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THE INHIBITION OF SUCCINATE DEHYDROGENASE BY OXALOACETATE

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SUMMARY

The inhibition of soluble succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) by oxaloacetate is preceded by a lag during which the degree of the inhibition gradually increases. A similar lag precedes a partial reversal of the inhibition by increasing the concentration of substrate.

Succinate dehydrogenase, which has been pretreated with oxaloacetate and from which most of the oxaloacetate has been removed, does not exhibit its full activity unless preincubated with succinate or malonate; preincubation with phosphate or micellar phospholipids is also partly effective.

The ability of soluble succinate dehydrogenase to reconstitute the succinate oxidase system is irreversibly lost after pretreatment with oxaloacetate.

It is suggested that oxaloacetate may either bind rather tightly with the enzyme or promote a reversible conformation change leading from the active form of the dehydrogenase to an inactive one.

INTRODUCTION

Oxaloacetate is known as a potent inhibitor of succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1)[1,2]. The inhibition is regarded as competitive[3]; however, there have been only few more detailed kinetic studies on this inhibition (*e.g.* ref. 4). In a preliminary communication[5], we have shown that oxaloacetate differs from other competitive inhibitors of succinate dehydrogenase in the sense that the inhibition by oxaloacetate is established and is also released, only slowly. This is manifested by the fact that the inhibition is preceded by a lag and that the enzyme which has been treated with oxaloacetate is inactive, or not fully active, unless preincubated with the substrate. ZEYLEMAKER AND SLATER[6] re-examined our results and found a biphasic inhibitory effect of oxaloacetate, an immediate inhibition with K_i between 1.7 and 6.3 μM and a secondary inhibition, which is established after a few minutes following the addition of oxaloacetate, with K_i of 0.6–0.7 μM. They disagree with us in the point that the effect of oxaloacetate is not instantaneous, but they fully

Abbreviations: PMS, phenazine methosulphate; DCIP, 2,6-dichlorophenolindophenol.

confirm our results showing that a constant degree of the inhibition is obtained only after a lag.

The present paper describes in more detail the effect of oxaloacetate on the soluble succinate dehydrogenase. Two explanations of the peculiar effect of oxaloacetate are discussed: (i) that oxaloacetate is rather firmly bound to the enzyme and (ii) that it promotes the transformation of the active succinate dehydrogenase into an inactive form (allosteric effect).

MATERIAL AND METHODS

Three procedures for the preparation of soluble succinate dehydrogenase have been used. (1) Crude soluble succinate dehydrogenase was obtained using alkaline extraction as described by KING[7], with the exception that frozen beef-heart sarcosomes and not the KEILIN–HARTREE[2] preparation were the starting material and that 1 μM rotenone was routinely added during the initial incubation of the sarcosomes with succinate. Both the heavy, as well as the light, fractions of sarcosomes, isolated as described by LÖW AND VALLIN[8], were equally suitable. (2) A crude succinate dehydrogenase was also obtained from acetone powder of beef-heart sarcosomes, as described by CERLETTI *et al.*[9]. (3) Highly purified succinate dehydrogenase was prepared as described by DERVARTANIAN AND VEEGER[4], except that beef-heart mitochondria or Keilin–Hartree preparation from beef hearts and not from pig hearts were the starting material. If not stated otherwise, dehydrogenase preparations obtained according to Procedure 1 were used throughout.

Determination of the activity of succinate dehydrogenase was routinely accomplished with phenazine methosulphate (PMS) as the primary and 2,6-dichlorophenolindophenol (DCIP) as the terminal electron acceptor[10]. The reduction of DCIP was followed spectrophotometrically at 600 nm. Only in some experiments was O_2 the terminal electron acceptor; its uptake was determined polarographically with the Clark electrode. Spectrophotometric measurements with ferricyanide as the primary electron acceptor were also made.

Reconstitution of the succinate oxidase system was carried out as described by KING[7,11]. Respiratory particles devoid of succinate dehydrogenase were prepared from beef-heart sarcosomes according to KING[12].

Oxaloacetate was determined by the use of the reaction:

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{malate dehydrogenase}} \text{malate} + \text{NAD}^+$$

The oxidation of NADH was measured either fluorimetrically[13] or, at high levels of oxaloacetate, spectrophotometrically[14].

