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Oxidation of malate by the mitochondrial succinate-ubiquinone reductase

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The purified succinate-ubiquinone reductase catalyzes the L- (or D-) malate: acceptor oxidoreductase reaction with K_m for malate of about $2 \cdot 10^{-3}$ M and initial V_{max} of 50 and 100 nmol per min per mg of protein for L- and D-stereoisomers, respectively (25 °C, pH 7.0). The reaction rate rapidly decreases both in the absence and presence of L-glutamate and L-glutamate-oxaloacetate transaminase added for trapping of oxaloacetate. Both keto and enol forms of oxaloacetate were found to be strong, slowly dissociating inhibitors of succinate dehydrogenase; the first-order rate constant for the enzyme inhibition by the enol form is about 3 times as high as that by the keto form. Oxidation of malate by succinate dehydrogenase in the presence of the oxaloacetate trapping system occurs at an indefinitely constant rate when enol-oxaloacetate, which is an immediate product of the reaction, is rapidly converted into the keto isomer – a substrate for transaminase. A quantitative kinetic scheme for malate oxidation by succinate dehydrogenase which includes two kinetically distinct enzyme-oxaloacetate complexes is proposed, and the specific role of the mitochondrial oxaloacetate keto–enol-tautomerase (EC 5.3.2.2) in the regulation of succinate dehydrogenase is suggested.

Introduction

The substrate specificity of succinate dehydrogenase (EC 1.3.99.1) is not absolute. The enzyme has been shown to catalyze the oxidation of a variety of haloid-substituted analogs of succinate [1–3]. The slow enzymatic oxidation of 3-nitropropionate to 3-nitroacrylate has also been shown to be involved in a suicide inhibition of succinate dehydrogenase by this toxic compound [4,5]. There are controversial reports in the literature on the ability of succinate dehydrogenase to oxidize malate. It has been shown that both D- and L-malate cause bleaching of a flavin in the partially purified enzyme [6] and give rise to the low-temperature EPR-detectable flavin semiquinone and reduced nonheme iron [7]. The formation of about 10 mol oxaloacetate per mol of the enzyme after anaerobic incubation of soluble succinate dehydrogenase with both malate stereoisomers has been demonstrated [8]. It has been

Abbreviations: carboxin, 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide; Mops, 4-morpholinepropanesulphonic acid; DCIP, 2,6-dichlorophenolindophenol; PMS, *N*-methylphenazonium sulphate; Wurster's blue, a semiquinone diimine radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD); Q_2, homolog of ubiquinone having two isoprenoid units in position 6 of the quinone ring.

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reported that crude preparations of the soluble succinate dehydrogenase catalyze the overall D- or L-malate: ferricyanide reductase reaction [6], although, presumably due to the strong inhibitory effect of the oxaloacetate formed, no more than about ten turnovers of the enzyme have been observed. Contrary, Hanstein et al. [9] have claimed that pure two-subunit succinate dehydrogenase shows no dehydrogenating activity when either D- or L-malate was used as the substrate.

Another group of observations relevant to the malate dehydrogenase activity of succinate dehydrogenase is concerned with the so-called 'activation–deactivation' phenomenon, first discovered by Kearney [10] and later on accounted for the presence of tightly bound oxaloacetate in deactivated preparations [11]. It has been found that succinate dehydrogenase activity rapidly decline in parallel with the formation of tightly bound oxaloacetate at the enzyme active site when the enzyme was incubated with low concentrations of D- or L-malate [11,12].

The malate dehydrogenase activity of the succinate dehydrogenase, when present, raises the possibility of the enzyme regulation by malate via a suicide inhibition mechanism, since L-malate is a natural constituent of the mitochondrial matrix where the substrate binding site of succinate dehydrogenase is located, and since oxaloacetate is known to be an extremely potent inhibitor of the enzyme.

