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STUDIES ON SUCCINATE DEHYDROGENASE

V. INHIBITION BY OXALOACETATE

W. P. ZEYLEMAKER, A. D. M. KLAASSE AND E. C. SLATER

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

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SUMMARY

1. Oxaloacetate inhibits succinate dehydrogenase in two ways; the primary inhibition occurs immediately, the other develops slowly.

2. Both the primary and secondary inhibitions are competitive, with K_i values of 4 μM and 0.4 μM, respectively.

3. As a result of the slow irreversibility of the secondary inhibition, oxaloacetate behaves as a stoicheiometric, pseudo-irreversible inhibitor, if the enzyme is pre-incubated with the inhibitor, and initial rates are measured.

4. From optical titrations of the enzyme with oxaloacetate a dissociation constant of 4 μM was found.

5. Stopped-flow measurements of the rate of formation of the spectrally detectable enzyme–oxaloacetate complex indicate that this reaction is much more rapid than the secondary inactivation reaction.

6. The results are explained by assuming the mechanism: $E + I \rightleftharpoons EI_{\mathrm{I}} \rightleftharpoons EI_{\mathrm{II}}$, in which the first equilibrium is rapidly established, and the second only slowly. The equilibrium constant of the first reaction is about 4 μM, and that of the second about 0.1 μM.

INTRODUCTION

Oxaloacetate is a powerful inhibitor of the succinate oxidation, as was first shown in 1936 by DAS[1]. PARDEE AND POTTER[2], using particulate preparations, showed that the inhibition is competitive in nature; this was confirmed by other authors with the soluble enzyme[3,4]. However, CHAPPELL[5] and WOJTCZAK and co-workers[6,7] reported that the inhibition of succinate dehydrogenase by oxaloacetate is of a mixed type. Upon addition of oxaloacetate the rate of succinate oxidation gradually decreases, but finally reaches a steady state. When initial reaction velocities were measured, a non-

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

competitive inhibition was obtained, and a typically competitive type of inhibition was found with the steady-state rate of oxidation. In the latter case an inhibitor constant of 0.2 μM was found. Inhibitor constants for the competitive inhibition ranging from 1.5 to 3 μM have been reported by various authors, using different types of preparations[2–4,8].

We have examined the mechanism of inhibition of succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) by oxaloacetate, using soluble succinate dehydrogenase[9]. A preliminary report has appeared[10].

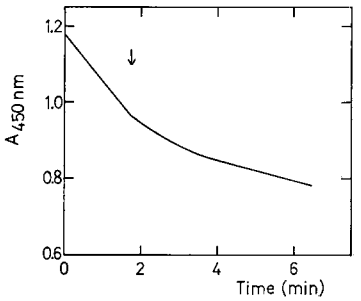


Fig. 1. Inhibition of succinate dehydrogenase by oxaloacetate. The reaction mixture contained 0.1 M phosphate buffer (pH 7.8), 1 mM EDTA, 1 mg/ml bovine serum albumin, 5 mM potassium ferricyanide and 10 mM succinate. The reaction was started by addition of enzyme to a final concentration of 0.24 μM. At the arrow oxaloacetate was added to a concentration of 12 μM.

RESULTS

Kinetic measurements

In Fig. 1 it is seen that succinate oxidation is inhibited immediately after addition of oxaloacetate, and the inhibition increases with time, until a constant value is reached. We found that, under the conditions of Fig. 1, the addition of 1 mM fumarate had no appreciable effect on the reaction rate in the absence of oxaloacetate, or on the degree of inhibition by oxaloacetate. Thus, the increasing inhibition can not be due to the formation of fumarate during the reaction. The primary and secondary inhibitions were further studied.