Solutions of oxaloacetate were prepared by dissolving oxaloacetic acid (Sigma or Boehringer) in water and by carefully neutralizing it with Tris. These solutions have always been freshly made.

Micellar phospholipids were prepared according to FLEISCHER AND KLOUWEN[15].

RESULTS

Kinetics of the inhibition and of its reversal

The difference between the inhibition of succinate dehydrogenase by oxalo-

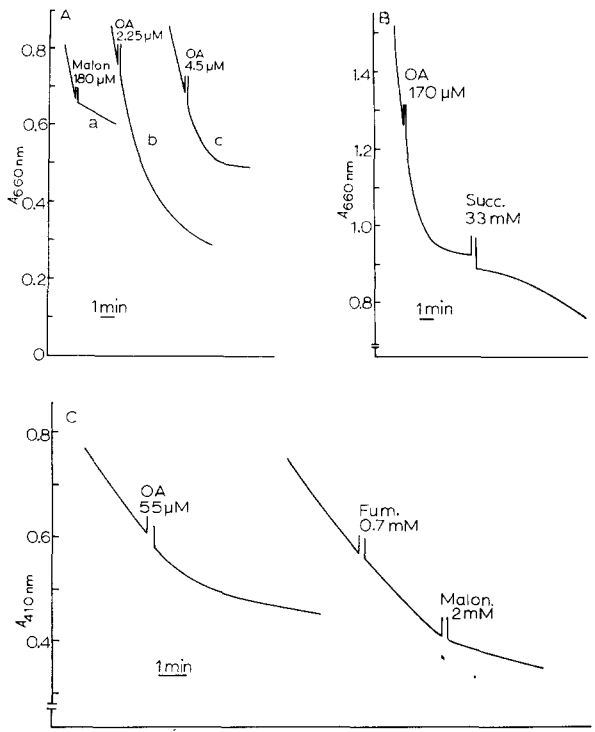

Fig. 1. Inhibition of soluble succinate dehydrogenase by oxaloacetate, malonate and fumarate. The assay medium contained 50 mM phosphate and 1 mM EDTA (pH 7.4); total vol. 3.0 ml. A and B. 2.2 mM PMS + 40–80 μM DCIP; crude enzyme (Procedure 1); temp., 30° (A) and 20° (B). C. 0.67 mM $K_3Fe(CN)_6$; purified enzyme (Procedure 3), 0.2 mg protein; temp., 28°. The enzyme was preincubated with succinate before assay for at least 10 min (A and B), and for 3 min (C). Succinate concn.: (A) 1.33 mM; (B) 3 mM (initial) and 33 mM (final); (C) 20 mM. Abbreviations: OA, oxaloacetate; Malon., malonate; Fum., fumarate; Succ., succinate.

acetate and by malonate is shown in Fig. 1A. It is seen that the effect of malonate is practically instantaneous, while the inhibition by oxaloacetate increases with time during a few minutes following the addition of the inhibitor. The higher the concentration of oxaloacetate is, the sooner the constant (inhibited) reaction rate is attained. However, even at concentrations of oxaloacetate high enough to inhibit the dehydrogenase almost completely, a lag can be observed (Fig. 1A, Trace c). Only with much higher concentrations of oxaloacetate was the effect almost instantaneous (not shown in Fig. 1). Not only the inhibition by oxaloacetate but also its partial reversal by an excess of substrate is preceded by a lag (Fig. 1B).

This pattern of oxaloacetate inhibition was observed with both the crude preparations of the enzyme obtained from fresh or acetone-dried mitochondria and the highly purified dehydrogenase. It was also independent of the electron acceptor and was observed with PMS (Figs. 1A and 1B) and ferricyanide (Fig. 1C) as well. The increasing inhibitory effect of oxaloacetate occurs when the enzyme is in contact with the inhibitor, regardless of whether the enzyme is or is not functioning during this time. This was shown (Fig. 2) when the enzyme was preincubated with succinate and oxaloacetate for varying periods of time and then the reaction was started by the addition

