curve 1 (open squares) this series of treatments restores the activability of the preparation by NADH completely. Hence, apparently the inhibition of the NADH-induced activation of succinate dehydrogenase by piericidin is entirely due to unspecifically bound inhibitor and its mechanism may involve equilibration of the CoQ_{10} pool.

The observation that in ETP_H, 97% inhibited by specifically bound piericidin as regards NADH oxidase activity, the activation of succinate dehydrogenase by NADH is not inhibited may seem paradoxical. This behavior becomes understandable, however, if one considers the fact that under these conditions sufficient electron flux from NADH to CoQ remains (0.054 μmole of NADH oxidized per min per mg) to reduce the endogenous CoQ_{10} pool (3–4 μmoles/mg of protein) in a matter of seconds when a combination of KCN and antimycin A is present to block the reoxidation of $CoQ_{10}H_2$ completely.

Energy of Activation and Thermodynamic Parameters. Kearney (1957) has reported an energy of activation of 36 kcal/mole for the activation of succinate dehydrogenase by succinate. The value for the deactivation process has never been determined, since the removal of bound substrate or competitive inhibitor is a time-consuming process, which precludes accurate measurement of the rate of the deactivation (Kimura *et al.*, 1967). Activation by NADH, on the other hand, permits rate studies of the deactivation process because one may rapidly oxidize all remaining NADH and $CoQ_{10}H_2$ by nonenzymatic reaction with PMS, whereupon spontaneous deactivation occurs.

Figure 6 (line A) is an Arrhenius plot of the NADH-induced activation and gives a value of 33.3 kcal/mole for the energy of activation, in substantial agreement with the value for activation by succinate. Line B represents the deactivation process. The energy of activation from three separate experiments (16.1, 16.3, and 16.6 kcal per mole) averaged 16.3 kcal/mole. These results permit calculation of the thermodynamic parameters of the process in both directions and of the differences in $\Delta H°$, $\Delta S°$, and $\Delta F°$ values between the two states

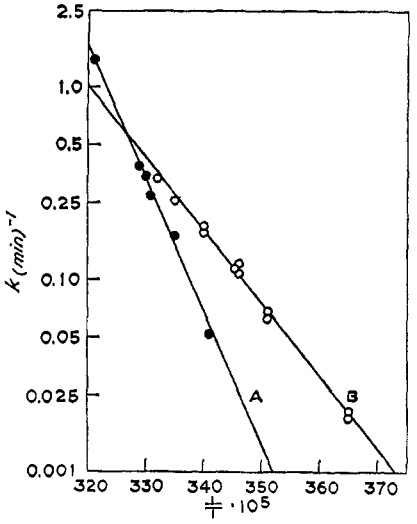


FIGURE 6: Arrhenius plot for the activation and deactivation of succinate dehydrogenase. (A) Activation of the enzyme at the temperatures indicated by NADH in the presence of 1 mM cyanide and 1 mμmole of antimycin A/mg. (B) Deactivation of the enzyme at various temperatures. In this experiment activation by NADH at 30° was allowed to proceed to maximum under the conditions of expt A; the sample was then rapidly chilled to 0° where no change in activity occurred for several hours. Samples were warmed to the temperatures indicated and PMS (82 μg/ml) was added to remove any remaining NADH by nonenzymatic oxidation, and aliquots were removed periodically to monitor the deactivation by succinate dehydrogenase assays at 25°. After complete deactivation in this manner, the enzyme could be fully activated again with malonate, showing that no inactivation of the dehydrogenase had occurred.

