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STUDIES ON SUCCINATE DEHYDROGENASE

IV. KINETICS OF THE OVERALL REACTION CATALYSED BY PREPARATIONS OF THE PURIFIED ENZYME

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SUMMARY

1. The kinetics of the reaction catalysed by succinate dehydrogenase (EC 1.3.99.1) have been studied. Double reciprocal plots (inverse of velocity against inverse of succinate concentration) at various concentrations of ferricyanide or phenazine methosulphate yield a series of straight lines converging in the third quadrant, when malonate is added.

2. Incubation with succinate of the enzyme prepared by the method of KEILIN AND KING[10] caused almost a doubling of the activity.

3. Both before and after activation with succinate, the catalytic-centre activity, measured at infinite substrate and acceptor concentrations, is the same with ferricyanide and phenazine methosulphate as acceptor.

4. At low concentrations of phenazine methosulphate, the rate of the reaction is directly proportional to the acceptor concentration.

5. From the effect of succinate concentration on the intensity of optical absorption of the enzyme at 460 mμ, a dissociation constant of 110 μM was calculated.

6. The data support a mechanism in which the product of reaction between enzyme and substrate is oxidized by electron acceptor before dissociation of the product, fumarate, from the enzyme. No evidence could be obtained of an alternative pathway at 25° sufficiently rapid to make a significant contribution to the kinetics of the enzyme.

7. The main effect of activation is to increase the rate of intramolecular conversion of the enzyme–substrate complex, formed by reaction between enzyme and substrate, into a second complex which reacts with acceptor, and the reverse reaction. The reaction of this second complex with acceptor is also accelerated.

8. The catalytic-centre activity of the activated enzyme, 130 sec^{-1} at 25°, agrees quite closely with that reported for particle-bound succinate dehydrogenase (about 200 sec^{-1} at 37°).

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INTRODUCTION

We have briefly reported the results of a study of the kinetics of the oxidation of succinate by ferricyanide, catalysed by purified succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) and shown that double reciprocal plots (inverse of velocity against inverse of succinate concentration) at various ferricyanide concentrations yield a series of straight lines, converging in the third quadrant[1]. This and the observation that fumarate is a competitive inhibitor of the enzymic reaction supported the mechanism previously proposed for the particle-bound enzyme[2] in which the product of reaction between enzyme and substrate is oxidized by the electron acceptor before dissociation of the reaction product, fumarate, from the enzyme. The values of the kinetic constants obtained, calculated from the experimental data with the purified enzyme on the basis of this mechanism, agreed quite closely, insofar as they could be compared, with those obtained for the particulate enzyme by SLATER AND BONNER[3], and THORN[4]. This is of interest, since, in many respects, *e.g.* stability, the isolated enzyme has properties differing from those of the particle-bound enzyme, differences that have recently been emphasized by BRUNI AND RACKER[5].

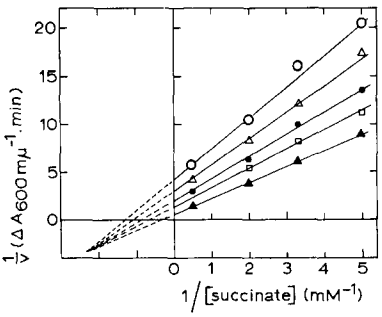
The preparation of succinate dehydrogenase used in all our previous studies[1,6–8] was made by the procedure of WANG *et al.*[9] as modified by KEILIN AND KING[10]. KEARNEY and co-workers[11–13] and KIMURA *et al.*[14] have shown that soluble succinate dehydrogenase is activated by incubation with succinate or competitive inhibitors, including inorganic phosphate. The activation is also found with particulate preparations[15]. Since the method of WANG *et al.*[9] includes preincubation of the particulate enzyme with succinate, we had assumed that enzyme made by this procedure would be fully activated, a conclusion that has justifiably been called into question by KIMURA *et al.*[14]. We have indeed found that incubation of our enzyme with succinate (but not with phosphate) causes almost a doubling of the activity. We have therefore repeated the previous kinetic measurements, using fully activated enzyme. At the same time we have compared the activity with ferricyanide and phenazine methosulphate as electron acceptor. Higher activities have been reported with the latter acceptor, even at infinite acceptor concentration[16], a result not be expected from the proposed mechanism[2].