In Fig. 2 initial rates were measured in the presence and absence of oxaloacetate. In disagreement with WOJTCZAK and co-workers[6,7] this primary inhibition is purely competitive. From the double-reciprocal plots K_i values of 4.3 and 4.5 μM may be calculated. Similar results were obtained over a large range of oxaloacetate concentrations (from 0.6 to 135 μM) and succinate concentrations (from 0.2 to 135 mM). The K_i values varied between 1.7 and 6.3 μM in 14 measurements, with an average value of 4.5 μM. In the experiment of Fig. 2, the reactions were started by the addition of enzyme to an otherwise complete reaction mixture, but it made no difference when the order of addition was the same as in Fig. 1.

The secondary inhibition was studied in the experiment shown in Fig. 3. This inhibition is also purely competitive. From the double-reciprocal plots K_i values of 0.35, 0.41 and 0.5 μM can be calculated. In four other experiments of this type K_i values were found between 0.25 and 0.6 μM, with a mean value of 0.4 μM.

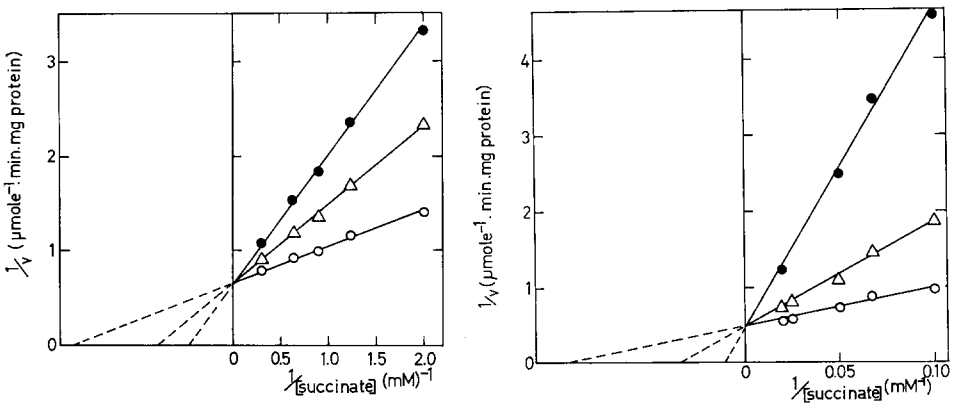


Fig. 2. Primary inhibition by oxaloacetate. Conditions as in Fig. 1, but with different concentrations of succinate. The reaction was started by addition of enzyme (final concentration, 0.32 μM). Initial reaction rates were measured. ○, no oxaloacetate; △, 5.4 μM oxaloacetate; ●, 10.8 μM oxaloacetate.

Fig. 3. Secondary inhibition by oxaloacetate. Conditions as in Fig. 1, with different concentrations of succinate. The reaction was started by addition of enzyme (final concentration, 0.44 μM). The reaction rate was recorded after the reaction velocity had become constant (*cf.* Fig. 1). ○, 18 μM oxaloacetate; △, 36 μM oxaloacetate; ●, 54 μM oxaloacetate. The K_m value without oxaloacetate was determined in a separate experiment, using lower succinate concentrations; a value of 0.31 mM was found.

Incubation experiments

Because the secondary inhibition occurs very slowly, we have carried out experiments in which the enzyme was incubated with oxaloacetate and the reactions started by the addition of the enzyme–oxaloacetate mixture to an otherwise complete reaction mixture. In these experiments, the concentrations of oxaloacetate were of