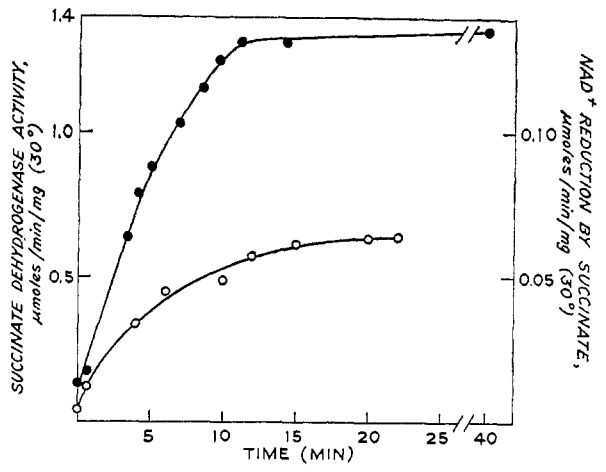


FIGURE 7: Effect of activation of succinate dehydrogenase on the energy-linked reduction of NAD by succinate. ETP_H was washed and resuspended in 0.18 M STM buffer (pH 7.4) as in previous experiments at 3.3 mg of protein concentration/ml. Activation by 16 mM succinate was carried out at 30° in the presence of 2 mμmoles of antimycin A/mg and 1 mM KCN. Samples withdrawn periodically were assayed at 30° for succinate dehydrogenase activity (solid circles) and for the ATP-driven reduction of NAD by succinate (open circles).

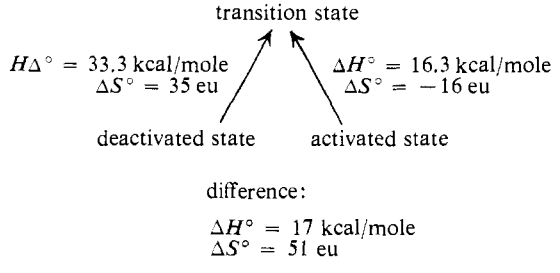
of the enzyme. The results are represented in Chart I. The difference in standard free energy between the activated and deactivated states is then $\Delta F^\circ = 17{,}000 - (298 \times 51) = 1800$ cal/mole.

Dependence of the Rates of Forward and Reversed Electron Transport on the Activation of Succinate Dehydrogenase. There is an abundance of evidence for the importance of activation in overall succinoxidase activity in various particulate preparations, including intact mitochondria (Kearney, 1957; Thorn, 1962; Kimura *et al.*, 1967). Thus far, however, no studies have been reported of the quantitative relations between the degree of activation of succinate dehydrogenase and the rate of electron flux in the respiratory chain, including energy-dependent reactions, in other words the extent of activation required before the dehydrogenase ceases to be rate limiting.

Figure 7 illustrates the effect of activation of succinate dehydrogenase on the rate of the ATP-driven reduction of NAD by succinate. It is seen that the latter process, although slow compared to the forward rate of electron flux in ETP_H, is strictly dependent on the state of activation of succinate dehydrogenase; in other words, despite the high turnover number of the dehydrogenase, the enzyme must be fully activated for maximal rate of electron flux to NAD. Figure 8 illustrates the influence of the state of activation of the dehydrogenase on the rate of reduction of endogenous cytochrome *b* by succinate in ETP_H. Aliquots of a preparation were activated with malonate for various periods at 24°, chilled to 0° to stop the activation, and after adding antimycin A (1 mμmole/mg) and cyanide (1 mM), assayed for succinate dehydrogenase activity with PMS (abscissa) and for the velocity of reduction of cytochrome *b* (ordinate). Over the range where the extent of activation was low, so that succinate dehydrogenase activity was rate limiting, a linear dependence was noted between the two activities compared in Figure 8. When over 50% of the dehydrogenase was in the activated state, further activation had relatively little effect on the rate of reduction of cytochrome *b* by succinate. Extrapolation of the linear part of the curve to zero further suggests that the deactivated (unactivated) form of the dehydrogenase is completely inactive in catalyzing either the reduction of PMS or of endogenous cytochrome *b* by succinate. This seems to settle the long-standing question (Kearney, 1957; Thorn, 1962) as to whether the deactivated enzyme is completely inactive or merely shows a much lower activity than the activated form.

Figure 9 compares the rates of reduction of endogenous CoQ_{10} by succinate before activation (about 10% activated) and after activation by malonate. Although the quantitative relation between degree of activation of the dehydrogenase and the rate of reduction of CoQ_{10} have not been explored, it is clear that activation of succinate dehydrogenase is needed for the reduction of CoQ_{10}.