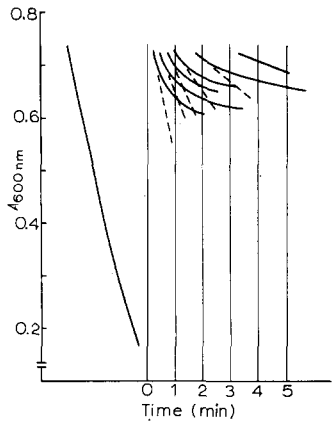

Fig. 2. Inhibition of succinate dehydrogenase by oxaloacetate; effect of preincubation of the enzyme with oxaloacetate. Assay medium as in Fig. 1; succinate, 20 mM; purified dehydrogenase same as in Fig. 1C but with PMS and DCIP as electron acceptors (concentrations as in Figs. 1A and 1B); temp., 24°. Oxaloacetate (final concn. 156 μM) was added at zero time and the reaction was started by the addition of DCIP and PMS at times indicated by the beginning of the traces. The trace to the left from zero time, no oxaloacetate added. Dashed lines indicate initial reaction rates.

of PMS. It is evident that the degree of the inhibition depends on the time of incubation of the dehydrogenase with oxaloacetate and not on the duration of the enzymatic reaction. This rules out the possibility that the increasing inhibition is produced by the reaction products or is caused by the inactivation of the enzyme by PMS or ferricyanide, which might be more inhibitory in the presence than in the absence of oxaloacetate. Fumarate, in a concentration twice as high as that which may appear if all ferricyanide present in the reaction medium is reduced, has only a slight inhibitory effect (Fig. 1C).

The peculiar patterns of the inhibition by oxaloacetate imply that special precautions must be taken when determining the inhibition constant. ZEYLEMAKER AND SLATER[6] determined the K_i values from both the initial and the final reaction rates after the addition of oxaloacetate and found both inhibitions to be competitive.

TABLE I

KINETIC CONSTANTS FOR SOLUBLE SUCCINATE DEHYDROGENASE

Expt. No.	*Enzyme preparation*	*Electron acceptor*	*Temp.*	K_m (μM)	K_i (μM) *for*	
					Oxaloacetate	*Malonate*
1	Crude*	PMS	25°	700		25
2	Crude*	PMS	30°	600	0.12	
3	Crude*	PMS	25°	420	0.30	
4	Crude*	PMS	25°	450	0.16	
5	Purified**	Ferricyanide	28°	850	0.35	

* Procedure 1.
** Procedure 3.

However, in our hands, the initial rate as determined by the tangential to the spectrophotometer record immediately (*i.e.* a few seconds) after addition of the inhibitor was equal to or very close to the uninhibited reaction rate (Fig. 1). Therefore, only the final (constant) reaction rates were taken for calculations or, even better, the enzyme had to be preincubated with the substrate and the inhibitor for at least 10 min before the reaction was started by the addition of the electron acceptor. In this way, plots typical for a competitive inhibition could be obtained, and K_i values from 0.12 to 0.35 μM were obtained (Table I). The K_m values were from 0.42 to 0.85 mM (Table I), and K_i for malonate (with PMS as electron acceptor) was 25 μM. When succinate dehydrogenase was preincubated with oxaloacetate and then succinate was added or when the concentration of succinate was increased, the concentration of oxaloacetate remaining unchanged, and patterns similar to a noncompetitive inhibition were observed. However, the inhibition constant could not be determined because the enzyme was quickly reactivated, especially in the presence of high concentrations of succinate.

The results described here suggest that succinate dehydrogenase either forms a rather stable complex with oxaloacetate or is transformed by oxaloacetate into an inactive form and that these processes and their reversal occur only slowly. Attempts to obtain the inhibited form of the enzyme and to decide between these two possibilities were carried out as follows. The crude enzyme preparation (obtained according to Procedure 1) was dialyzed or passed through Sephadex G-25 in order to make it free of succinate and phosphate. The enzyme taken up into 0.05 M Tris–HCl buffer (pH 7.4), containing 1 mM EDTA, was then incubated for 50 min with oxaloacetate (8 mM in most experiments). Then, oxaloacetate was removed. In earlier experiments this was