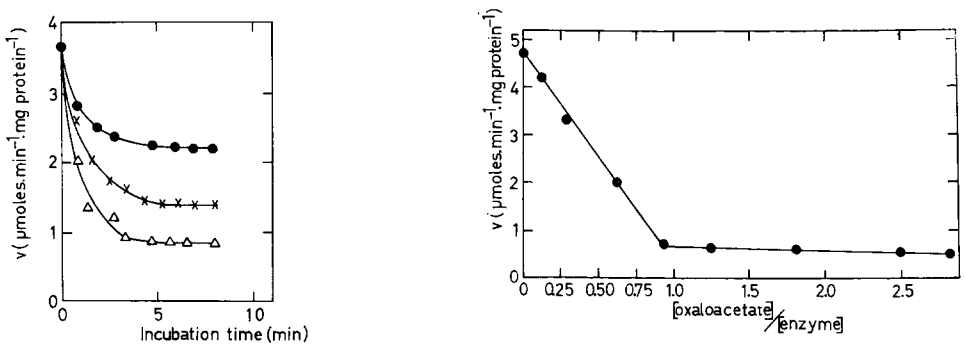


Fig. 4. Incubation of succinate dehydrogenase with oxaloacetate. The enzyme, in a concentration of 11 μM, was preincubated with different concentrations of oxaloacetate. The reaction medium was as in Fig. 1. The reaction velocity was measured after different incubation times. (The reactions were started by addition of the enzyme–oxaloacetate mixture). The oxaloacetate concentrations during the preincubation were: ○, 2.8 μM; ×, 5.5 μM; △, 11.1 μM.

Fig. 5. Titration of the enzyme activity with oxaloacetate. An enzyme solution (concentration 8.0 μM) was preincubated with different concentrations of oxaloacetate. The enzyme activity was measured after maximal inactivation had been reached (10 min, *cf.* Fig. 4). The reaction mixture contained 2.5 mM potassium ferricyanide and 20 mM succinate, and other constituents as in Fig. 1. The reactions were started by the addition of 0.2 ml of the enzyme–oxaloacetate mixture.

the same order of magnitude as the enzyme concentration. In Fig. 4 we see that in this case the activity of the enzyme reaches a minimum value after about 5 min incubation with oxaloacetate. In the experiment shown in Fig. 5 the enzyme was titrated with oxaloacetate. The enzyme activity decreases linearly with increasing oxaloacetate concentration, until the enzyme concentration is reached; there is then a sharp bend, after which the activity decreases very slowly with further increase of the oxaloacetat concentration. This indicates that oxaloacetate behaves as an irreversible, stoicheiometric inhibitor (*cf.* refs. 11–13), binding with the enzyme in a 1:1 ratio. This is also illustrated by the experiment of Fig. 6; here different amounts of enzyme were incubated with a constant concentration of oxaloacetate. It is seen that in the presence of oxaloacetate, the slope of the line of velocity *versus* enzyme concentration is about one-eighth of that without oxaloacetate. When, however, the enzyme concentration is higher than the oxaloacetate concentration, these slopes differ only by 20%. The same result was obtained in a similar experiment by ACKERMAN AND POTTER[13], who studied the effect of different irreversible inhibitors including oxaloacetate on particulate succinate oxidase preparations. It is important to note that in Fig. 5 the deflection point occurs before the reaction velocity is zero, and also that in Fig. 6 the slope of the line relating reaction rate and enzyme concentration is not initially zero in the presence of oxaloacetate. This is in contrast to the picture one would expect for a simple irreversible inhibitor (*cf.* ref. 11).

In the experiments of Figs. 5 and 6, after incubation of the enzyme with oxaloacetate, the reaction rates were measured in the presence of a high concentration of succinate; in a next experiment, the results of which are shown in Fig. 7, the succinate concentration was varied. It is seen that, when the oxaloacetate concentration during