CHART I



Discussion

The reversible activation of succinate dehydrogenase described in this paper appears to be in every respect examined, identical with the activation of the enzyme by substrates and

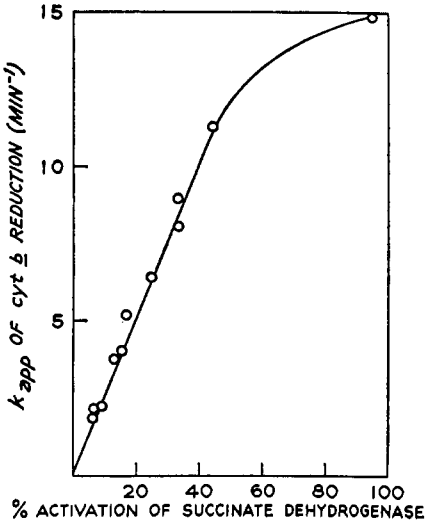


FIGURE 8: Relation of the extent of activation of succinate dehydrogenase to the rate of the succinate–cytochrome *b* reaction in ETP_H. The particles were washed and resuspended in 0.18 M STM buffer as in previous experiments at 2 mg/ml of protein concentration, activated for varying periods with 0.33 mM malonate at 24° in order to obtain samples with different degrees of activation. Samples were periodically removed, rapidly chilled to 0°; antimycin A (1 mμmole/mg) and KCN (1 mM) were added to prevent oxidase activity and succinate dehydrogenase activity was determined as in Figure 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.

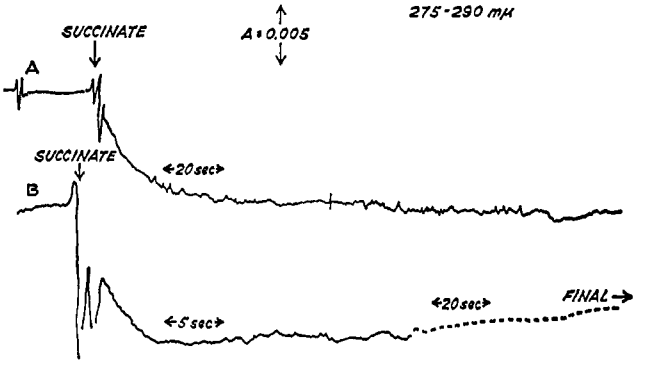


FIGURE 9: Effect of activation of succinate dehydrogenase on the rate of reduction of endogenous CoQ_{10} by succinate. (A) An ETP_H preparation, washed and resuspended in 0.18 M STM buffer at 1 mg/ml of protein concentration, was treated with 5 mμmoles of antimycin A/mg of protein plus 1 mM KCN. At the point indicated by the arrow 33 μM malonate and immediately thereafter 5 mM succinate were added and the kinetics of CoQ_{10} reduction were followed at room temperature at 275 − 290 mμ. This sample served as the control without activation, since malonate was not permitted to activate the enzyme prior to start of the reduction of CoQ_{10}. In part B, the particles were activated with 133 μM malonate for 5 min at 38° in 0.78-ml volume in 0.18 M STM buffer (pH 7.4). The preparation was then cooled to room temperature, diluted to 3 ml with 0.18 M STM buffer (pH 7.4), resulting in 33 μM malonate concentration; 5 mM succinate was added (arrow) and the reduction of CoQ_{10} recorded as above. Note the difference in time scales in expt A and B and the fact that in B the chart speed was slowed from 5 to 20 sec per in. in the dashed portion. The final level (horizontal arrow) indicates the absorbance 3 min after the addition of succinate.

competitive inhibitors. Not only are the specific activity and the K_m for PMS identical for the enzyme activated by NADH and by succinate or malonate, but the energy of activation is also the same whether initiated by succinate or NADH.