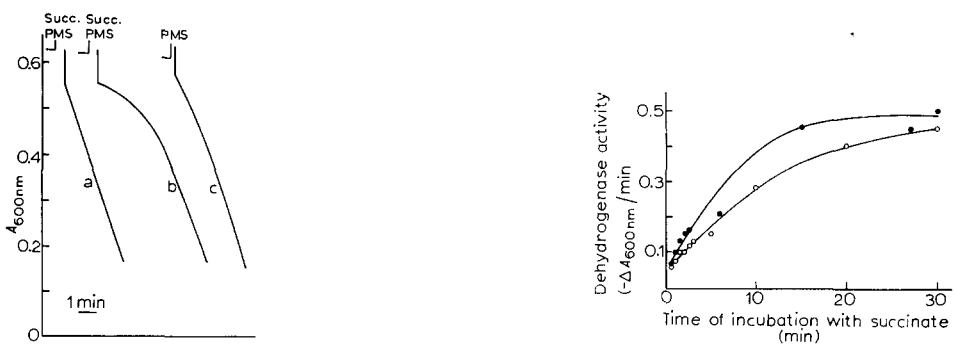

Fig. 3. Reactivation by Sephadex filtration of succinate dehydrogenase inhibited by oxaloacetate. Succinate dehydrogenase (crude), free of succinate, was incubated 30 min with 10 mM oxaloacetate and was passed twice through Sephadex G-25 column. a, control, not treated with oxaloacetate and Sephadex; b, after preincubation with oxaloacetate and Sephadex filtration; c, same as b but incubated 9 min with 30 mM succinate before addition of PMS. The enzyme in b and c contained oxaloacetate at final concentration in the assay medium 1.3 μM. The volume of the enzyme solution in b and c was twice of that in a; this approximately counterbalances the dilution of the enzyme preparation during Sephadex filtration. Assay medium was as in Figs. 1A and B; succinate (Succ.) concn. 30 mM; temp. 30°.

Fig. 4. Reactivation of succinate dehydrogenase by succinate. The enzyme was preincubated with 4 mM oxaloacetate and then dialyzed for 2 days against 20 mM Tris–HCl + 1 mM EDTA (pH 7.4) at 0°. Before the assay, the enzyme was incubated for various periods of time at 20° with two concentrations of succinate. PMS–DCIP assay procedure; assay medium, 20 mM Tris–HCl + 1 mM EDTA (pH 7.4); temp. 20°. ○—○, 3.3 mM succinate; ●—●, 33 mM succinate. Enzyme activity expressed in absorbance change ($-\Delta A_{600\ nm}$) per min.

TABLE II

REACTIVATION OF SUCCINATE DEHYDROGENASE BY SEPHADEX FILTRATION

Succinate dehydrogenase prepared by Procedure 1 and made free of succinate by subsequent Sephadex filtration was incubated with 8 mM oxaloacetate and subjected to repeated Sephadex filtration. Enzyme activity was measured with PMS and DCIP as electron acceptors and expressed in absorbance change ($-\Delta A_{600\ nm}$) per min per ml of enzyme solution (corrected for dilution during Sephadex filtration). Initial activity is the reaction rate immediately after addition of succinate to the assay medium; final activity, the constant rate which was established after a few minutes of incubation with succinate. This experiment is similar, but not the same, as that shown in Fig. 3. Assay conditions as in Fig. 3.

	Enzyme activity ($-\Delta A_{600\ nm}$ *(per min per ml)*		*Final concn. of oxaloacetate in the assay medium* (μM)
	Initial	*Final*	
Before treatment	1.41	1.41 (100%)	0
After incubation with oxaloacetate	0	0 (0%)	533
After 1 filtration	0	0.068 (5%)	27
After 2 filtrations	0.285	0.502 (35%)	17
After 3 filtrations	0.488	0.791 (56%)	0.9

attained by first incubating the preparation with either an excess of NADH or glutamate (there are sufficiently high activities of malate dehydrogenase (EC 1.1.1.37) and glutamate–oxaloacetate transaminase (EC 2.6.1.1) in the crude preparation of succinate dehydrogenase to account for effective removal of oxaloacetate) and then dialyzing or filtering on Sephadex to remove the reaction products and the excess of NADH. It appeared, however, that the dialysis or Sephadex filtration alone was as effective in removing oxaloacetate as was the complete procedure. Therefore, in further experiments the preincubation with NADH or glutamate was omitted.