RESULTS

Effect of varying succinate and acceptor concentrations, using phenazine methosulphate as acceptor

As already discussed elsewhere[2,1,17] the addition of a competitive inhibitor in order to bring the K_m for substrate into the region of that for acceptor increases the usefulness of the kinetic analysis of the overall reaction catalysed by an enzyme. As in the previous experiments with ferricyanide as acceptor[1], malonate was used as a competitive inhibitor with phenazine methosulphate as acceptor. The results obtained with four different succinate and five different acceptor concentrations are plotted in Figs. 1 and 2. As in the previous study[1], partially activated succinate dehydrogenase was used in this experiment. The results obtained with phenazine methosulphate are similar to those with ferricyanide.

From the intercept of these curves on the two axes, the velocities at fixed



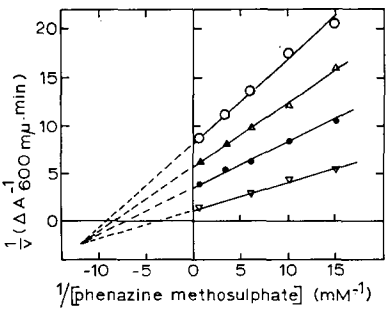


Fig. 1. Effect of varying succinate and acceptor concentration on rate of oxidation of succinate by phenazine methosulphate, plotted as $1/v$ *versus* 1/[succinate]. 90 μM malonate was present. The concentrations of phenazine methosulphate were 0.067 (○), 0.10 (△), 0.167 (●), 0.30 (□) and 2.0 (▲) mM, respectively. The concentration of enzyme-bound flavine was 0.027 μM. Enzyme as prepared was used.

Fig. 2. Same data as in Fig. 1 plotted as $1/v$ *versus* 1/[phenazine methosulphate]. The concentrations of succinate were 0.2 (○), 0.3 (△), 0.5 (●) and 2 (▽) mM, respectively.

acceptor and infinite substrate concentration (V) and the K_m values for succinate (taking into account the presence of the competitive inhibitor malonate with a K_i of 18 μM, see ref. 6) at various acceptor concentrations may be calculated. From secondary plots of K_m against V (intercept on ordinate $= k_{-1}/k_1$, see ref. 2), of K_m/V against $1/a$ (intercept on ordinate $= 1/k_1 e$, see refs. 17, 18) and of $1/V$ against $1/a$ (intercept on ordinate $= 1/k_4 e$, see refs. 17, 18; slope $= 1/k_3 e$), where a is the concentration of acceptor (A) and e the enzyme concentration, the velocity constants for the mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \tag{1}$$

$$ES + A_{ox} \xrightarrow{k^2} EP + A_{red} \tag{2}$$

$$EP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P \tag{3}$$

may be calculated (see ref. 18). These constants are listed in Table I, in which previously published values are also assembled. The k_2 for phenazine methosulphate is higher than that for ferricyanide.

Effect of activation on kinetic constants

Incubation with succinate of the enzyme prepared by the method of KEILIN AND KING[10] caused almost a doubling of the activity (Table II). Phosphate had no effect under the conditions of our experiments. However, as pointed out by KEARNEY[12], this process is affected markedly by the enzyme concentration. The effect of activation on the kinetics of the enzyme with ferricyanide as acceptor is shown in Fig. 3. The secondary plots are shown in Figs. 4–6, from which the data listed in Table III were calculated. From these data, the kinetic constants given in Table IV may be calculated. For the enzyme as prepared, the values of k_1 and k_3 are somewhat lower than in the previous study[1], whereas the values for k_{-1} and k_4 are in good agreement. The main

TABLE I

RATE CONSTANTS FOR SUCCINATE DEHYDROGENASE

The rate constants are defined for the mechanism given by Eqns. 1–3 (see text).

	This study	*Ref. 1*	*Ref. 2*	*Ref. 4*
Preparation	Soluble*	Soluble*	Particulate**	Particulate**
Acceptor	PMS***	$K_3Fe(CN)_6$	MB§ and O_2	MB§ and O_2
Temperature	25°	25°	38°	38°
k_1 ($M^{-1} \cdot sec^{-1}$)	$9 \cdot 10^4$	$5.1 \cdot 10^4$	$7 \cdot 10^4$	$3.5 \cdot 10^4$
k_{-1} (sec^{-1})	6.3	1.8	2	1.1
k_3 ($M^{-1} \cdot sec^{-1}$)	$8.5 \cdot 10^4$	$1 \cdot 10^4$		
k_4 (sec^{-1})	56	65	32	17
k_{-1}/k_1 (μM)	70	35	30	31

* As prepared.