If the activation induced by NADH the latter compound is clearly not the direct activator since rhein, which inhibits its oxidation, also prevents its activating effect on succinate dehydrogenase. The demonstration that reversible removal of CoQ_{10} by pentane extraction is accompanied by reversible loss of activation of the membrane-bound succinate dehydrogenase by NADH, coupled with the observation that externally added $CoQ_{10}H_2$ and NADH activate the enzyme to about the same extent make it very likely that $CoQ_{10}H_2$ is the actual activator, or at least capable of reducing some other component which may itself be the direct activating agent. The identity of such a component is open to speculation. It would have to be modulated by the redox state of the quinones and in physical contact with the succinate dehydrogenase. Keeping this in mind, we shall for the sake of clarity, continue to refer to the phenomenon as activation by $CoQ_{10}H_2$. Thus NADH serves only as a reducing agent for endogenous CoQ_{10} and it may be predicted that the substrates of other respiratory chain-linked flavoproteins which are capable of reducing the CoQ_{10} pool would act in a like manner.

Thus it appears that succinate dehydrogenase is subject to at least two types of regulation. One is activation by substrates (Kearney, 1957) and the second is activation by way of reduced CoQ_{10}. At present it is not known whether the CoQ_{10} reducing site on the enzyme is identical with the combining site of $CoQ_{10}H_2$ in activation or whether the latter occurs at a separate, allosteric site. Thenoyltrifluoroacetone blocks electron transfer between succinate dhydrogenase and the CoQ_{10} pool but does not affect activation by CoQ_{10}. This difference does not permit the conclusion that a separate regulatory site exists for $CoQ_{10}H_2$, since the inhibitor mentioned is an iron-chelating agent and may block electron transport to CoQ_{10} by virtue of chelating with certain nonheme irons, without preventing combination of the enzyme with CoQ_{10}.

Rossi *et al.* (1970) have recently shown that on removal of CoQ_{10} from membrane preparations the kinetic properties of succinate dehydrogenase change to those characteristic of the soluble enzyme, and that on reincorporation of CoQ_{10} the original properties of the membrane-bound enzyme reappear (TTF and KCN sensitivity). These effects of CoQ_{10} are different from the phenomenon described here in that they do not involve activation of the enzyme, and the oxidized quinone is the modifying agent. The effects must be regarded as static, as the CoQ content of mitochondria is presumably not subject to rapid change. Activation and deactivation of succinate dehydrogenase, reflecting the redox state of CoQ_{10} as described here, should be regarded as a dynamic effect with possible regulatory implications. It cannot be determined at this time whether the influence of CoQ described by Rossi *et al.* (1970) and the activation by way of $CoQ_{10}H_2$ result from combination at the same specific combining site.

Another point which deserves comment is the effect of piericidin A on the NADH-induced activation of succinate dehydrogenase. When piericidin is added to ETP_H without taking precautions to prevent binding at unspecific sites (Horgan *et al.*, 1968), it inhibits the activation as expected from the interruption of electron transport from NADH to CoQ_{10}. When unspecifically bound piericidin is removed, leaving only specifically bound inhibitor in the preparation, activation of succinate dehydrogenase is not inhibited, despite the fact that in both cases about 97% of NADH oxidation is

blocked under these conditions. As mentioned in the text, this situation is understandable if one considers the fact that reoxidation of CoQ_{10} is completely blocked by antimycin and cyanide and the reduction of CoQ_{10} is still faster in the 97% inhibited system than is the activation process. Further, there is no reason to believe that $CoQ_{10}H_2$ is oxidized when acting as an activator, so that continuous reduction of the CoQ_{10} pool is not required. The conclusion is inevitable that unspecifically bound piericidin interferes with activation of succinate dehydrogenase in a manner unrelated to the mechanism by which piericidin (actually, specifically bound piericidin) is commonly thought to act: interruption of electron flux beween the nonheme irons of NADH dehydrogenase and the CoQ_{10} pool. The mode of action of unspecifically bound piericidin in the activation process may well be interference with redox reactions between CoQ_{10} molecules (*i.e.*, equilibration of the CoQ_{10} pool). This hypothesis was foreshadowed in our earlier studies on the differential effects of unspecifically bound piericidin on electron transport from NADH to external CoQ, and the cytochrome system (Gutman *et al.*, 1970) and has been discussed in more detail elsewhere (Singer and Gutman, 1971). The hypothesis received considerable support from the demonstration that CoQ_{10} is not a "mobile coenzyme" in the fabric of the inner membrane in free equilibrium with other redox components, as has been postulated (Klingenberg, 1968), but a highly compartmentalized entity (Gutman *et al.*, 1971a), so that one may visualize that unspecifically bound piericidin might interfere with redox reaction among CoQ_{10} molecules in the NADH and succinate dehydrogenase compartments, respectively. An alternative possibility is that $CoQ_{10}H_2$ is bound at different sites on succinate dehydrogenase in electron flux and in activation and that unspecifically bound piericidin interferes with binding only at the latter site at these concentrations because it has no effect on electron transport between the dehydrogenase and CoQ_{10}.