The concentration of oxaloacetate added to the enzyme was high enough to inhibit completely its activity, but after a 24-h dialysis or after a single Sephadex filtration, the activity was partly restored. However, the reaction rate was not constant but increased in time (Fig. 3, Trace b). The initial activity was usually several times lower than the activity which was established after a few minutes. This lag could be eliminated by preincubating the enzyme with succinate (Fig. 3, Trace c). Fluorimetric determinations showed that the preparation still contained small quantities of oxaloacetate. On prolonged dialysis (up to three days) or during repeated Sephadex filtrations, these traces of oxaloacetate could be gradually removed, and the enzyme activity was restored (Table II). A full reactivation was, however, never obtained because of an irreversible partial inactivation produced by the dialysis or the filtration. As long as any detectable oxaloacetate was retained, the initial activity of succinate dehydrogenase observed immediately after the addition of substrate was submaximal and increased during the incubation (Table II).

The activation by succinate of succinate dehydrogenase previously pretreated with oxaloacetate is shown in Fig. 4. It is seen that the rate of reactivation depends on the concentration of succinate, while the final activity is practically independent. The reactivation could also be produced by malonate, which is even more effective than succinate, by phosphate and by micellar phospholipids (Table III). Malonate which

TABLE III

REACTIVATION OF SUCCINATE DEHYDROGENASE INACTIVATED BY PRETREATMENT WITH OXALOACETATE

Pretreatment of the enzyme was carried out as described in the legend to Fig. 4. Activation was carried out at 20°; assay conditions were as in Fig. 4; concentration of succinate, if not stated otherwise, was 3.3 mM. The reaction was started by the addition of electron acceptors (DCIP and PMS) and succinate (except for the sample with succinate as the activator where it was started by the addition of electron acceptors only). The initial reaction rate was measured and expressed in percentage of the initial activity of the control sample without any activation.

Activator	*Activation procedure*	*Activity (% of the control)*
Succinate (20 mM)	Preincubation for 10 min	171
Malonate (40 mM)	Preincubation for 10 min and removal of malonate by dialysis	250
Phosphate (50 mM)	Preincubation for 30 min	150
Brain phosphatidylethanolamine (2 mg/3.0 ml)	Preincubation for 15 min	126

is a potent competitive inhibitor of succinate dehydrogenase itself should be removed by dialysis before assay. Phosphate and phospholipids were less effective activators than were succinate or malonate and never produced a full reactivation, even after a prolonged preincubation. Contrary to what has been observed by CERLETTI *et al.*[9], micellar phospholipids did not activate, in our hands, freshly prepared succinate dehydrogenase, regardless of whether it was prepared from frozen (Procedure 1) or from acetone-dried (Procedure 2) mitochondria, provided that the enzyme was prepared in the presence of rotenone and did not contain any oxaloacetate. However, phospholipids (brain phosphatidylethanolamine and mitochondrial phospholipids) did activate the dehydrogenase previously partially inactivated by pretreatment with oxaloacetate.

It has been observed by KEARNEY[16] that soluble or particulate succinate dehydrogenase develops its full activity only after preincubation with either succinate, malonate, fumarate or phosphate. In the present study, the crude enzyme obtained according to Procedures 1 or 2 needed not to be activated, as it was obtained in the presence of succinate. However, the purified preparation (Procedure 3) developed its full activity only after preincubation with succinate or malonate. Succinate dehydrogenase obtained according to Procedure 1, but without rotenone, contained a small amount of oxaloacetate (about 60 nmoles/ml). If rotenone was included from the first step of preparation, *i.e.* during the incubation of mitochondria with succinate and phosphate, the content of oxaloacetate was below the limits of estimation. The enzyme prepared from acetone powder (Procedure 2) contained a negligible amount of oxaloacetate, below 1 nmole/ml, regardless whether the acetone powder was made in the presence or in the absence of rotenone. The content of oxaloacetate in the purified succinate dehydrogenase (Procedure 3) could not be determined because of the small amount of the preparation available.