** The concentration of succinate dehydrogenase was not determined, but assumed to be equal to that of cytochrome *c*. In fact, the concentration of the enzyme is at most 40% of that of cytochrome *c* (see Table II of ref. 32), so that these values should be multiplied by at least 2.5.

*** Phenazine methosulphate.

§ Methylene blue.

TABLE II

EFFECT OF INCUBATION WITH SUCCINATE AND PHOSPHATE ON ACTIVITY OF ISOLATED SUCCINATE DEHYDROGENASE

10 mg/ml succinate dehydrogenase pre-incubated for 5 min at 38°.

Conditions during pre-incubation	*Succinate dehydrogenase activity measured under standard conditions (μmoles succinate/min per mg protein)*
0.1 M Phosphate	3.2
0.3 M Phosphate	2.8
0.1 M Phosphate + 20 mM succinate	6.0

TABLE III

INTERCEPTS IN SECONDARY PLOTS FROM KINETIC DATA FOR SUCCINATE DEHYDROGENASE WITH FERRICYANIDE AS ACCEPTOR, FOR PARTIALLY AND FULLY ACTIVATED ENZYME

Enzyme was activated by incubation at a concentration of 10 mg protein/ml with 20 mM succinate for 5 min at 38°. The activated enzyme and a control preparation were diluted in 0.1 M phosphate to the same concentration before determination of the kinetics at 25°. The primary data are shown in Fig. 3, the secondary plots in Figs. 4–6. Concentration of enzyme flavine, 0.42 μM.

Fig.	*Plot*	*Intercept on*	*Enzyme as prepared*	*Activated enzyme*
4	K_m *versus* V	Ordinate	63 μM	60 μM
5	K_m/V *versus* $1/a$	Ordinate	75 sec	66 sec
6	$1/V$ *versus* $1/a$	Ordinate	$4.5 \cdot 10^4 M^{-1} \cdot sec$	$1.85 \cdot 10^4 M^{-1} \cdot sec$
		Slope	490 sec	310 sec

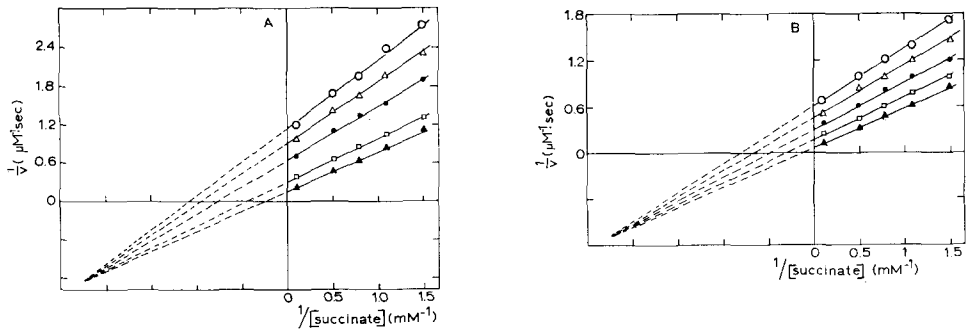


Fig. 3. Effect of activation on kinetics of succinate dehydrogenase with ferricyanide as acceptor. The effect of varying succinate and acceptor concentrations on rate of oxidation is plotted as $1/v$ *versus* 1/[succinate]. 140 μM malonate was present. The concentrations of $K_3Fe(CN)_6$ were 0.5 (○), 0.67 (△), 1.0 (●), 2.0 (■) and 5.0 (▲) mM, respectively. The concentration of enzyme-bound flavine was 0.42 μM. A, enzyme as prepared. B, activated as is described in Table III.

TABLE IV

RATE CONSTANTS FOR SUCCINATE DEHYDROGENASE, WITH FERRICYANIDE AS ACCEPTOR, FOR PARTIALLY AND FULLY ACTIVATED ENZYME

Enzyme was activated by incubation at a concentration of 10 mg protein/ml with 20 mM succinate for 5 min at 38°. The activated enzyme and a control preparation were diluted in 0.1 M phosphate to the same concentration before examination of the kinetics. From the data given in Figs. 3–6, the rate constants were calculated for the mechanism given by Eqns. 1–3.