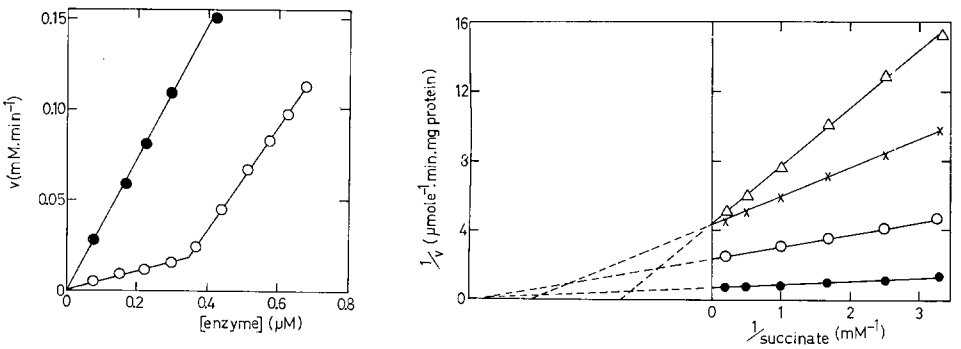


Fig. 6. Titration of oxaloacetate with different concentrations of enzyme. Conditions as in Fig. 5, but here the oxaloacetate concentration was constant and the enzyme concentration was varied. The reactions were started by adding 0.1 ml of the enzyme–oxaloacetate mixture to 2.9 ml of reaction mixture. On the abscissa the final enzyme concentrations during measurement of enzyme activity are given. The final oxaloacetate concentration was 0.35 μM. ●, no oxaloacetate during preincubation; ○, 10.5 μM during preincubation, *i.e.* 0.35 μM during measurement of enzyme activity.

Fig. 7. Lineweaver–Burk plots after incubation with oxaloacetate. An enzyme solution (concentration 50 μM) was incubated with different concentrations of oxaloacetate, until maximal inactivation was reached. The reaction mixture was the same as in Fig. 5, except that different succinate concentrations were used. The oxaloacetate concentrations during the preincubation were: ●, no oxaloacetate; ○, 30 μM oxaloacetate; ×, 90 μM oxaloacetate; △, 180 μM oxaloacetate.

the preincubation is lower than the enzyme concentration, only V (the maximal velocity at infinite succinate concentration) is lowered, without any change in the K_m for succinate. If, however, the oxaloacetate concentration is higher than the enzyme concentration, there is also an effect on K_m, and further increase of oxaloacetate causes no further change in V. Apparently, the inhibition is non-competitive when the oxaloacetate concentration is less than that of the enzyme, and is mixed competitive–non-competitive with concentrations of oxaloacetate that exceed that of the enzyme.

Spectral measurements

Addition of oxaloacetate to succinate dehydrogenase causes spectral changes, particularly the appearance of a broad absorption band with a maximum at 600 nm (*cf.* refs. 4, 14 and 15). Fig. 8 shows a titration curve, from which a dissociation constant of the enzyme–oxaloacetate complex (K_D) of 4 μM may be calculated. In 5 similar experiments K_D values were obtained between 2.8 and 4.6 μM, with a mean value of 4.1 μM.

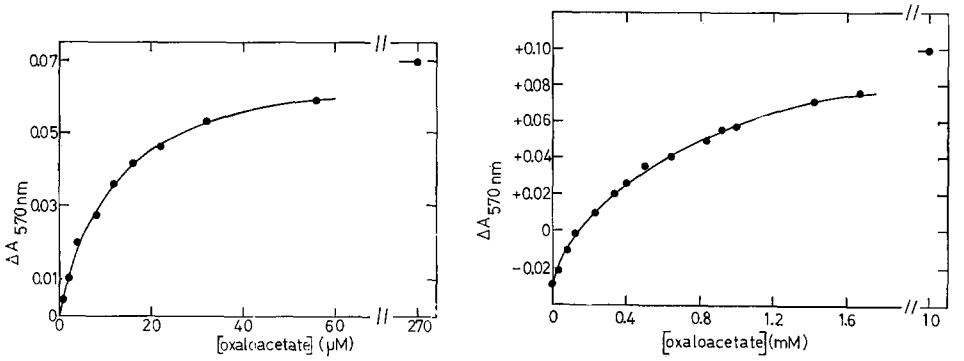


Fig. 8. Spectrophotometric titration of succinate dehydrogenase with oxaloacetate. To 1.8 ml of enzyme (concentration 17.5 μM) in a 1-cm cell various amounts of 1 mM oxaloacetate were added from a Hamilton microsyringe. Finally an excess oxaloacetate was added in the form of a 10 mM solution. The total volume added was 0.3 ml. The absorbances were corrected for dilution. The initial absorbance was 0.186.

