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Direct demonstration of *enol*-oxaloacetate as an immediate product of malate oxidation by the mammalian succinate dehydrogenase

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Rapid malonate-sensitive transitory formation of *enol*-oxaloacetate followed by slow ketonization of the product was observed after addition of malate to the mammalian succinate-ubiquinone reductase in the presence of electron acceptor. The initial rate of *enol*-oxaloacetate production was equal to that of malate oxidation. Oxaloacetate *keto–enol* tautomerase had no effect on the initial rate of *enol*-oxaloacetate production nor on the kinetics of malate oxidation; the enzyme drastically accelerated the ketonization of the product. The solubilized and partially purified membrane-bound flavine adenine dinucleotide-dependent malate dehydrogenase from *Acetobacter xylinum* catalyzed oxidation of L- and D-malate without formation of *enol*-oxaloacetate as an intermediate of the reaction.

Succinate dehydrogenase; Malate dehydrogenase; Oxaloacetate; Tautomerism, *keto–enol*; Suicide inhibition; Bovine heart mitochondria; *Acetobacter xylinum*

1. INTRODUCTION

The mammalian succinate dehydrogenase (EC 1.3.99.1) being an intrinsic component of the inner mitochondrial membrane catalyzes oxidation of succinate by ubiquinone with the formation of fumarate and ubiquinol during the operation of the Krebs cycle in the mitochondrial matrix. The enzyme is a subject of feedback control by oxaloacetate (OA) which is a product of the malate dehydrogenase (EC 1.1.1.37) reaction ([1–5] and references cited therein). OA can also be formed as a product of L- or D-malate oxidation by the succinate dehydrogenase itself [6]. The latter process leads to a suicide inhibition of the enzyme since the first-order rate constant for the activation of oxaloacetate-inhibited succinate dehydrogenase is extremely low (0.02 min^{-1} at 25°C [4]). The evidence has been presented that *enol*-OA is an immediate product of L- or D-malate oxidation at the succinate dehydrogenase active site [6] and that transformation of metabolically inactive *enol*-OA into *keto*-isomer is catalysed by the specific mitochondrial OA *keto–enol* tautomerase (OAT; EC 5.3.2.2) [7]. The latter enzyme activity is widely distributed in mammalian and avian organs, plants and bacteria [8]. However, no metabolic sources of *enol*-oxaloacetate except for suicide malate oxidation by the mammalian succinate dehydrogenase are known so far.

The purpose of the present studies was twofold. However strong the circumstantial evidence for *enol*-OA production [7], it seemed desirable to show directly the formation of *enol*-OA during malate-ubiquinone reductase reaction catalyzed by the mammalian succinate dehydrogenase. It was also of interest to find out whether or not *enol*-isomer is a product of malate oxidation by the family of membrane-bound flavin-dependent malate dehydrogenases widely distributed in microorganisms [9–11]. We were able to show directly a transitory formation of *enol*-OA during malate oxidation by the mammalian succinate dehydrogenase; no *enol*-oxaloacetate was identified as a product of the membrane-bound malate dehydrogenase from *Acetobacter xylinum*.

Abbreviations: OA, oxaloacetate; OAT, 37 kDa oxaloacetate *keto–enol* tautomerase; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Q_2, homolog of ubiquinone having two isoprenoid units in position 6 of the quinone ring; PMS, *N*-methylphenazonium sulphate; DCIP, 2,2-dichlorophenolindophenol; MOPS, 4-morpholinepropanesulphonic acid

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2. MATERIALS AND METHODS

Succinate-ubiquinone reductase [12] free of succinate and tightly bound OA [13] and 37 kDa oxaloacetate *keto–enol* tautomerase [7] were prepared from bovine heart mitochondria according to the published procedures. FAD-dependent malate dehydrogenase from *Acetobacter aceti* subsp. *xylinum* cells was partially purified by the original method which included solubilization of the membranes with CHAPS, gel filtration through the column with Ultragel AcA-34 and concentration of the soluble enzyme in high-pressure ultrafiltration cell. The specific activities of the final preparations with 0.4 mM ferricyanide or 10 μM Q_2 as the electron acceptors were 2.7 and 2.5 μmol of malate oxidized per min per mg of protein, respectively at 24°C, pH 7.5. The complete procedure for preparation of the enzyme will

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be published elsewhere. All spectral measurements were performed at 24°C using Hitachi-557 spectrophotometer. Protein was determined by Lowry procedure [14]. Q_2 and PMS were from Ferak (FRG); Triton X-100, and MOPS were from Serva (Germany); CHAPS and FAD were from Sigma (USA); DCIP was from BDH (UK); Ultragel AcA-34 was from LKB-ABF (France); L-malate dehydrogenase (EC 1.1.1.37) was from Reanal (Hungary). Other chemicals were of the highest quality commercially available.