The purpose of the present studies was to investigate in more detail the malate dehydrogenase activity of succinate dehydrogenase and to relate it to the known parameters of the enzyme–oxaloacetate interaction [13]. We will show that purified succinate-ubiquinone reductase oxidizes D- and L-malate and that the enol-oxaloacetate is an immediate product formed at the enzyme-active site. When the oxidation of malate by the enzyme is coupled with L-glutamate/oxaloacetate transamination, the key step which controls the succinate dehydrogenase activity is the tautomerism of oxaloacetate. In the subsequent paper [44] the isolation procedure and the properties of mitochondrial oxaloacetate keto–enol-tautomerases (EC 5.3.2.2) are described. The preliminary account of the results has been published elsewhere [14].

Materials and Methods

Bovine heart succinate-ubiquinone reductase was prepared [15], activated and made free of contaminating succinate [16] according to the published procedures. The soluble reconstitutively active succinate dehydrogenase was isolated and stored as described [17]. The succinate dehydrogenase activity was measured at 25 °C in a standard assay mixture comprising 5 mM Tris/Cl^- (pH 8.0), 20 mM succinate, 0.2 mM EDTA, 1 mM cyanide (potassium salts), 0.004% Triton X-100, 2 mM PMS and 0.05 mM DCIP. The malate dehydrogenase activity of the succinate dehydrogenase preparations was measured at 25 °C in an assay mixture comprising L- or D-malate, Mops (pH 7.0; the concentrations are indicated in the figure legends), 0.2 mM EDTA, 1 mM cyanide (potassium salts), 0.004% Triton X-100, and proper amounts of the electron acceptors (Q_2, Wurster's Blue or PMS plus DCIP). The details are described in the figure and table legends.

We found that different batches of commercial preparations of L-malate (Sigma, U.S.A.; Serva, Austria; Fluka, Switzerland) are considerably contaminated with succinate (1–7% of dry weight). Thus, L-malate was purified by ion-exchange chromatography. 4.5 ml of 0.5 M L-malate (Fluka, Switzerland), pH 2.5 was passed through a column (3 × 50 cm) packed with Dowex 1 × 8 (Serva, Austria) equilibrated with HCl (pH 2.5) at room temperature. The fractions containing L-malate were combined and concentrated by evaporation.

Protein was determined by the procedure of Lowry et al. [18]. Wurster's Blue was prepared from TMPD according to Michaelis and Granik [19]. Oxaloacetate, PMS, Tris, Mops, TMPD and D-malate were obtained from Sigma (U.S.A.), NAD^+, NADH, L-glutamate and pig heart L-malate dehydrogenase (EC 1.1.1.37) were from Reanal (Hungary), Triton X-100 was from Loba Chemie (Austria), PMS was from Lowson (U.K.), DCIP was from General Biochemicals (U.S.A.), Q_2 was from Ferak (West Berlin), *N*-ethylmaleimide was from BDH (U.K.) Carboxin was a kind gift from Prof. H. Lyr (Institute of Plant Protection Research, G.D.R.). L-Glutamate oxaloacetate transaminase from pig heart (EC 2.6.1.1) was kindly provided by Dr. Yu.M. Torchinsky (In-

stitute of Molecular Biology, U.S.S.R. Academy of Sciences). Other chemicals were of the highest quality commercially available.

Results

Kinetics of malate oxidation catalyzed by succinate-ubiquinone reductase

Fig. 1A (curve 1) demonstrates the time-course of Wurster's Blue reduction in the presence of 1 mM D-malate and a proper amount of purified succinate-ubiquinone reductase. The reaction rapidly deviates from the zero-order pattern; this deviation can not be prevented by an increase of the substrate or the electron acceptor concentrations, or both. The same time-course was seen with L- or D-malate used as the substrates, when PMS (2 mM) plus DCIP (0.05 mM) or Q_2 (10 μM) were added as acceptors. The succinate-acceptor reductase reaction rate was perfectly constant during any reasonable time under the same conditions. In order to assure that the reaction shown in Fig. 1 is indeed the malate-acceptor oxidoreductase catalyzed by succinate dehydrogenase, the following experiments were performed.