Fig. 9. Spectrophotometric titration of succinate dehydrogenase with oxaloacetate, in the presence of 10 mM succinate. To 1.8 ml of enzyme (concentration, 29 μM; initial absorbance at 570 mμ, 0.326), 0.2 ml 100 mM succinate was added. This caused a decrease in absorbance of 0.029. Different amounts of 10 mM oxaloacetate were then added from a Hamilton microsyringe. Finally an excess oxaloacetate was added in the form of a 0.2 M solution. The total volume added was 0.5 ml. When oxaloacetate alone was added in excess, an absorbance increase of 0.099 was found.

In another type of experiment the enzyme was first saturated with succinate (see Fig. 9). This causes an absorption decrease with a maximum at 460 nm, but at the wavelength used in Fig. 9 (570 nm) the decrease is very small (*cf.* refs. 4 and 15). The enzyme–succinate complex was then titrated with oxaloacetate. The advantage of this procedure over that shown in Fig. 8 is that the concentrations of oxaloacetate added are much higher than the enzyme concentration, and therefore it is not necessary to correct the oxaloacetate concentrations for the amount of oxaloacetate bound to the enzyme. In this system the following equilibria occur:

$$E + S \rightleftharpoons ES \qquad (K_S)$$

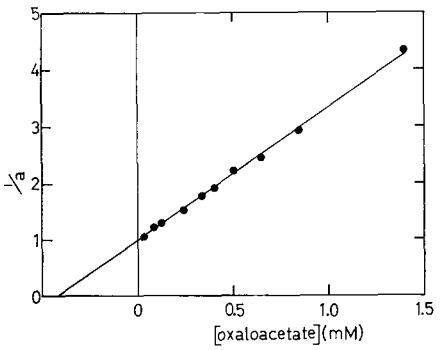
$$E + O \rightleftharpoons EO \qquad (K_O)$$

where, E, S and O represent enzyme, succinate and oxaloacetate, respectively, and K_S and K_O are the dissociation constants of the enzyme–succinate and enzyme–oxaloacetate complexes, respectively. Under the conditions in Fig. 9, virtually all the enzyme is bound in these complexes. When a fraction (a) of the enzyme is present as (ES), then the following relation holds:

$$1/a = 1 + (K_S \cdot [O]/K_O \cdot [S])$$

Since we know the absorbance of E, ES and EO, the value of a may be calculated for every point in the titration curve. In Fig. 10 a plot is shown of $1/a$ against $[O]$. The intercept of this graph on the abscissa is equal to $-K_O[S]/K_S = 0.42$ mM. The value of K_S was determined from a titration of the enzyme with succinate, followed at 460 nm; we found $K_S = 0.1$ mM (*cf.* refs. 15–17). Since $[S] = 10$ mM, $K_O = 4.2\ \mu$M.

The rate of formation of the enzyme–oxaloacetate complex was studied in a stopped-flow apparatus (for the details of the procedure see ref. 18). In Fig. 11 it is seen that under conditions where the reaction leading to irreversible inactivation has a $t_{1/2}$ of 1 min (see Fig. 4), the spectrally detectable enzyme–oxaloacetate complex is formed in a reaction with a $t_{1/2} = 0.8$ sec.



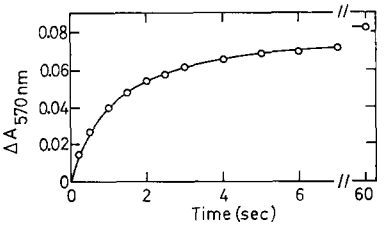


Fig. 10. Plot of $1/a$ against [oxaloacetate], calculated from Fig. 9 (for details see the text).

Fig. 11. Rate of formation of the spectral detectable enzyme–oxaloacetate complex. At $t = 0$ 15 μM enzyme was mixed with 20 μM oxaloacetate in a stopped-flow apparatus. The increase in absorbance was recorded in time (for the details of the procedure see ref. 18).

DISCUSSION

The results may be explained by assuming the following mechanism for the interaction between succinate dehydrogenase and oxaloacetate:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(p)}{EI_I} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{(q)}{EI_{II}}$$

Since the secondary inhibition sets in slowly, both k_2 and k_{-2} are small relative to k_1 and k_{-1}. The instantaneous inhibition by oxaloacetate (see Fig. 2) is explained by the formation of EI_I. Thus $K_i = k_{-1}/k_1 = 4.5\ \mu$M.

