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STUDIES ON SUCCINATE DEHYDROGENASE

VII. THE EFFECT OF TEMPERATURE ON THE SUCCINATE OXIDATION

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SUMMARY

1. At temperatures below 11°C soluble succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) is strongly inhibited by high concentrations of ferricyanide. This inhibition is reversed by high concentrations of succinate.

2. At 15°C and lower the enzyme shows an intermediary plateau region in the saturation curves with succinate.

3. An Arrhenius plot with a breakpoint at 18°C is found with both particulate and soluble succinate dehydrogenase. The Arrhenius plot for the non-activated enzyme shows a second breakpoint at 27°C.

4. A possible explanation for the breaks in the Arrhenius plots is offered in terms of temperature-dependent conformational changes in the enzyme.

INTRODUCTION

When the velocity of an enzymic reaction is measured at different temperatures and a graph is made of log v against $1/T$, the resulting Arrhenius plot often shows one or more breakpoints[1]. These breaks may be caused by a change in the rate-limiting step of the enzyme-catalysed reaction with change in temperature[2], or by the transition of the enzyme from one conformational state to another, differing in activation enthalpy[3,4] or catalytic-centre activity[5,6]. An effect of temperature on the interaction between enzyme and solvent is also a possible explanation[7].

The Arrhenius plots for the oxidation of succinate by oxygen, catalysed by bacterial[8,9] or particulate heart preparations[10] have been reported to show no discontinuities. However, WANG *et al.*[11] found that purified succinate dehydrogenase, with ferricyanide as acceptor, gives a discontinuous Arrhenius plot with two downward bends at 17°C and 26°C. CERLETTI *et al.*[12,13], on the other hand, using phenazine methosulphate as acceptor, found only one upward bend at 25°C (temperatures lower than 15°C were not tested).

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We have studied the effect of temperature on the oxidation of succinate by oxygen, catalysed by the Keilin and Hartree heart-muscle preparation, and by ferricyanide catalysed by the purified dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1). We have also investigated how the Arrhenius plot is affected by activation of the enzyme (*cf.* refs. 14–16).

RESULTS

Effect of temperature on the succinate oxidase system

Fig. 1 shows that the Arrhenius plot for the Keilin and Hartree succinate oxi-

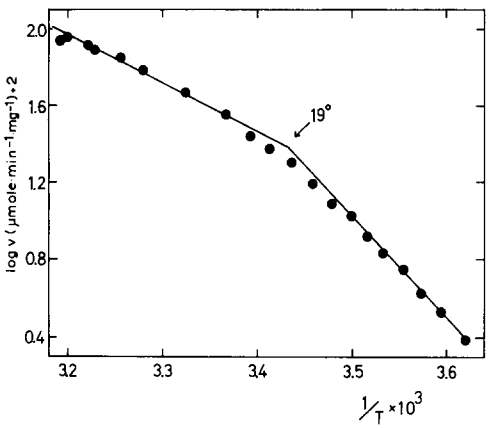


Fig. 1. The effect of temperature on succinate oxidase. The medium contained Keilin and Hartree heart-muscle preparation, 0.55–2 mg; 35 μM cytochrome *c*; 20 mM succinate; 0.1 M phosphate buffer (pH 7.6); 1 mM EDTA; volume, 1.5 ml.

dase system consists of two straight lines, with a transition at 19°C. In three other experiments transition temperatures between 17°C and 19°C were found. The Arrhenius activation energies are 12 kcal/mole above 18 C°, and 24 kcal/mole below 18°C.

Kinetics of soluble succinate dehydrogenase at different temperatures

In an attempt to determine the effect of temperature on the rate constants of the individual reaction steps of the enzyme-catalysed reaction, the kinetic analysis previously described[16] was repeated at different temperatures. Malonate, added in the previous study in order to increase the accuracy of the calculations, was omitted, because of the effect of temperature on the degree of inhibition.

Fig. 2 shows some typical examples out of a series of experiments carried out at 12 different temperatures with non-activated enzyme. Similar results were obtained with enzyme activated by incubation with succinate. From Fig. 2 it is clear that the Lineweaver–Burk plots are straight lines at the higher temperatures only; at 11°C and 3.3°C there is a strong inhibition by high concentrations of ferricyanide. This inhibition is abolished by increasing the succinate concentration.