3. RESULTS AND DISCUSSION

When succinate dehydrogenase catalyzes oxidation of L- or D-malate in the presence of suitable electron acceptor, L-glutamate, and L-glutamate-oxaloacetate aminotransferase (EC 2.6.1.1) the rapid suicide inhibition of the former enzyme is prevented by OAT. This observation strongly suggests that *enol*-OA is an intermediate of the overall aspartate formation [6]. The enolic form of OA has an absorption maximum at 260 nm [15] and since the rate of ketonization under certain conditions is relatively low [16], the formation of *enol*-OA can be followed spectrophotometrically. However, in practice such a registration is hampered because of strong absorbance change of any suitable electron acceptor at 260 nm during its oxidoreduction. This difficulty was overcome using water-soluble ubiquinone homolog Q_2 as the electron acceptor for succinate-ubiquinone reductase, as depicted in Fig. 1. The differential spectrum of $Q_{ox}-Q_{red}$ shows equal absorbance at 260 and 290 nm, whereas significant absorbance of *enol*-OA is seen at this wavelength pair ($\Delta A_{260-290}$). Thus an increase of *enol*-OA can be followed using dual wavelength spectrophotometry without overlap caused by the quinone reduction. The linear dependence of the initial $\Delta A_{260-290}$ on the concentration of OA added from dry dioxane solution (100% of *enol*-isomer [17]) followed by slow ketonization was observed (the results are not shown) and the molar extinction coefficient for *enol*-OA $\epsilon_{260-290}$ = $8.5 \times 10^3\ M^{-1} \cdot cm^{-1}$ was calculated. No change of the absorbance at this wavelength pair was observed when sodium borohydride was added to the aqueous Q_2 solution.

Fig. 2 shows the time course of $\Delta A_{260-290}$ along with that of DCIP reduction (independent measurements in a separate sample) during D-malate oxidation by the succinate-ubiquinone reductase. The initial rates of DCIP reduction (0.07 μmol of malate oxidized per min per mg of protein) and that of *enol*-OA production (0.07 μmol per min per mg of protein using $\epsilon_{260-290}$ *enol*-OA = $8.5 \times 10^3\ M^{-1} \cdot cm^{-1}$) were determined. Both DCIP reduction and *enol*-OA formation were abolished by 20 mM malonate. More significantly, no changes of $\Delta A_{260-290}$ were observed when the reaction was started by 5 mM succinate thus suggesting that the reduction of the enzyme chromophores (flavin, iron-sulfur centers [12]) did not contribute to the $\Delta A_{260-290}$ changes observed. Rapid *enol*-OA formation after ad-

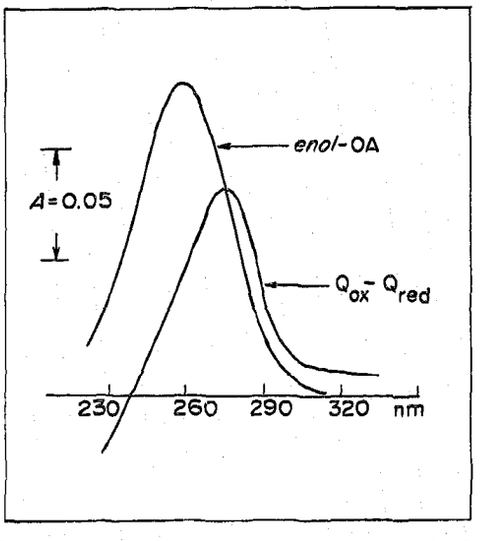

Fig. 1. Optical spectra of *enol*-oxaloacetate and Q_2. Solid oxaloacetate was dissolved in dry diethyl ether (final concentration was 25 μM, 67% of *enol*-isomer [7]). Q_2 (12 μM) was dissolved in the mixture containing 2 mM MOPS, pH 7.5, 0.2 mM potassium EDTA and 0.004% Triton X-100. Few crystals of sodium borohydride were added to one cuvette. The mixture was rapidly stirred and left for 2 min before the differential spectrum was recorded.