Rate constant	*Activated enzyme*	*Enzyme as prepared*	
		This study	*Ref. 1*
k_1 ($M^{-1} \cdot sec^{-1}$)	$3.6 \cdot 10^4$	$3.2 \cdot 10^4$	$5.1 \cdot 10^4$
k_{-1} (sec^{-1})	2.2	2.0	1.8
k_3 ($M^{-1} \cdot sec^{-1}$)	$0.77 \cdot 10^4$	$0.5 \cdot 10^4$	$1 \cdot 10^4$
k_4 (sec^{-1})	130	53	65
k_{-1}/k_1 (μM)	63	60	35

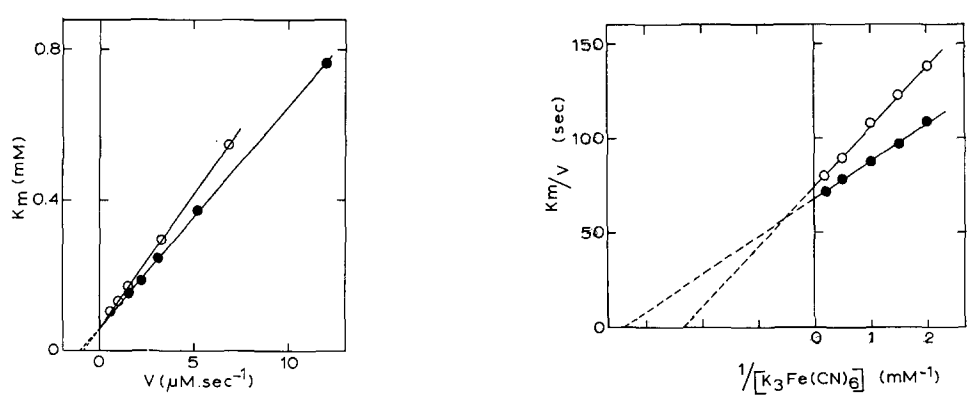


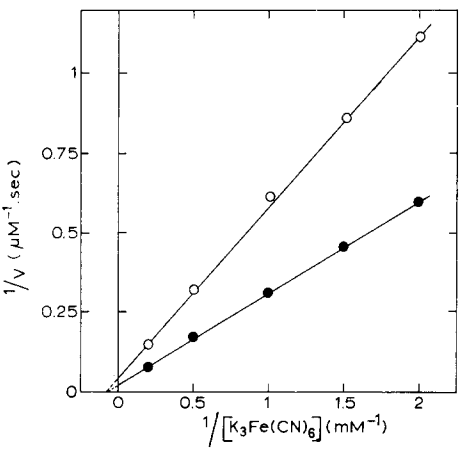
Fig. 4. Data of Fig. 3 plotted as K_m at fixed acceptor concentration *versus* V (infinite succinate concentration) at various acceptor concentrations. ○, enzyme as prepared; ●, activated.

Fig. 5. Data of Fig. 3 plotted as K_m/V *versus* 1/[acceptor]. ○, enzyme as prepared; ●, activated.

effect of activation appears to be on k_4, the catalytic-centre activity at infinite substrate and acceptor concentrations.

Comparison of ferricyanide and phenazine methosulphate as acceptor

The rate constants for the partially activated enzyme assembled in Tables I and III show that the value of k_4 is independent of the acceptor. Fig. 7 shows that this is also the case with the fully activated enzyme.



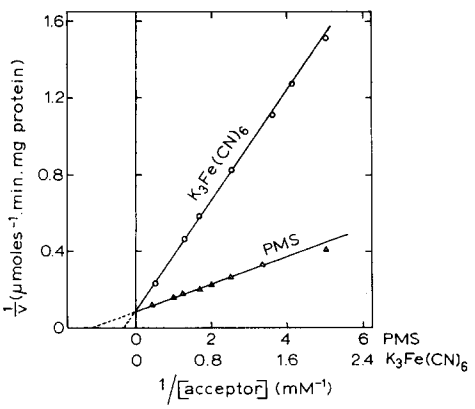


Fig. 6. Data of Fig. 3 plotted as $1/V$ *versus* 1/[acceptor]. ○, enzyme as prepared; ●, activated.