(i) The reaction was found to be completely inhibited by malonate (curve 3) with the same K_i value as the succinate-acceptor reductase activity was.

(ii) The amount of Wurster's Blue reduced in the reaction was measured, and the reaction was stopped by the addition of 0.5 mM dithiothreitol (instant nonenzymatic reduction of the dye); 5 mM L-glutamate and 5 μg of L-glutamate-oxaloacetate transaminase were then added, the mixture was incubated for 5 min and the amount of α-oxoglutarate formed was determined, using 1 mM NAD^+ and the α-oxoglutarate dehydrogenase complex in the presence of 70 μM thiamine pyrophosphate and 25 μM coenzyme A [20]. It was found that the amount of Wurster's Blue reduced in the reaction (two electron equivalents) was equal to the amount of α-oxoglutarate determined in the mixture. Thus, oxaloacetate was identified as the product of the reaction shown in Fig. 1.

(iii) Although the preparations of succinate-ubiquinone reductase used throughout this study are highly purified [15], it seemed to be conceivable that the malate dehydrogenase activity shown in Fig. 1 was due to some contaminating enzyme. To exclude this possibility, the correlation between irreversible inactivation of succinate- and malate-acceptor reductase activities was studied, using site-directed inhibition of succinate dehydrogenase by *N*-ethylmaleimide [21,22]. Fig. 2 demonstrates that the ratio between the residual fractions of both activities during treatment of the enzyme with the inhibitor remains constant. Moreover, both activities were protected by saturating concentrations of malonate which is known as a specific competitive inhibitor of suc-

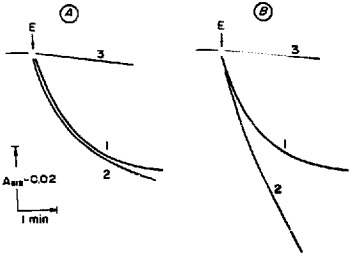

Fig. 1. Time-course of D-malate oxidation by purified succinate-ubiquinone reductase. The reaction was started by an addition of the enzyme (50 μg/ml) to a standard reaction mixture (see Materials and Methods) containing 1 mM D-malate, 5 mM L-glutamate and 40 μM WB. (A) 1 mM Mops (pH 7.0) was used as a buffer. (B) 0.25 M Mops (pH 7.0) was used as a buffer. Curves 1, no other addition; curves 2, L-glutamate-oxaloacetate transaminase (140 μg/ml) was added; curves 3, 20 mM malonate was added.

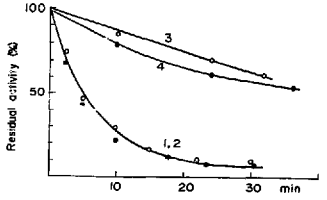

Fig. 2. Inhibition of the succinate dehydrogenase (●) and D-malate dehydrogenase (○) activities of succinate-ubiquinone reductase by *N*-ethylmaleimide. Succinate-ubiquinone reductase (0.5 mg/ml) was incubated at 25 °C for the time indicated on abscissa in a mixture containing: 20 mM phosphate (pH 7.8), 0.2 mM EDTA (potassium salts) and 0.05 mM *N*-ehylmaleimide. The proper amount of the enzyme was withdrawn and the initial D-malate (1 mM) and succinate (1 mM) dehydrogenase activities were determined in 0.25 M Mops (pH 7.8), as described in the legend to Fig. 1. Curves 1 and 2, no other addition to the incubation mixture; curves 3 and 4, 50 μM potassium malonate was added.

cinate dehydrogenase [13,23]. Qualitatively, the same results as those shown in Fig. 1 were obtained when purified two-subunit soluble succinate dehydrogenase was used as the enzyme preparation.