The discovery of the activation of succinate dehydrogenase by NADH in membrane preparations has certain important implications. First, by providing a practical and readily controlled means of removing the activator rapidly, it has permitted a study of the kinetics of the deactivation process. From comparison of the temperature dependence of the activation and deactivation processes it has been possible to calculate the thermodynamic constants characterizing the two states of the enzyme. Thus it has become clear that the activation of the dehydrogenase is characterized by a relatively small free-energy change but a large entropy change (ΔS° = 51 eu), compatible with the previously postulated conformation change in the enzyme (Kearney, 1957). Of this conversion of the unactivated enzyme into the excited state appears to account for the major part of the entropy change (ΔS° = 35 eu) and the rest is generated during the conversion of the latter to the activated form. The enthalpy change between the unactivated form and the excited state is 33 kcal/mole, while its transition to the catalytically active state liberates 16 kcal/mole.

The calculated free-energy change for the conversion of the unactivated to the activated enzyme is 1.2 and 3 kcal per mole at 37 and 0°, respectively, corresponding roughly to inactive: active enzyme ratios of 10:1 and 170:1 at these two temperatures. Nevertheless, in the entire temperature range where the process has been measured (20–37°) conversion of the unactivated to activated enzyme appears to go to completion, as judged by the fact that the same activity is reached with all activators tested. Since this is unexpected from the free-energy changes calculated, it appears likely that an additional exergonic step is involved in the activation. This may be the stabilization of the activated form by the activating agent, as originally postulated by Thorn (1962). According to this hypothesis the unactivated and activated forms of the enzyme are in equilibrium but only the latter binds the activator and is stabilized thereby, shifting the equilibrium to the right. This hypothesis would also explain the spontaneous and nearly complete deactivation consequent on oxidation of the bound $CoQ_{10}H_2$ with PMS, since this treatment removes the binding energy of the activator–activated enzyme complex and permits rapid shift of the equilibrium toward inactive enzyme, in accord with the measured free-energy change.

The second point concerns the role of activation as a possible control mechanism in the Krebs cycle. It is clear from the observed rates that at 37° major increases or decreases in succinate dehydrogenase activity can occur in a few seconds as a result of the activation or its reversal, which are reflected in electron flux in both forward and reversed electron transport. In this regard succinate dehydrogenase exhibits characteristics commensurate with the fine regulation of metabolic flux. Until now, however, it has been difficult to see what compound might act as a regulator under physiological conditions. Succinate and fumarate are not known to undergo sufficiently pronounced changes in concentration during metabolic flux to bring about major changes in the degree of activation of the enzyme. The findings reported here suggest the CoQ_{10}:$CoQ_{10}H_2$ ratio as a likely candidate for the physiological regulator. The redox state of the CoQ_{10} pool is known to undergo major changes in the transition between metabolic states (Kröger and Klingenberg, 1966; Klingenberg, 1968) and is expected to respond rapidly to changes in the steady-state concentrations of NADH and of other substrates of the respiratory chain-linked flavoproteins. Current investigations are aimed at examining the degree to which the operation of the Krebs cycle and associated phosphorylation are governed by the state of activation of the dehydrogenase in tightly coupled mitochondria.