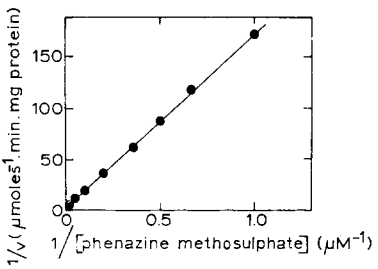
Fig. 7. Comparison of phenazine methosulphate and $K_3Fe(CN)_6$ as acceptor, plotted as $1/v$ *versus* 1/[acceptor]. The enzyme was activated as described in the legend to Table III. The succinate concentration was 10 mM; no malonate was present. ○, 0.13 mg/ml succinate dehydrogenase, varying ferricyanide concentrations; △, 5 μg/ml succinate dehydrogenase, varying phenazine methosulphate (PMS) concentrations.

Rate measurements at low concentrations of acceptor

In order to test a mechanism proposed by others (see DISCUSSION), it became important to determine the linearity of the relationship between rate and acceptor concentration. This is best done at low acceptor concentration, where, according to the mechanism given in Eqns. 1–3, the rate is approximately proportional to the acceptor concentration. Low acceptor concentrations are most conveniently used with phenazine methosulphate, since, in the test system used, dichlorophenol indophenol is used as final acceptor, and the concentration of phenazine methosulphate is constant. Fig. 8 shows that the rate is proportional to acceptor concentration between 1 and 100 μM phenazine methosulphate. The k_3 calculated from the slope equals $4 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$ (*cf.* $8.5 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$ in Table I).

Dissociation constant of the enzyme–substrate complex

According to the mechanism given by Eqns. 1–3, K_D, the dissociation constant of the *ES* complex is equal to k_{-1}/k_1. For the partially activated enzyme, this equals 70 μM (Table I) with phenazine methosulphate and 60 μM (Table III) with ferricyanide. For the fully activated enzyme the value is 64 μM (Table III) with ferricyanide. These values are all appreciably higher than the 35 μM with ferricyanide and partly activated enzyme reported previously[1].



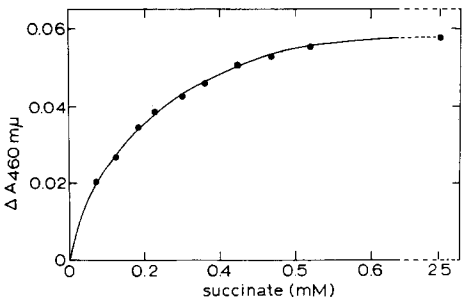


Fig. 8. Relationship between velocity of enzyme reaction and acceptor concentration measured at low concentrations of phenazine methosulphate. Enzyme as prepared was used. The succinate concentration was 1 mM; no malonate was present. 0.17 mg/ml succinate dehydrogenase.

Fig. 9. Determination of K_D for succinate dehydrogenase. The effect of successive additions of succinate on the absorbance at 460 mμ was determined, using 4.2 mg/ml succinate dehydrogenase as prepared. The enzyme was dissolved in 0.1 M phosphate buffer (pH 7.8), containing 1 mM EDTA and 0.1% serum albumin. The $A_{460\ m\mu}$ was corrected for dilution of the solution by the addition of succinate, and the original absorbance substrated. A Zeiss PMQ II spectrophotometer was used.

The dissociation constant was measured by three independent methods, *viz.* (a) the effect of succinate concentration on the intensity of optical absorption of the enzyme at 460 mμ; (b) competition between succinate and various competitive inhibitors, measured by the effect of succinate on the intensity of the bands characteristic of the enzyme–inhibitor complexes[6] (see refs. 1, 18); (c) effect of succinate concentration on the intensity of the semiquinone signal recorded by electron-spin spectroscopy[8] (see refs. 1, 18).

The first method is the most accurate of the three. From the curve shown in Fig. 9, the K_D was calculated according to the formula: $K_D = ((e/p) - 1)(s - p)$, where e is the total enzyme concentration, p is the concentration of ES, and s the total concentration of succinate. The mean value calculated in this way was 110 μM. The other methods gave values varying between 72 and 123 μM.