Taken together, these results unambiguously show that succinate dehydrogenase is capable of malate oxidation. Some kinetic parameters of the reaction are given in Table I.

An obvious reason for a rapid decline of the enzyme activity during malate oxidation (Fig. 1) is the accumulation of oxaloacetate which is a potent active site-directed inhibitor of succinate dehydrogenase [13,24–31]. However, the initial turnover of the enzyme in the malate dehydrogenase reaction (Table I) is incompatibly higher than the first-order rate constant for the dissociation of the dehydrogenase-oxaloacetate complex (0.02 min^{-1} and 0.2 min^{-1} for oxidized and reduced succinate dehydrogenase, respectively [13]). Moreover, the same time-course of the reaction was observed, when malate oxidation occurred in the presence of L-glutamate and transaminase (Fig. 1, curve 2). Thus, a possible explanation of quantitative discrepancies seemed to lie either in the difference between the enzyme-product complex formed as an intermediate of the malate dehydrogenase reaction and that formed after incubation of succinate dehydrogenase with oxaloacetate, or in the absence of inhibition of succinate dehydrogenase by enol-oxaloacetate, which certainly might be expected as the reaction product.

Interaction of succinate dehydrogenase with the tautomers of oxaloacetate

It is well established that in aqueous solutions oxaloacetate exists as a mixture of several species consisting predominantly of keto and enol forms [31–33]. In a solid state or in ether or dioxane solutions this compound is mainly enol, whereas in aqueous solutions at neutral pH about 80% of oxaloacetate is the keto form [31,33]. The rate of interconversion between two forms depends on many factors (ionic strength, metal ion chelation, general acid-base catalysis) and can be relatively low (up to the minute scale) [34,35]. Taking into account the mechanism by which succinate dehy-

TABLE I

KINETIC PARAMETERS OF OXIDATION OF MALATE BY THE MITOCHONDRIAL SUCCINATE-UBIQUINONE REDUCTASE

25 °C, pH 7.0, 0.25 M Mops.

Substrate	K_m (mM)	V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Turnover number [a] (min^{-1})	K_i for succinate-ubiquinone reductase [b] (mM)
L-malate	2.2	0.05	8.5	2.2
D-malate	1.5	0.10	17	not determined
Succinate	0.1	10.0	1700	

[a] Calculated on the basis of covalently bound flavin content of 6 nmol per mg [15].

[b] Determined by the Dixon method [43].

drogenase removes hydrogen atoms from the substrate (transelimination [36]), it should be anticipated that enol-oxaloacetate is an immediate product of L- or D-malate oxidation at the enzyme-active site. Then a question arises as whether enol oxaloacetate is an inhibitor of succinate dehydrogenase. If not, the results shown in Fig. 1 could simply be explained, assuming that the product of malate oxidation is non-inhibitory enol which slowly interconverts into strongly inhibitory keto-isomer. Thus, we decided to compare the relative inhibitory potencies of keto- and enol-tautomers in the succinate dehydrogenase reaction. Fig. 3 demonstrates the kinetics of oxaloacetate ketonization under the same conditions as that used for malate dehydrogenase activity assay. The method used for the determination of the isomerization rate is based on the absolute specificity of L-malate dehydrogenase for the keto form of oxaloacetate [37]. An instant decrease of absorbance at 340 nm after oxaloacetate addition from dry ether solution corresponds to the amount of ketone, whereas a subsequent slow process is due to slow ketonization of enol. In agreement with the published data we observed a strong acceleration of tautomeric interconversion when the buffer concentration was increased from 1 mM (curve 1) up to 0.25 M (curve 2). The

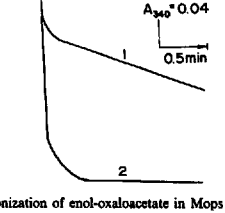

Fig. 3. Ketonization of enol-oxaloacetate in Mops buffer (pH 7.0). 5 μl of the solution of oxaloacetate in dry diethyl ether (OA) were added to 2 ml of a mixture containing: Mops (curve 1, 1 mM, curve 2, 0.25 M), 0.2 mM EDTA, 0.2 mM NAD·H, and malate dehydrogenase (25 μg/ml); pH of 1 mM Mops was adjusted to make pH 7.0 after addition of 50 μM oxaloacetate.