Reconstitution of succinate oxidase

Soluble succinate dehydrogenase can react only with ferricyanide and certain

N-alkylphenazines (phenazine metho- and ethosulphates) as electron acceptors[17]. However, if prepared as described by KING[7] (Procedure 1 of the present paper), this enzyme can recombine with respiratory particles deprived of succinate dehydrogenase, but containing the other components of the respiratory chain, and can reconstitute the complete succinate oxidase system, transporting electrons from succinate to molecular oxygen[7,11,18]. The reconstitutively active dehydrogenase can be obtained only if the enzyme is isolated in the presence of succinate[11]. We have observed that if the enzyme is made free of succinate after the isolation, *e.g.* by subsequent Sephadex filtration, it also loses its reconstitutive activity.

In order to examine the effect of oxaloacetate on the reconstitution ability, a series of experiments was carried out in which the enzyme was incubated 2 min with oxaloacetate, oxaloacetate was then removed by transamination with glutamate, and finally succinate dehydrogenase-deficient particles were added and oxygen uptake was measured polarographically. The nonreconstitutive activity was determined by adding 2 mM KCN followed by PMS and by measuring the O_2 uptake. As shown in Table IV and Fig. 5, the ability of succinate dehydrogenase to reconstitute the suc-

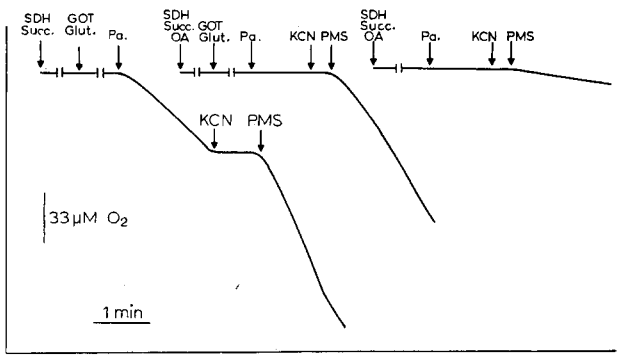

Fig. 5. Reconstitution of the succinate oxidase system. Polarographic traces of oxygen uptake. The assay medium contained 50 mM phosphate and 1 mM EDTA (pH 7.4); temp. 30°; total vol. 3.0 ml. Additions (and abbreviations): succinate dehydrogenase, crude preparation (Procedure 1) (SDH), containing succinate (Succ.) at final concentration in the assay medium 9 mM; oxaloacetate (OA), 0.6 mM; glutamate (Glut.), 6.7 mM; glutamate–oxaloacetate transaminase (GOT); succinate dehydrogenase-deficient particles (Pa.), 3 mg protein; KCN, 1 mM; PMS, 2.2 mM. Time lapse between the addition of succinate dehydrogenase + succinate (or succinate dehydrogenase + succinate + oxaloacetate) and that of glutamate + glutamate–oxaloacetate transaminase was 2 min; between glutamate + glutamate–oxaloacetate transaminase and succinate dehydrogenase-deficient particles, 3 min; and between succinate dehydrogenase + succinate + oxaloacetate and succinate dehydrogenase-deficient particles (last trace), 5 min.

cinate oxidase system was irreversibly lost after a short contact with oxaloacetate, in spite of the subsequent removal of oxaloacetate by transamination. Contrary to this, the activity with the artificial electron acceptor PMS was partly restored. The enzyme preparation used usually contained enough glutamate–oxaloacetate transaminase, so that the omission of externally added transaminase had only a little effect on the recovery of succinate dehydrogenase activity (nonreconstitutive). However, the addition of glutamate was obligatory (Table IV). The irreversible inactivation of the reconstitution was caused by oxaloacetate and not by the products of transamination, aspartate or α-oxoglutarate. As shown in Table IV, Expt. 2, aspartate and α-oxo-

TABLE IV

EFFECT OF OXALOACETATE ON THE RECONSTITUTION OF THE SUCCINATE OXIDASE SYSTEM

All conditions as in Fig. 5; concentrations of aspartate and oxoglutarate, 0.7 mM each.