DISCUSSION

The mechanism of action of succinate dehydrogenase expressed by Eqns. 1–3 is the minimum hypothesis necessary to explain (i) the non-parallel reciprocal plots of Figs. 1–3; (ii) the independence of the activity at infinite substrate and acceptor concentrations on the nature of the acceptor; (iii) competitive inhibition by fumarate[6].

The calculation of the rate constants applicable to this mechanism from the intercepts of the secondary plots on the ordinate or abscissa is inevitably not very accurate, especially when the intercepts are small. When this is taken into account, the differences between the various values obtained for k_1 ($3.3 \cdot 10^4$–$9 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$), k_{-1} (1.8–6.3 sec^{-1}), k_3 with ferricyanide ($0.54 \cdot 10^4$–$1 \cdot 10^4$ $M^{-1} \cdot sec^{-1}$) and k_{-1}/k_1 (35–70 μM) reported here and previously[1] probably fall within the experimental error. The values for k_4, the catalytic-centre activity at infinite substrate and acceptor concentration, vary only between 53 and 65 sec^{-1}.

In the previous report, the discrepancy between the value for K_D of the ES compound calculated from the kinetics of the overall reaction and that directly de-

termined with substrate amounts of enzyme was explained by postulating that the *ES* compound reacts with ferricyanide in two discrete steps. Although this is undoubtedly the case, since a termolecular reaction is ruled out by the data of Fig. 8 (*cf.* refs. 1, 18), we now think it is much more likely that only the first oxidation step is rate-limiting. According to the data published in this paper the discrepancy between the two values of K_D is only about 1.7-fold. In view of the experimental error and the large difference in the enzyme concentration under the conditions of the two measurements of K_D, it is doubtful whether the difference is significant.

However, as discussed earlier[1], it seems rather likely that two enzyme–substrate compounds are formed, first an enzyme–succinate compound (analogous to those formed with competitive inhibitors), followed by intramolecular hydrogen transfer to give a fumarate-reduced enzyme compound. Eqn. 1 should, then, be expanded to

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_{\mathrm{I}} \tag{1a}$$

$$ES_{\mathrm{I}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_{\mathrm{II}} \tag{1b}$$

Indeed, preliminary spectrophotometric experiments with the stopped-flow apparatus (W. P. ZEIJLEMAKER, unpublished experiments) indicate that the initial rapid reaction between enzyme and succinate, in the absence of acceptor, is independent of the concentration of succinate down to 1 mM. A similar independence on substrate concentration (down to 0.5 mM) of the reaction velocity was obtained by following the reaction by ESR spectroscopy, using the rapid-freeze technique[19]. These independent measurements indicate that the intermediate detectable by both light and ESR spectrometry is ES_{II}, and that at the concentration of succinate used in the present study, the conversion of ES_{I} into ES_{II} would be rate-limiting.

The kinetic equation for the expanded mechanism given by Eqns. 1a, 1b, 2 and 3 is

$$v = \frac{\dfrac{k_2k_3k_4ae}{k_{-2}k_4 + k_3k_4a + k_2k_3a}}{1 + \dfrac{1}{s}\left[\dfrac{k_{-1}k_{-2}k_4 + k_{-1}k_3k_4a + k_2k_3k_4a}{k_1k_{-2}k_4 + k_1k_3k_4a + k_1k_2k_4 + k_1k_2k_3a}\right]}$$

The intercepts of the secondary plots are: (i) on ordinate in plot of K_m *versus* V, $k_{-1}/k_1 \cdot [k_{-2}/k_2 + k_{-2})]$; (ii) on ordinate in plot of K_m/V *versus* $1/a$, $(k_{-1} + k_2)/k_1k_2e$; (iii) on ordinate in plot of $1/V$ *versus* $1/a$, $(1/k_2 + 1/k_4)\ 1/e$. The slope of the latter plot is $(1/k_3e)\ (1 + k_{-2}/k_2)$.

In terms of the expanded mechanism, K_D calculated from Fig. 9 equals $k_{-1}/k_1 \cdot [k_{-2}/(k_2 + k_{-2})]$, *i.e.* it is the same as the intercept on the ordinate in the plot of K_m *versus* V. The expanded mechanism does not, therefore, explain the discrepancy between this intercept and the dissociation constant calculated from Fig. 9. However, as already mentioned, this may lie within the experimental error.