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Calcium Binding to the Sarcoplasmic Reticulum of Rabbit Skeletal Muscle*

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ABSTRACT: Passive calcium binding to the sarcoplasmic reticulum of rabbit skeletal muscle has been studied. Previous results obtained by Carvalho, and Cohen and Selinger are reevaluated in view of artifacts which may arise due to endogenous, exchangeable calcium in the preparations. By washing or treating membrane preparations with Chelex-100, and by carrying out binding assays by equilibrium dialysis against a relatively large amount of free calcium, we can distinguish three types of calcium binding sites on the membranes. About 80–90% is nonspecific and display a dissociation constant for calcium of about 0.32 mM. The two remaining sites are designated as calcium specific since the calcium binding to these sites persists in the presence of 0.6 M KCl and 1 mM $MgCl_2$.

In the absence of KCl and $MgCl_2$, the sites of highest affinity bind about 10–20 nmoles of calcium/mg of protein and display a dissociation constant of 0.4 μM; the sites of lower affinity bind about 130 nmoles of calcium/mg of protein with a dissociation constant of 13 μM. In the presence of KCl and $MgCl_2$, the dissociation constants for both the high- and the low-affinity sites increase about threefold. About 10 and 90 nmoles of calcium are bound per mg of protein to the high- and low-affinity sites, respectively. The lanthanide ions La^{3+}, Gd^{3+}, and Yb^{3+}, which are known to display a very high affinity toward anionic sites because of their high charge density, are nevertheless relatively poor inhibitors of calcium binding to the specific sites, illustrating the high degree of specificity for calcium at these sites. La^{3+} inhibition of calcium binding at the calcium specific sites is of the competitive type with a K_i of 6.5 μM.

The relaxation process in skeletal muscle involves the removal of calcium from myofibrils by the sarcoplasmic reticulum (Weber *et al.*, 1963; Weber, 1966). Isolated preparation of these membranes provides a simple system for studying ion transport, since their major function is the active translocation of calcium ions (Martonosi and Feretos, 1964). In the presence of ATP, magnesium, and a "trapping" agent such as oxalate, these vesicular preparations can transport large quantities of calcium into their internal space against a large concentration gradient (Hasselbach and Makinose, 1961; Hasselbach and Makinose, 1963; Weber, 1966).

Present concepts of the mechanism of active transport require that a preliminary and essential step for calcium translocation is the binding of the ion to the membrane. A knowledge of the number of calcium binding sites, their specificity, and relative affinities, is essential to an understanding of this process and its relationship to active transport. Aspects of this problem have been investigated by Carvalho (1966) and more recently by Cohen and Selinger (1969). In this report we present results on calcium binding which differ in part from the results obtained by these investigators. In addition, our studies have been extended to include the effects of some lanthanides on passive calcium binding.

Materials and Methods

Materials. Chelex-100 (100–200 mesh) was obtained from Bio-Rad Laboratories. ^{45}Ca was purchased from New England Nuclear as $CaCl_2$ in 0.5 N HCl (6.15 mCi/mg of Ca). Lanthanum chloride ($LaCl_3 \cdot 6H_2O$) was obtained from Fischer. Gadolinium sulfate ($Gd_2(SO_4)_3 \cdot 8H_2O$) and ytterbium sulfate ($Yb_2(SO_4)_3 \cdot 8H_2O$) were purchased from Alfa Inorganics. All other reagents were biochemical grade.

Preparation of Sarcoplasmic Reticulum. Membranes of the sarcoplasmic reticulum were prepared according to the procedure of Martonosi *et al.* (1968). After isolation and purification the preparation was stored frozen at −70° in 0.15 M KCl, 0.01 M histidine-Cl (pH 7.4), and 0.5 M sucrose at a protein concentration of 20–25 mg/ml. Prior to the binding experiments, suspensions of sarcoplasmic reticulum were prepared following two different procedures. (1) Membranes were washed two times by centrifugation with 0.01 M histidine-Cl (pH 6.5) containing 10^{-3} M $MgCl_2$ and 0.6 M KCl (buffer A).

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