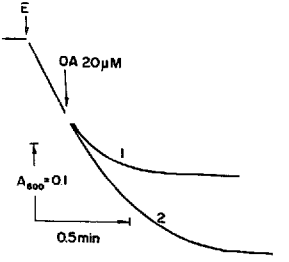

Fig. 4. Inhibition of succinate dehydrogenase by the enol and keto forms of oxaloacetate. 20 μM oxaloacetate was added where indicated from dry diethyl ether (curve 1) or acid (pH 2.0) aqueous (curve 2) solutions to the assay cuvette containing: 5 mM Tris/Cl⁻ (pH 8.0), 2 mM succinate, 0.2 mM EDTA (potassium salts), 0.05 mM DCIP, 2 mM PMS, 0.004% Triton X-100 and a proper amount of the enzyme.

[ketone]/[enol] ratio in Mops buffer at pH 7.0, 25°C was found to be 5; this value is in good agreement with the results reported by others [31–33]. The low rate of oxaloacetate ketonization at low buffer concentration is advantageous for a comparison of relative efficiencies of ketone and enol forms in succinate dehydrogenase inhibition. Fig. 4 demonstrates the time-course of the succinate-ubiquinone reductase inhibition by oxaloacetate added as enol (67%) or as ketone (95%). As can be evident from Fig. 3, the ratio between the two tautomers was not significantly changed during the time needed to inhibit the reaction. Thus, the pseudo-first-order rate constants for the inhibition of succinate dehydrogenase by the enol and ketone forms could be determined. The inhibition patterns shown in Fig. 4 give linear semilogarithmic plots (the results are not shown). When the ratio between the added enol and keton was varied using different solvents where oxaloacetate was added from, the linear dependence of the first-order inhibition rate constant on the enol/ketone ratio was obtained (Fig. 5). The intercepts of the straight line with the left (100% ketone) and right (100% enol) ordinates give a difference 3-times greater in the relative inhibition potencies for the two species. It is worthwhile mentioning that neither succinate

ubiquinone reductase nor soluble two-subunit succinate dehydrogenase catalyze keto–enol-tautomerization of oxaloacetate as was determined in the assay system shown in Fig. 3.

Since under certain conditions the succinate dehydrogenase inhibition by any tautomers of oxaloacetate proceeds faster than that of the isomers interconversion it might be conceivable that the inhibited succinate dehydrogenase-oxaloacetate (ketone) and succinate dehydrogenase-oxaloacetate (enol) complexes are different. Fig. 6 shows that both complexes are identical at least in terms of the dissociation rate. Thus, we may conclude that both keto and enol forms of oxaloacetate are powerful, very slowly dissociating inhibitors of succinate dehydrogenase and that the bimolecular rate constant for enol association with the enzyme active site is 3-times higher than that for the ketoform.

Tautomeric interconversion of oxaloacetate and malate dehydrogenase activity of succinate dehydrogenase

The results presented in the previous sections leave the question about the apparent discrepancy

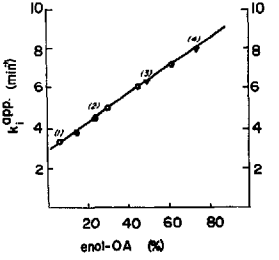

Fig. 5. Rate of the succinate dehydrogenase inhibition as a function of ketone and enol content in oxaloacetate. 20 μM oxaloacetate was added to the assay system as described in Fig. 4 from: (1) acid (pH 2.0) aqueous solution; (2) neutral aqueous solution; (3) ethanol solution; (4) dry diethyl ether solution. (○), oxaloacetate was added from dry diethyl ether solution to the assay system and the reaction was started by the addition of succinate-ubiquinone reductase at the moment when the enol/ketone ratio was as indicated on abscissa. The content of enol was determined by L-malate dehydrogenase assay as shown in Fig. 3.