Insufficient data are available to allow a calculation of all the rate constants corresponding to the expanded mechanism. Since the catalytic-centre activity is 53 sec^{-1}, the $t_{\frac{1}{2}}$ for the formation of ES_{II} in stopped-flow and rapid-freezing experiments should be less than 0.69/53 sec = 13 msec. In fact, a somewhat higher value,

20 msec, was obtained in the stopped-flow experiments, but the discrepancy is certainly not outside the experimental error. It seems reasonable, then, to conclude that, at infinite substrate and acceptor concentrations, the conversion of ES_I to ES_{II} is rate-limiting for the overall reaction, or in other words that $k_4 \gg k_2$. It follows that $k_2 = 53$ sec^{-1}. If k_{-1} and k_{-2} are small, k_1 and k_3 will be the same as given in Table IV. If they are not small, k_1 and k_3 will be greater than these values.

These calculations are applicable to the enzyme as prepared by the modified method of WANG *et al.*[9]. In the absence of a value for K_D, it is not possible to make such calculations for the enzyme activated by pre-incubation with substrate. However, certain conclusions can be drawn.

In the first place, it seems likely that the activation is not an all-or-none phenomenon in which inactive molecules present in the preparation become activated, with a consequent increase in e (*cf.* ref. 12). If this were the case, the intercept on the ordinate of the K_m/V *versus* $1/a$ plot, $(1/k_1e)(1 + k_{-1}/k_2)$, would have been decreased by a factor of 2.5, since V, which is proportional to e, is increased 2.5-fold. In fact, the intercept was decreased by only 10%.

Activation had no appreciable effect on the intercept with the ordinate in the K_m *versus* V plot. Thus

$$\frac{k_{-1}}{k_1}\left(\frac{k_{-2}}{k_2 + k_{-2}}\right) = \frac{k_{-1}/k_1}{1 + k_2/k_{-2}}$$

is not affected by the activation. Various explanations are possible:

(a) none of the rate constants k_1, k_{-1}, k_2 or k_{-2} are affected by the activation;
(b) k_1 and k_{-1} are changed by the same factor;
(c) k_2 and k_{-2} are changed by the same factor, which may be the same as or different from that in (b);
(d) a combination of (b) and (c).

The secondary plot that is most affected by the activation is the $1/V$ *versus* $1/a$ (Fig. 6). The intercept on the ordinate, which becomes $1/k_2e$ when $k_4 \gg k_2$, increases 2.5-fold. Since we have already concluded that e does not change, k_2 and, according to the argument in the above paragraph, k_{-2} must have increased 2.5-fold. The slope, $(1/k_3e)(1 + k_{-2}/k_2)$, decreases by a factor of 1.6. Since we have already concluded that no change takes place in e or k_{-2}/k_2, it follows that k_3 must have increased 1.6-fold. Practically the same value is obtained from the effect of activation on the slope of the K_m/V *versus* $1/a$ plot, $k_{-1}k_{-2}/k_1k_2k_3e$, which is decreased by a factor of 1.8. The intercept on the abscissa in this plot, $k_3\,(k_{-1} + k_2)/k_{-1}k_{-2}$, is increased 1.5 times. From this it may be calculated that k_{-1}, and therefore k_1, are increased by only about 10% by activation.

Thus, the main effect of activation is to increase the rate of the intramolecular conversion of ES_I and ES_{II} into one another, and the rate of oxidation of ES_{II}. Since k_2 and k_{-2} are equally affected, the equilibrium constant of the reaction $ES_I \rightleftharpoons ES_{II}$ is not affected. The increase in both k_2 and k_{-2} suggests that activation affects the $\Delta G^{\ddagger}$ of the transition states, without affecting the ΔG of the reaction. Thus, it is likely that the activation is accomplished by changes in the $\Delta S^{\ddagger}$.

GAWRON and co-workers[20,21] propose that two pathways compete for what we call ES_{II} in Eqn. 1b. One pathway is identical with that given by our Eqns. 2 and 3.