The plots of $1/v$ against 1/[succinate] (Fig. 3) are non-linear. The saturation curves (v against s) at 15°C and lower show an intermediary plateau region that is absent at the higher temperatures (see Fig. 4).

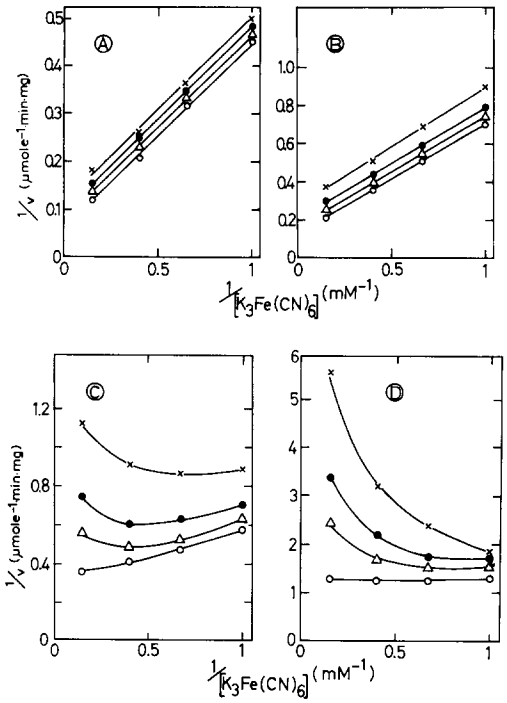
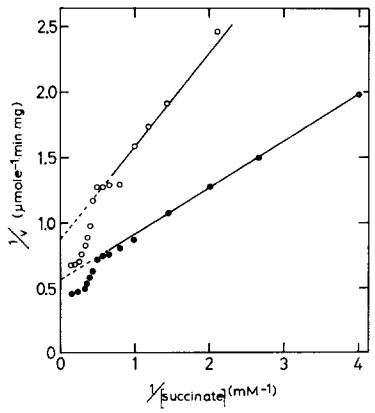


Fig. 2. Kinetics of soluble succinate dehydrogenase at different temperatures. The medium contained: succinate dehydrogenase, 0.1–0.5 mg/ml; 0.1 M phosphate buffer (pH 7.8); 1 mM EDTA; 1 mg/ml bovine serum albumin. The succinate concentrations were: ×, 0.5 mM; ●, 1 mM; △, 2 mM; ○, 5 mM. The ferricyanide concentrations were varied as indicated. The temperatures were: A, 42°C; B, 25°C; C, 11°C; D, 3.3°C.



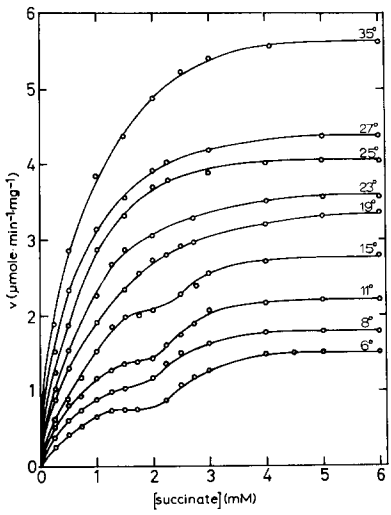


Fig. 3. Double-reciprocal plots with succinate. The ferricyanide concentration was 6 mM. Other conditions as in Fig. 2. ●, 11°C; ○, 6°C.

Fig. 4. Saturation curves with succinate. The ferricyanide concentration was 6 mM. Other conditions as in Fig. 2.