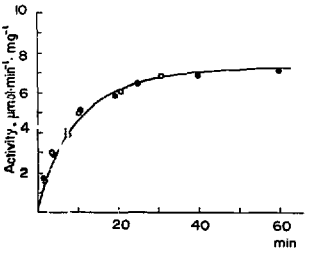

Fig. 6. Activation of succinate-dehydrogenase inhibited by the enol and keto isomers of oxalaoacetate. Succinate-ubiquinone reductase (0.15 mg/ml) in 5 mM Tris/Cl$^-$, 0.2 mM EDTA was incubated for 0.5 min at 25 °C with 10 μM oxaloacetate added from acid aqueous (●) or dry diethyl ether (○) solutions. At zero time the samples were diluted for activation in the assay cuvettes containing: 5 mM Tris/Cl$^-$ (pH 7.8), 0.2 mM malonate (potassium salts), 0.004% Triton X-100. After dilution the final concentrations of the enzyme and oxaloacetate were 0.4 μg/ml and $2.5 \cdot 10^{-8}$ M, respectively. The succinate dehydrogenase activity was assayed at the time indicated on abscissa after simultaneous addition of 50 mM succinate, 2 mM PMS and 0.05 mM DCIP to the assay cuvette.

between relatively rapid initial turnover of succinate dehydrogenase in the malate dehydrogenating reaction $k_{cat} \geq 8.5$ min^{-1}, Table I) and an extremely slow dissociation of the enzyme-oxaloacetate complex ($k_{off} = 0.02$ min^{-1} [13]) unanswered. It is established that the keto form of oxaloacetate is the substrate for L-glutamate-oxaloacetate transaminase [38]. It seemed of interest to find out whether the accelerating of keto–enol-tautomerization would affect the malate dehydrogenase activity of succinate dehydrogenase coupled with transamination of oxaloacetate. As depicted in Fig. 1B, transamination of oxaloacetate at high ionic strength, i.e., under the conditions where enol interconversion to ketone is rapid (Fig. 3), does prevent the rapid decline of the malate dehydrogenase activity of succinate dehydrogenase. In contrast, transaminase does not affect the time-course of the reaction at low ionic strength, where the tautomerisation of oxaloacetate is slow. These results clearly demonstrate that enol oxaloacetate is an immediate product of D- or L-malate oxidation at the enzyme active site. Fig.

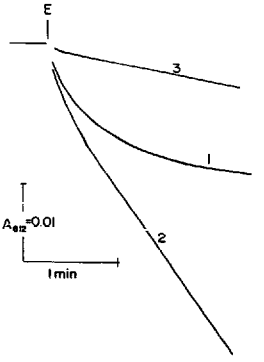

Fig. 7. The effect of oxaloacetate keto–enol-tautomerase on a suicide inhibition of the malate dehydrogenase activity of succinate-ubiquinone reductase. L-Malate (2 mM) dehydrogenase activity was assayed in 1 mM Mops (pH 7.8) in the presence of 1 mM L-glutamate and L-glutamate-oxaloacetate transaminase (65 μg/ml) as described in Fig. 1. Curve 1, no other additions; curve 2, 200 μg/ml of oxaloacetate keto-enol tautomerase – prepared as described in the accompanying paper (Ref. 44) were added; curve 3, as curve 2, 20 mM malonate was added.

7 shows that no inhibition of the enzyme during malate oxidation is observed, when ketonization of oxaloacetate at low ionic strength is accelerated by the purified oxaloacetate keto-enol tautomerase (EC 5.3.2.2) prepared from bovine heart mitochondria according to the procedure described in the accompanying paper.