When the second equilibrium has been established, as in Fig. 3, the following rate equation applies:

$$v = \frac{V}{1 + \frac{K_m}{s}\left\{1 + \frac{[I]}{K_j}\left(1 + \frac{1}{K_l}\right)\right\}} \quad (1)$$

where V is the velocity at infinite succinate concentration (s), K_m is the Michaelis constant for succinate, $[I]$ is the free oxaloacetate concentration, and $K_l = k_{-2}/k_2 = p/q$. The apparent inhibition constant is equal to $K_j/(1+1/K_l)$. From the value of this inhibition constant, calculated from Fig. 3, *viz.* 0.4 μM, and putting $K_j = 4.5\ \mu$M, it follows that $K_l = 0.1$.

In the experiments in which enzyme was pre-incubated with low concentrations of oxaloacetate, the following equilibrium equations are applicable

$$(e - p - q)(i - p - q) = K_j p \quad (2)$$

$$p = K_l q \quad (3)$$

where e and i are the total concentrations of enzyme and oxaloacetate, respectively. From Eqns. 2 and 3, it follows that

$$p^2(1 + 1/K_l)^2 - p\{K_j + (e + i)(1 + 1/K_l)\} + ei = 0 \quad (4)$$

Under the conditions of the experiments shown in Figs. 4 and 5, $(e+i)(1+1/K_l)$ is much greater than K_j, so that Eqn. 4 simplifies to

$$p^2(1 + 1/K_l)^2 - p(e + i)(1 + 1/K_l) + ei = 0 \quad (5)$$

which yields

(i) for $i < e$, $p = i/(1 + 1/K_l)$, *i.e.* $q = i/(1 + K_l)$ and $p + q = i$

(ii) for $i < e$, $p = e/(1 + 1/K_l)$, *i.e.* $q = e/(1 + K_l)$ and $p + q = e$

Thus, when the oxaloacetate concentration is lower than that of the enzyme, all the oxaloacetate is bound to the enzyme, and when the oxaloacetate concentration exceeds that of the enzyme, all of enzyme is bound to oxaloacetate.

In these experiments, a high concentration of succinate was used, sufficient rapidly to convert EI_I to ES, but, because of the low value of k_{-2}, little EI_{II} is converted to ES. The activity measured, therefore, refers to the concentration of enzyme not bound as EI_{II} at the end of the preincubation. In Fig. 5 the activity at the intersection point is lower than that of the uninhibited reaction by a factor of 7.5, corresponding with $K_l = 0.13$. In Fig. 6, in the presence of oxaloacetate, the slope of the first part of the line is one seventh that in the absence of oxaloacetate, corresponding with $K_l = 0.14$. Finally, in Fig. 7 it is seen that, after incubation with oxaloacetate, the maximal velocity V can be lowered by maximally a factor of 6.8, giving $K_l = 0.15$. These values of K_l are all in reasonable agreement with the value calculated from the experiment of Fig. 3, *viz.* 0.1.

The competitive part of the inhibition in Fig. 7 is due to the oxaloacetate that is not bound in EI_{II}. Knowing the concentrations of enzyme and oxaloacetate, and K_l, the concentration of free oxaloacetate can be calculated, and from the effect on

K_m we can calculate K_j. From the experiment we find $K_j = 4.7$ and 5.3 μM; these values are in good agreement with that calculated from Fig. 2.

The spectral changes occurring upon addition of oxaloacetate to the enzyme must be ascribed to the formation of EI_I, and we may conclude that the transition $EI_I \rightarrow EI_{II}$ causes no further spectral changes. This conclusion is based on the observations that: (i) the spectral titrations gave a dissociation constant of 4 μM, which agrees with the value of K_j calculated from kinetic experiments; (ii) in the stopped-flow experiments it was found that the rate of formation of the spectrally detectable enzyme–oxaloacetate complex was much greater than the rate of the difficultly reversible inactivation.