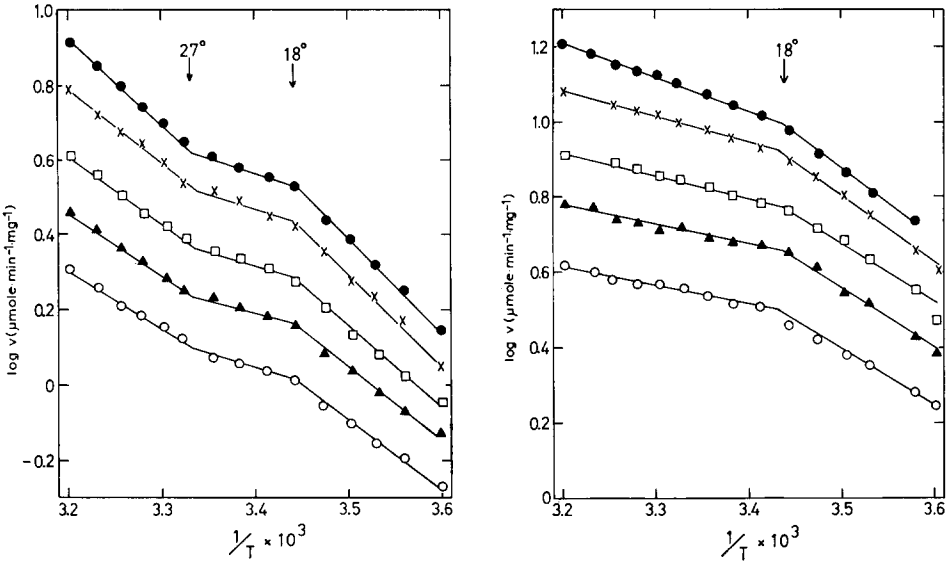


Fig. 5. Arrhenius plots for non-activated succinate dehydrogenase. The succinate concentration was 0.2 M. The ferricyanide concentrations were: ○, 1 mM; ▲, 1.5 mM; □, 2.5 mM; ×, 4 mM; ●, 6 mM. Other conditions as in Fig. 2.

Fig. 6. Arrhenius plots for activated succinate dehydrogenase. Conditions as in Fig. 5. The enzyme was activated before the experiment by incubation at a concentration of 10 mg/ml with 20 mM succinate for 5 min at 38°C under anaerobic conditions.

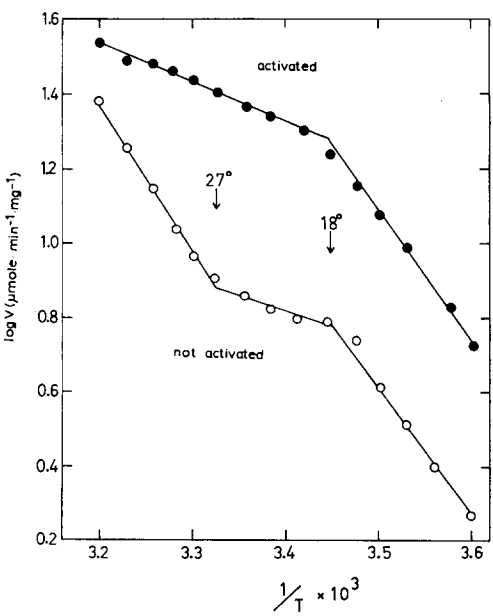
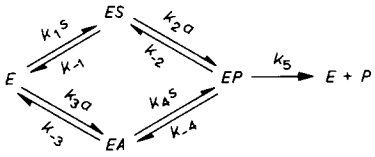


Fig. 7. Arrhenius plots for maximal velocities (at infinite ferricyanide concentration) obtained from the data of Figs. 5 and 6.

The anomalous kinetics make it impossible to determine rate constants at different temperatures. Therefore it was decided to determine only the maximal velocity at infinite succinate and ferricyanide concentrations, by measuring the reaction rates at a concentration of succinate (0.2 M) very nearly sufficient to saturate the enzyme, and extrapolating to infinite ferricyanide concentration. Figs. 5 and 6 show a series of Arrhenius plots obtained at different ferricyanide concentrations, before and after activation of the enzyme. In Fig. 7 the Arrhenius plots for maximal velocities are shown.

DISCUSSION

At low temperatures the kinetics of soluble succinate dehydrogenase show two types of anomalies: (1) a marked inhibition by ferricyanide, except in the presence of high concentrations of succinate; (2) saturation curves (v against [succinate]) that show an intermediary plateau region. Since there is no evidence that succinate dehydrogenase, which is a monomer[11,17], contains more than one succinate-binding site, it is unlikely that the mechanism proposed by TEIPEL AND KOSHLAND[18] for such cases, which requires multiple binding sites, is applicable. An alternative-pathway mechanism (*cf.* refs. 19 and 20), which can also give rise to substrate inhibition and sigmoidal saturation curves[21], is the more likely. Such a mechanism may be represented schematically by