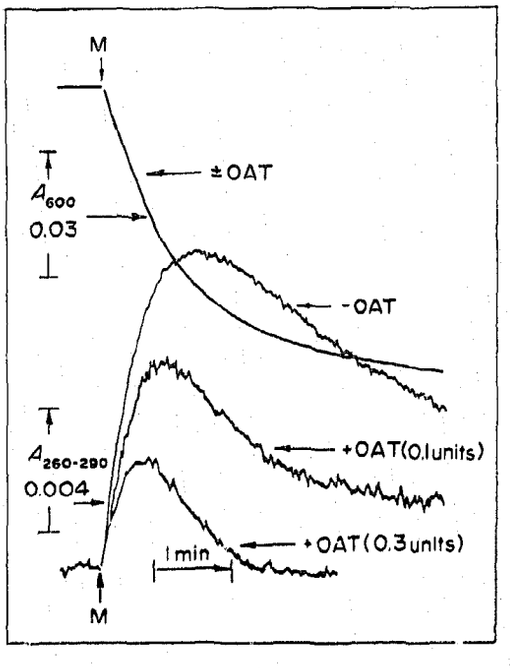

Fig. 2. Time course of D-malate oxidation and *enol*-oxaloacetate formation by succinate-ubiquinone reductase. The enzyme (80 μg of protein) was added to 2 ml of the mixture containing: 2 mM MOPS, pH 7.5, 0.2 mM EDTA, 0.004% Triton X-100. The reaction was started by 10 mM D-malate (M) where indicated. 2 mM PMS and 50 μM DCIP were added when D-malate-DCIP reductase (A_{600}, upper curve) was recorded. 10 μM Q_2 as an electron acceptor was present when *enol*-oxaloacetate ($A_{260-290}$) was recorded. Bovine heart mitochondrial 37 kDa-oxaloacetate-*keto–enol* tautomerase (OAT) was added to the assay system where indicated. Both changes of A_{600} and that of $A_{260-290}$ were completely abolished by 20 mM malonate.

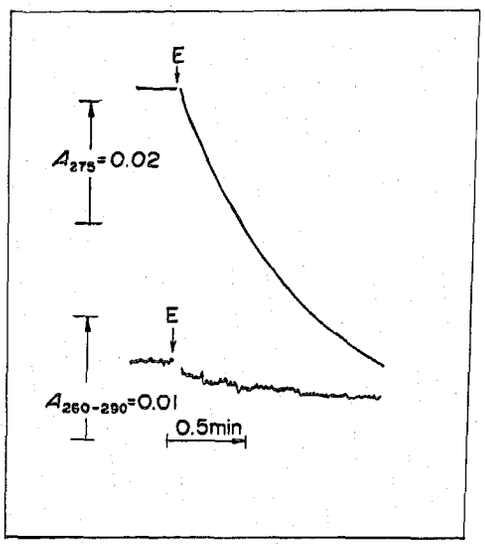

Fig. 3. Time-course of malate oxidation and oxaloacetate production by *Acetobacter xylinum* malate dehydrogenase. The reaction was started by the addition of 5 μg of the soluble dehydrogenase (E) to the mixture containing: 2 mM MOPS, pH 7.5, 0.2 mM EDTA, 0.004% Triton X-100, 10 μM Q_2 and 15 mM D- or L-malate. Before the experiments the enzyme (1.6 mg/ml) was preincubated with 0.6 mM FAD for 10 min at 25°C.

dition of D-malate was followed by a slow decrease of $\Delta A_{260-290}$ presumably due to strong inhibition of the enzyme activity by *enol*-OA formed (note that rapid decline of DCIP reduction occurs) and because of spontaneous ketonization of the product. The amount of *enol*-OA produced corresponds to 14 turnovers of D-malate oxidation per mol of the enzyme (calculated in terms of the covalently bound flavin [12]). The mitochondrial 37 kDa-OAT [7] added to the assay cuvette changed neither the initial rate of *enol*-OA production nor the time course of DCIP reduction. However, as expected, it did change the pattern of a transitory *enol*-OA formation by decreasing maximal $A_{260-290}$ amplitude and increasing the ketonization rate. Thus the results shown in Fig. 2 demonstrate unambiguously that *enol*-OA is an immediate product of malate oxidation at the succinate dehydrogenase active site.

Taking into account some similarities between the mammalian succinate dehydrogenase and FAD-dependent membrane-bound malate dehydrogenase (EC 1.1.99.?) of microorganisms [9–11] we have speculated [7] that *enol*-OA may be the product of the latter enzymes. Thus, it was of interest to identify the tautomeric form of OA produced by the FAD-dependent malate dehydrogenase from *Acetobacter xylinum*. The data presented in Fig. 3 show that no *enol*-OA is formed during malate oxidation by this enzyme. It seemed possible that the dehydrogenase from *Acetobacter xylinum* initially produces *enol*-OA and *keto*-OA finally forms as a result of the subsequent intramolecular proton transfer reaction at the enzyme active site as has been suggested for pyruvate kinase (EC 2.7.1.40) mechanism [18]. We found no catalysis of OA *enol–keto* tautomerization by the malate dehydrogenase from *Acetobacter xylinum*. In accord with this we also showed that OAT did not accelerate NADH-ferricyanide reductase reaction catalysed by the coupled operation of the mammalian NAD^+-dependent malate dehydrogenase (EC 1.1.1.37) and *Acetobacter xylinum* FAD-dependent malate dehydrogenase (EC 1.1.99.?) in the presence of limited amount of either D- or L-malate. Thus it is safe to conclude that in contrast to the mammalian succinate dehydrogenase the malate dehydrogenase from *Acetobacter xylinum* oxidizes malate with a formation of *keto*-OA as the reaction product.

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