From our results it is clear that oxaloacetate behaves as a "pseudo-irreversible" inhibitor of succinate dehydrogenase (*cf.* ref. 11). This is especially evident from the experiments of Figs. 5–7. This irreversibility is due to the fact that the equilibrium between EI_I and EI_{II} is established very slowly, so the enzyme stays "frozen" in the EI_{II}-complex, when initial rates are measured. A similar case has been reported by KERNOHAN[19] for the inhibition of carbonic anhydrase by sulfonamides.

According to the differences between K_j and K_i, the difference in binding energy between the two complexes is about 1.4 kcal/mole. The readily reversible primary complex of succinate dehydrogenase is probably due to the interaction between the two carboxylate groups and cationic groups in the enzyme (*cf.* refs. 3, 8). It is possible that the secondary complex is the result of interaction between the enzyme and the carbonyl function in the oxaloacetate. An interaction of this type is suggested by the finding by HELLERMAN *et al.*[3] that the monoethyl carboxylic ester of oxaloacetate is a competitive inhibitor of succinate dehydrogenase, with a K_i of about 40 μM. The slow interconversion of the two complexes may be explained in two ways. (i) The reaction represents a change in conformation of the enzyme to one with a higher affinity towards oxaloacetate. In this respect it is interesting to note that succinate dehydrogenase can exist in two different conformations, with different catalytic activities; by incubation with succinate or competitive inhibitors ("activation"), the enzyme can be converted in the active form[20,21]. However, the slow inactivation by oxaloacetate is given both with enzyme preparations that were not activated and with those fully activated by incubation with succinate[17]. There are also indications for a temperature-dependent conformational change in succinate dehydrogenase (W. P. ZEYLEMAKER AND H. JANSEN, unpublished). The rate of inactivation by incubation is not very dependent on temperature: at 10° the half time of the reaction was 1.3 min; at 25° 1 min,and at 35° 0.6 min. The low activation energy of the inactivation reaction makes it unlikely that a large part of the enzyme molecule is involved in any conformational change. (ii) The slow inactivation reaction is caused by a tautomerization of oxaloacetate, from the keto to the enol form (*cf.* refs. 22 and 23). The free acid in solid form is completely in the enol form, but when it is dissolved in water ketonization occurs; under the conditions of our experiments, at pH 7.8, 80–85% of the acid is in the keto form (*cf.* refs. 24–27). The tautomerization is indeed a very slow reaction, with a half time of several minutes[24,25]. The slow inactivation by oxaloacetate might be due to a more firm binding of the tautomer present in lower concentration, *i.e.* the enol form.

A definite conclusion about the nature of the slow secondary inactivation can not yet be drawn.

METHODS

Succinate dehydrogenase was isolated from pig heart by the method of WANG *et al.*[9], as modified by KEILIN AND KING[28] and DERVARTANIAN AND VEEGER[4]. The preparation was kept in small amounts in liquid nitrogen, and was always used within 15 min after thawing.

Activity measurements were carried out with ferricyanide as electron acceptor, as described before[17]. When measured under standard conditions[9,17] the activity was between 3 and 4.5 μmoles succinate/min per mg protein. The activity could be increased by a factor of 2 when the enzyme was first incubated with succinate[20,21].

Kinetic measurements were carried out as previously described[17], with ferricyanide as electron acceptor.

The temperature in all experiments was 25°.

The enzyme concentration was determined on the basis of the FAD content of the preparations (*cf.* ref. 17). Protein was determined by the biuret method, after precipitation with 5% trichloroacetic acid.

Oxaloacetate was dissolved in 0.1 M phosphate buffer, and neutralized carefully. The oxaloacetate concentration was measured enzymatically, using malate dehydrogenase and NADH.

Potassium ferricyanide was from Merck, succinate and malate dehydrogenase from Boehringer, oxaloacetate from Fluka and from Sigma. All other chemicals were from British Drug Houses.

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