It is assumed that the order of reaction of the enzyme with succinate (S) and the acceptor (A) is random. If the reaction rate *via* the upper pathway is equal to v_s and that *via* the lower pathway to v_a, then the following rate equations can be derived:

$$v_s = \frac{e}{\frac{k_3}{k_1k_2} + \frac{(k_{-1} + k_2a)}{(k_{-3} + k_4s)}\left(\frac{1}{s} + k_4\right) + \frac{1}{k_5} + \frac{1}{k_2a} + \frac{k_{-1}}{k_1k_2as} + \frac{1}{k_1s}}$$

$$v_a = \frac{e}{\frac{k_1}{k_3k_4} + \left(\frac{k_{-3} + k_4s}{k_{-1} + k_2a}\right)\left(\frac{1}{a} + k_2\right) + \frac{1}{k_5} + \frac{1}{k_4s} + \frac{k_{-3}}{k_3k_4as} + \frac{1}{k_3a}}$$

The total reaction rate is then equal to $v_t = v_s + v_a$.

The complexity of these rate equations permits only a qualitative approach. The value of v_s is dependent upon a/s, a higher value for this ratio leading to a lower velocity, and this will lead to inhibition of the enzyme activity when $v_s > v_a$. Thus, at a fixed value of s, a will lower v_s and the resultant inhibition will be overcome by raising s. Similarly, v_a will be lowered by succinate and increased by adding ferricyanide, under conditions when v_s is already near maximum.

Fig. 8 shows how a saturation curve of the type shown in Fig. 4 may be a composite curve of two components.

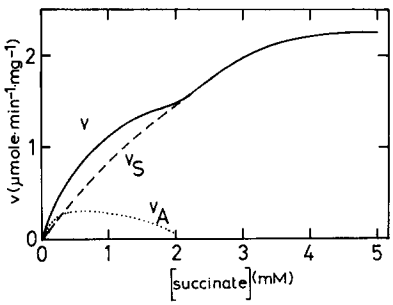


Fig. 8. Possible composition of a saturation curve from Fig. 4.

At higher temperatures the plateau in the saturation curves becomes less significant, presumably due to a decreasing contribution of v_a. This could mean that at higher temperatures the affinity of the enzyme for ferricyanide is decreased.

The experiments shown in Figs. 5 and 6 were carried out at a very high succinate concentration, in which case v_a will make little contribution to the total reaction rate, and there will also be little inhibition by ferricyanide. The maximal velocity will then be proportional to only one rate constant, *viz.* k_5. This mechanism is, however, a simplified case (*cf.* ref. 16). From Figs. 5 and 6 it appears that the slope of the Arrhenius plots increases with increasing ferricyanide concentration. This means that at low ferricyanide concentrations another rate-limiting step with a lower activation energy is operating. By extrapolation it is possible to determine the value of the slope of the Arrhenius plot at zero acceptor concentration, from which can be calculated the activation energy of the rate-limiting oxidation of reduced enzyme by ferricyanide. Within the experimental error, the activation energy for the non-activated enzyme in the ranges 3–17°C and 27.5–40°C, and for the activated enzyme in the range 2–17°C, calculated in this way is the same, *viz.* 7.0 kcal/mole. This is in agreement with the earlier conclusion[16,22] that activation increases only the rate of intramolecular transfer of reducing equivalents from substrate bound to the enzyme to acceptor groups in the protein.

The breakpoint at 18°C in the Arrhenius plots is found with both the purified enzyme and the succinate oxidase system. In Table I a summary is given of the calculated activation energies. The difference in the experimental activation energies

TABLE I

ACTIVATION ENERGIES FOR OXIDATION OF SUCCINATE

System	*Temperature region (°C)*	E_a	
		kcal/mole	*kJ/mole*
Succinate oxidase	3–19	24	100
	19–41	12	50
Soluble enzyme, activated	2–17	16	67
	17–40	4.8	20
Soluble enzyme, non-activated	3–17	18	75
	17–27.5	4.6	19
	27.5–40	19	80

between activated and non-activated (16 and 18 kcal/mole, respectively) appears to be greater than to be expected from the observed difference in catalytic-centre activity in the region 2–17°C. If this is true, the entropy of transition of the rate-limiting step in the reaction catalysed by the activated enzyme, *e.g.* the intramolecular transfer of reducing equivalents, is about −5 cal/mole per °K lower, which differs