Discussion

The results presented in this paper leave no doubt that mammalian succinate dehydrogenase in the presence of electron acceptors is capable of the overall oxidation of L- and D-malate. This conclusion is in accord with the findings of Dervartanian and Veeger [6] and contradicts the claim of Hanstein et al. on the inability of purified soluble succinate dehydrogenase to oxidize malate [9]. The peculiar pattern of malate oxidation by succinate dehydrogenase is that L-malate, being a natural constituent of the mitochondrial matrix, acts as a suicide inhibitor of the enzyme. This behaviour raises several interesting points.

The first point we would like to consider concerns the apparent discrepancy between the initial turnover of the enzyme in the reaction, where the succinate dehydrogenase-oxaloacetate complex is undoubtfully an intermediate of the overall malate oxidation and the well-documented slowness of the enzyme-oxaloacetate complex dissociation [13]. The minimal hypothesis which accounts for the experimental observation is given below (Scheme I):

$$M + E \overset{K_s}{\leftrightarrow} E\cdot M \xrightarrow[k_{cat}]{A_{ox}\ A_{red}} E\cdot O_c \overset{K_p}{\leftrightarrow} E + O_c \overset{K_t}{\leftrightarrow} O_k \qquad (I)$$
$$K_i^* \updownarrow$$
$$(E\cdot O)^*$$

where M is D- or L-malate, E is succinate dehydrogenase, A_{ox} and A_{red} are oxidized and reduced electron acceptors, respectively, O_c and O_k are the enol and keto forms of oxaloacetate, K_s and K_p are the dissociation constants for $E\cdot M$ and $E\cdot O_c$, respectively, K_t is the equilibrium constant for oxaloacetate tautomerization, K_i^* is the equilibrium constant for the interconversion of the intermediate $E\cdot O_c$ complex into the dead-end slowly dissociating complex $(E\cdot O)^*$ and k_{cat} is the pseudo-first-order rate constant which is a measure of catalytic activity (turnover number) of the enzyme. Each equilibrium constant is a combination of two rate constants; for example K_i^* equal to k_{-i}^*/k_{+i}^*, etc.

Since k_{cat} (8.5 and 17 min^{-1} for different stereoisomers, Table I) is quite small as compared with that for succinate oxidation (1700 min^{-1}), it seems very likely that the apparent K_m values for L- and D-malate are true equilibrium constants, K_s. This is substantiated by the finding that the K_i value for L-malate in the reaction of succinate oxidation determined by the method of Dixon [43] is the same as K_m for L-malate (Table I). The enol-oxaloacetate is a structural analog of malate; thus it may be assumed that $K_s \approx K_p$. With this assumption the individual rate constants $k_{\pm p}$ and $k_{\pm i}^*$ can easily be approximated. We have previously determined the dissociation constant (K_d) for the formation of a slowly dissociating succinate dehydrogenase-oxaloacetate complex which is equal to approx. $2\cdot10^{-8}$ M [13]. According to Scheme I, $K_d = K_p \cdot K_i^*$; this gives $K_i^* = 10^{-5}$.