In the alternative pathway, the product dissociates from the reduced enzyme before the latter is oxidized by acceptor (*cf.* ref. 22). The relative activities of the two pathways were calculated from the deviation from linearity of the plot of the reciprocal of the velocity against the reciprocal of the acceptor concentration. According to GAWRON *et al.*[21], the pathway that we propose is more than 15 times faster at 30° than the alternative pathway, when the oxidation of succinate by ferricyanide is catalysed by soluble succinate dehydrogenase. In our experiments, no departure from linearity was found between 1 μM and 2 mM phenazine methosulphate, or between 0.5 mM and 5 mM ferricyanide. There is, then, no evidence from our studies that the alternative pathway proposed by GAWRON *et al.*[20,21] or by VITALE AND RITTENBERG[22] is quantitatively important under our conditions, but we cannot exclude a slow reaction of ES_{II} by this pathway. Non-linear plots at temperatures below 15°, to be reported in detail in a later publication, suggest that the alternative pathway might be important in the conformation of the enzyme existing at low temperatures.

The catalytic-centre activity of the activated enzyme, 130 sec^{-1} at 25°, agrees quite well with that reported for particle-bound succinate dehydrogenase (180–220 sec^{-1} at 37°, calculated from ref. 23). The studies reported in this paper support our previous conclusion that the kinetics of the isolated enzyme resemble closely those of the mitochondrial-bound enzyme.

METHODS

Succinate dehydrogenase was isolated from pig heart as previously described[5], at 0° and in an atmosphere of nitrogen. The preparation was kept in small amounts in liquid nitrogen. The enzyme was always used within 15 min after thawing and its activity was tested by the assay procedure of WANG *et al.*[9], using a reaction mixture containing 20 mM succinate, 6 mM ferricyanide, 0.1 M phosphate buffer (pH 7.8), 1 mM EDTA and 1 mg/ml serum albumin. The preparations used contained 160 units[9] (3.2 μmoles/min per mg protein) before activation and the activity was increased by a factor of 2 by activation. This was carried out by incubation at 38° for 5 min of a solution containing 10 mg/ml enzyme, 20 mM succinate, 1 mM EDTA and 0.1 M phosphate buffer (pH 7.8).

The kinetic measurements were carried out at 25° in 0.1 M phosphate buffer (pH 7.8) containing 1 mM EDTA and 1 mg/ml serum albumin. The rate of reduction of ferricyanide was measured at 420 mμ ($\Delta\varepsilon_{mM}$ (oxidized *minus* reduced), 1.03) or, with concentrations above 2 mM, at 450 mμ ($\Delta\varepsilon_{mM}$ (oxidized *minus* reduced), 0.262). The reduction of phenazine methosulphate was coupled with that of 2,6-dichlorophenol indophenol[24–28], which was measured at 600 mμ ($\Delta\varepsilon_{mM}$ (oxidized *minus* reduced), 21). It was found that the reaction rate is independent of indophenol concentration between 0.01 and 1 mM. A concentration of 0.08 mM was used. At this level the direct reduction, in the absence of phenazine methosulphate, was negligible (the K_m for dichlorophenol indophenol, in the presence of 5 mM succinate, is about 5 mM). Phenazine methosulphate solutions were kept in the dark and added to the cuvette immediately before starting the reaction by addition of enzyme. When necessary a correction for the slow non-enzymic reaction between the dyestuffs[28] was made. In disagreement with KING[28], it was found that the rate of reduction of the dichlorophenol indophenol, when corrected for the non-enzymic reaction, was proportional to

the enzyme concentration. The spectrophotometric measurements were made with a Cary 14 recording spectrophotometer, or with a Zeiss PMQ II instrument, either read directly or fitted with a Photovolt Varicord 43 logarithmic recorder. Readings began within 10 sec of adding the enzyme. Initial rates, which were constant for 1–3 min, were measured.

Protein was determined by the biuret method, after precipitation with 5% trichloroacetic acid.

Flavin not extractable with acid was determined either spectrophotometrically or fluorimetrically. The enzyme was first precipitated with 5% trichloroacetic acid and the precipitate digested with a mixture of trypsin and chymotrypsin, as described by KING *et al.*[29] and CERLETTI *et al.*[30]. After digestion, FAD was hydrolysed to FMN by treatment with 10% trichloroacetic acid for 16 h at 38° (*cf.* ref. 31). Enzyme concentrations are expressed in terms of flavin determined in this way.

Potassium ferricyanide and 2,6-dichlorophenol indophenol were from Merck; succinate and malonate (sodium salts) from Boehringer; phenazine methosulphate, bovine serum albumin and FMN from Sigma. All other chemicals were from British Drug Houses.

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