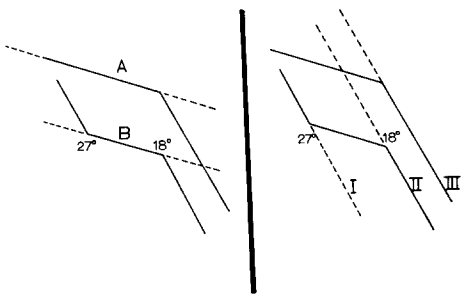


Fig. 9. Two different ways of extrapolating the Arrhenius plots of Fig. 7.

from expectations expressed in previous work[16]. However, the inaccuracy of the extrapolated values obtained with this enzyme is such that definite conclusions should not be drawn from this difference.

The results reported with the purified enzyme may be explained by assuming a temperature-dependent equilibrium between different conformations of the enzyme, differing in catalytic-centre activity (*cf.* refs. 6, 23). Incubation with succinate shifts the equilibrium towards the more active form. On this basis, the Arrhenius plots in Fig. 7 may be interpreted in two possible ways, as illustrated in Fig. 9. (1) There are two conformations, A and B, with high and low catalytic activity, respectively. At temperatures above 27°C the equilibrium shifts towards conformation A, and prior incubation with succinate stabilizes form A. In this case the regions above 27°C and below 18°C are transition regions. This would mean, of course, that a third conformation exists, stable at low temperatures. We have ,however, found no indications of a third breakpoint, in experiments at temperatures as low as 2°C. (2) There are three conformations, I, II and III, with transition regions between 18°C and 27°C for the non-activated enzyme and above 18°C for the activated. Conformation I has a lower catalytic-centre activity than Conformation II.

The first explanation seems the simpler. The equilibrium constant $K = [B]/[A]$ has been calculated for the points in the transition region between 18°C and 27°C, and from the van 't Hoff plot (Fig. 10) the values of ΔH and ΔS for the conformational transition were found to be −41 kcal/mole and −130 cal/mole per °K, respectively. On the basis of the second possibility, it may be calculated that for the transition I → III, $\Delta H = -32$ kcal/mole and $\Delta S = -113$ cal/mole per °K, and for the transition I → II, $\Delta H = -52$ kcal/mole and $\Delta S = -165$ cal/mole per °K.

The identical values of the activation energies (7 kcal/mole) at zero ferricyanide indicates that the second explanation is the more likely. If the first explanation were correct one would expect that the activation energies would increase on lowering the ferricyanide concentration.

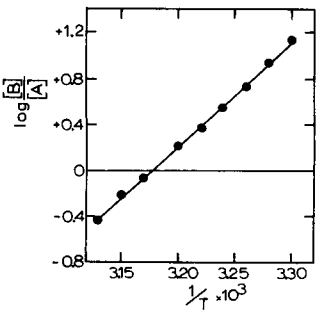


Fig. 10. Van 't Hoff plot for the equilibrium between conformations A and B (*cf.* Fig. 9), determined from the transition region above 27°C in the Arrhenius plot of the non-activated enzyme (Fig. 7).

From our results it may be concluded that the activation of succinate dehydrogenase is a conformational change in the enzyme, whereby the activation enthalpy remains unchanged, but the catalytic-centre activity is increased. The activated and non-activated forms are in a temperature-dependent equilibrium (*cf.* ref. 21).

EXPERIMENTAL

Succinate dehydrogenase (succinate:(acceptor)oxidoreductase, EC 1.3.99.1) was isolated from pig heart by the method of WANG *et al.*[11] as previously described[16]. Reaction velocities were measured as described previously[16]. Succinate oxidase activity was measured by following the oxygen consumption in an Oxygraph (Gilson Medical Electronics), fitted with a Clark-type electrode. The temperature of the reaction media was kept constant with a 'Colora' cold-thermostat and checked after the reaction with a telethermometer supplied by Yellow Springs Instrument Co. The reaction mixtures for the polarographic experiments were saturated with air at each temperature. The oxygen concentrations were calculated using data from the literature[24–27]. Protein was estimated by the biuret method ,after precipitation with 5% trichloroacetic acid.

Succinate was from Boehringer, bovine serum albumin from Sigma, potassium ferricyanide from Merck. All other chemicals were from the British Drug Houses.

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