Expt. No.	*Pretreatment*		*Activity (relative values)*	
	2 min	*3 min*	*Reconstituted**	*With PMS***
1	—	Glutamate + glutamate–oxaloacetate transaminase	100	100
	Oxaloacetate	—	0	0
	Oxaloacetate	Glutamate + glutamate–oxaloacetate transaminase	0	65
	Oxaloacetate	Glutamate	0	46
2	—	Glutamate + glutamate–oxaloacetate transaminase	100	100
	Oxaloacetate	—	0	0
	Oxaloacetate	Glutamate + glutamate–oxaloacetate transaminase	0	35
	Oxaloacetate	Glutamate	0	33
	Oxaloacetate	Glutamate–oxaloacetate transaminase	0	0
	—	Aspartate + glutamate	95	
	—	α-Oxoglutarate + glutamate	68	68
	—	α-Oxoglutarate	0	11

* Succinate → O_2.

** Succinate → PMS → O_2.

glutarate had no or little effect on the reconstitution, provided that glutamate was present, probably in order to prevent the formation of minute amounts of oxaloacetate by a reversed transamination (with endogenous aspartate or α-oxoglutarate).

DISCUSSION

The peculiar pattern of the inhibition of succinate dehydrogenase by oxaloacetate, as characterized by the increasing degree of inhibition in time, is a unique feature not observed, to the authors' knowledge, with other enzymes and their competitive inhibitors nor with other competitive inhibitors of succinate dehydrogenase. It has been repeatedly observed[19–21] that the inhibitory effect of oxaloacetate on succinate oxidation in mitochondria is not instantaneous but is preceded by a lag. Factors such as permeability of mitochondrial membranes to oxaloacetate and processes which remove oxaloacetate from mitochondria may be partly responsible for this phenomenon (*cf.* ref. 21). However, it seems probable that the unique property of succinate dehydrogenase to be inhibited only slowly by low concentrations of oxaloacetate may also be involved.

First reported by us[5], the inhibitory effect of oxaloacetate, increasing with time, has been confirmed by ZEYLEMAKER AND SLATER[6]. However, in disagreement with us, these authors report an immediate, although relatively small, inhibition, while in our experiments there was almost no immediate effect. The other point in which our results are in disagreement with those of ZEYLEMAKER AND SLATER[6] is that we have

observed a constant level of the inhibition only after a lag of 30 sec to a few minutes, while ZEYLEMAKER AND SLATER claim to be able to distinguish a linear, although short, portion of the trace immediately following the addition of oxaloacetate. They also report that spectral changes of succinate dehydrogenase[4] as produced by oxaloacetate are complete within 4 sec after the addition of oxaloacetate. These discrepancies require a further critical examination. It may be interesting to note here that, according to observations of DERVARTANIAN AND VEEGER[4], spectral changes of purified succinate dehydrogenase as produced by oxaloacetate markedly differ from changes caused by malonate and other competitive inhibitors. This may also indicate that the nature of the inhibition by oxaloacetate is different.

As shown in the present study, the increasing inhibition of succinate oxidation after the addition of oxaloacetate is due to the effect of oxaloacetate itself and not to the reaction products or the electron acceptors. The peculiar kinetics of the inhibition by oxaloacetate can only be observed with low (micromolar) concentrations of oxaloacetate or, rather, within certain limits of the ratio between oxaloacetate and succinate (and probably the enzyme protein). At high oxaloacetate concentrations, the inhibition is practically instantaneous. This is probably the reason why this lag has been overlooked by many investigators.

The inhibition constant for oxaloacetate determined in the present investigation is several times lower than that found by PARDEE AND POTTER[3] and DERVARTANIAN AND VEEGER[4]. ZEYLEMAKER AND SLATER[6] found that K_i for the immediate inhibition varied between 1.7 and 6.3 μM and for the secondary (final) one between 0.6 and 0.7 μM. The latter values are in a fairly good agreement with those reported by us for the final (constant) inhibition phase, namely between 0.12 and 0.35 μM. K_m and K_i values for malonate determined in the present study are similar to those values found by others[4,16–18,22].

The reactivation of succinate dehydrogenase inhibited by oxaloacetate, as described in the present paper, is similar to the activation of soluble succinate dehydrogenase observed by KEARNEY[16]. It may be speculated that both processes have a common background and that, in fact, the enzyme isolated by KEARNEY was already inhibited due to a previous contact with oxaloacetate.