Since k^*_{-i} is known (0.02 min^{-1} [13]), the value of the first-order rate constant, k^*_{+i}, calculated as $k^*_{+i} = k^*_{-i}/K_i^* = 2 \cdot 10^3$ min^{-1} can be obtained. The lower limit for the first-order-rate constant of the enzyme-dicarboxylate complex formation can be estimated from the K_d value for the oxidized enzyme-fumarate complex (approx. $2 \cdot 10^{-4}$ M [13]) and from the turnover number of succinate-ubiquinone reductase ($5 \cdot 10^3$ min^{-1} at 25 °C [15]); thus k_{+p} can be approximated as $2 \cdot 10^7$ $min^{-1} \cdot M^{-1}$. If the assumed value of K_p is approx. 10^{-3} M, lower limit for the value of $k_{-p} = K_p \cdot k_{+p}$ may be approximated as $2 \cdot 10^4$ min^{-1}. An important consequence of such quantitative consideration is that if succinate dehydrogenase interacts with malate in the absence of oxaloacetate-utilizing systems, the enzyme would be rapidly inactivated via the suicide inhibition mechanism. In contrast, in the presence of an oxaloacetate trapping system the enzyme can be maintained in an active state for a long time, since the ratio $k_{-p}/k^*_{+i} = 20$ permits only one inactivation act per 20 turnovers. Indeed, such a behavior was observed. (Fig. 1A and B).

Since succinate dehydrogenase dicarboxylate binding site operates in the mitochondrial matrix, where the steady-state concentration of L-malate is rather high [20], a direct contact between the enzyme and the suicide inhibitor seems very probable. Scheme I emphasizes an important role of oxaloacetate utilizing enzymes in the maintenance of succinate dehydrogenase in an active state. An important point is that intramitochondrial oxaloacetae-utilizing enzymes, in particular, L-glutamate-oxaloacetate transaminase used as a model in this study, utilize the keto isomer of oxaloacetate as the substrate [38]. In 1968 Annett and Kosicki described the enzyme which catalyzed tautomeric interconversion of oxaloacetate [38]. The physiological function of this enzyme is unknown [38,39], since no enzymes utilizing or producing enol-oxaloacetate were known so far. To our knowledge, succinate dehydrogenase oxidizing L-malate is the first example of such an enzyme in mammalian tissues. An important role of oxaloacetate keto–enol-tautomerase in regulation of the succinate dehydrogenase activity thus becomes evident. The L-malate dehydrogenase activity of succinate dehydrogenase under optimal conditions is quite low, therefore its contribution to the overall operation of the Krebs cycle can hardly be significant. However, under certain conditions this activity coupled with oxaloacetate keto–enol-tautomerase and malate dehydrogenase may be of importance for maintaining a proper $NAD \cdot H/NAD^+$ ratio in the mitochondrial matrix.

The last point to be considered here is the nature of two kinetically distinct complexes $E \cdot O_c$ and $(E \cdot O)^*$ depicted in Scheme I. The existence of two enzyme-oxaloacete complexes has been postulated by Zeylemaker et al. [28] who have reported on the biphasic inhibition of soluble succinate-ferricyanide reductase oxidizing 10 mM succinate by added 12 μM oxaloacetate. In line with the data of Wojtzcak et al. [27] we were unable to distinguish two steps in the inhibition in the assay system similar to that employed by Zeylemaker et al. [28] no matter whether keto or enol isomer of oxaloacetate were added (Fig. 4). Taking into account the rate constants in Scheme I approximated as discussed above, it may be predicted that the 'rapid' $E \cdot O_c$ and the 'slow' $(E \cdot O)^*$ complexes can be separated only by rapid reaction measuring techniques. On the other hand, their existence is evident from other experimental approaches employed in the present study; it is also in line with the data of Ackrell et al. [40] on the mechanism of the enzyme inactivation by oxaloacetate. In contrast with Gutman [41] and Bonomi et al. [42], we believe that in the deactivated state, which is identical to the $(E \cdot O)^*$ complex, oxaloacetate is bound to the active site sulfhydryl group by a thiosemiacetal bond as has been discussed elsewhere [29,30]. The higher efficiency of enol in comparison with the keto-form in the reaction leading to $(E \cdot O)^*$ does not contradict our chemical model. As depicted in Scheme I, the apparent pseudo-first-order inactivation rate constant in the presence of either tautomers is equal to k^*_{+p}/K_p. At present it would not be judicious to account for the higher reactivity of enol in the inhibition by either increased k^*_{+p} or by decreased K_p as compared to ketone.

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