It seems likely that the effect of phospholipids as described here is of a different nature than that studied by CERLETTI *et al.*[9]. Both processes differ in two respects: (1) Various phospholipids were equally active in the system described here, while only acidic phospholipids were active under experimental conditions of CERLETTI *et al.*[23]; (2) the activating effect was produced in our system not only by phospholipids but also, and even better, by succinate or malonate, whereas the activation observed by CERLETTI was specific to phospholipids and differed from the activating effect of succinate[23].

The peculiar behaviour of succinate dehydrogenase towards oxaloacetate may be explained by two hypotheses: (1) that oxaloacetate is a *sensu stricto* competitive inhibitor of succinate dehydrogenase, forming reversibly a complex with the enzyme, but the formation and the splitting of this complex occur only slowly; (2) that the enzyme exists in two forms, active and inactive, and that the transition from the active form into an inactive one is promoted by oxaloacetate, while the reverse process is induced by succinate or certain other substances. The existence of two forms of succinate dehydrogenase exhibiting high and low activities, respectively, has been already postulated by the group of SINGER[24] and supported by recent kinetic studies

of ZEYLEMAKER AND JANSEN[25]. According to the second hypothesis, as proposed above, oxaloacetate would not be a true competitive inhibitor, although the kinetics of the inhibition may imitate a competitive inhibition, the ratio of the active to the inactive forms of the enzyme being controlled by the ratio of succinate to oxaloacetate.

The first hypothesis postulates a relatively stable complex between oxaloacetate and the dehydrogenase. The binding site of succinate dehydrogenase for succinate presumably contains two positively charged groups, able to attract the two carboxylic groups of succinate. Malonate, a three-carbon-atom analogue of succinate, probably fits even better to this binding site, as indicated by the K_i value which is 20–30 times lower than the K_m value. On the contrary, unsaturated and substituted dicarboxylic four-carbon-atom acids, such as fumarate, methylene succinate, D-chlorosuccinate and malate, are weak inhibitors[4,16,26]. Therefore it seems puzzling why oxaloacetate which is the carbonyl derivative of succinate is such a potent inhibitor. It should be supposed that in this case not only the two carboxyls, but also the carbonyl group or the enol structure which may be formed, is involved in the binding with the enzyme. This additional binding would also be responsible for the slowness of the formation and of the splitting of the enzyme–inhibitor complex. It may be speculated that the acid-labile SH group or the non-heme iron is involved in this binding. However, this might also be any other moiety of the enzyme protein, even not necessarily essential for the enzymatic reaction. Succinate, malonate and, to a lesser degree, phosphate and phospholipids compete for the positive binding sites, thus enabling the other binding to be slowly split. The stability of the dehydrogenase–oxaloacetate complex should be, however, not high enough to sustain repeated Sephadex filtration or prolonged dialysis against Tris buffer.

The other hypothesis assumes that conformation changes in the enzyme protein are induced by oxaloacetate. These changes may be reversed by succinate, by its analogues, *e.g.* malonate or fumarate, or by certain other substances probably showing an affinity to the substrate binding site of the enzyme, *e.g.* phospholipids and phosphate (inhibition of succinate dehydrogenase by phosphate has been observed by SLATER AND BONNER[27]). The transition from the inactive to the active states of the enzyme must, however, occur also spontaneously, as the reactivation also proceeds, at least partly, on simply removing oxaloacetate. In consequence, experiments described here do not allow us to decide unequivocally between these two hypotheses. We failed more directly to demonstrate the existence of a stable complex of succinate dehydrogenase with oxaloacetate or to isolate an inactive form of the enzyme which would be stable enough in the complete absence of oxaloacetate. Further work and perhaps a new experimental approach to this problem are needed.

At present there is one more direct experimental evidence for changes in the molecule of succinate dehydrogenase caused by a transient contact with oxaloacetate, namely the loss of the ability of the enzyme to reconstitute the complete succinate oxidase system. This change is, however, irreversible and is thus not similar to the reversible inactivation of the dehydrogenase activity. The hypothesis of conformation changes produced by oxaloacetate in succinate dehydrogenase is also compatible with suggestions expressed elsewhere and based on the behavior of succinate dehydrogenase in aged mitochondria[28].

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