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Identification of the high-molecular-mass mitochondrial oxaloacetate *keto-enol* tautomerase as inactive aconitase

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The homogeneous bovine heart mitochondrial high-molecular-mass oxaloacetate *keto-enol* tautomerase [(1988) Biochim. Biophys. Acta 936, 10–19] is shown to be an iron-sulfur protein as revealed by the enzyme spectral properties and direct chemical determination of non-heme iron and acid-labile sulfur. The protein is capable of catalysing the aconitase reaction after treatment with ferrous ions under anaerobic conditions. Treatment of the 'activated' protein with *N*-ethylmaleimide results in the simultaneous irreversible loss of the oxaloacetate *keto-enol* tautomerase and aconitase activities. The effects of some substrates and inhibitors on both activities show that the same catalytic site is involved in the oxaloacetate tautomerase and aconitase reactions. It is concluded that the protein previously described as a 80 kDa oxaloacetate *keto-enol* tautomerase is inactive aconitase.

Oxaloacetate tautomerase, *keto-enol*; Aconitase; Iron-sulfur protein; (Bovine heart mitochondria)

1. INTRODUCTION

The rate of tautomeric interconversion between *keto*- and *enol*-isomers of oxaloacetate under certain conditions can be much lower than the oxaloacetate turnover in the enzyme-catalyzed reactions [1–4]. Although no metabolic sources of the *enol*-isomer have so far been found, the specific enzyme named oxaloacetate *keto-enol* tautomerase (EC 5.3.2.2) has been found and partially purified from porcine kidney extracts [5]. Recently we have described the malate dehydrogenase activity of succinate dehydrogenase (EC 1.3.99.1), and the *enol*-isomer of oxaloacetate has been identified as the reaction product [6]. Subsequently, two highly purified proteins, with apparent molecular masses of 37 and 80 kDa, capable of the oxaloacetate *keto-enol* tautomerase activity were isolated from bovine heart mitochondrial matrix [7]. Both mitochondrial enzymes were found to be quite different from that previously obtained from porcine kidney extracts [5,8]. We have pointed out that the proteins described may either be the unique mitochondrial oxaloacetate *keto-enol* tautomerases, or their tautomerase activity is the partial reaction of certain enzymes capable of oxaloacetate binding [7]. In this report it will be shown that the larger 80 kDa protein is identical to the mitochondrial inactive aconitase.

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Abbreviations: OAT-2, 80 kDa oxaloacetate *keto-enol* tautomerase; NEM, *N*-ethylmaleimide

2. MATERIALS AND METHODS

Electrophoretically homogeneous 80 kDa oxaloacetate keto-enol tautomerase (OAT-2) was prepared from the mitochondrial matrix as described [7]. The tautomerase activity (enol → ketone direction) was measured at 25°C using a coupled malate dehydrogenase (EC 1.1.1.37) assay [7] in a mixture containing 2 mM Tris-Cl^-, 0.2 mM NAD·H, proper amounts of malate dehydrogenase and 35 μM *enol*-oxaloacetate added from a freshly prepared solution of solid acid in dry acetone (the pH of the assay mixture after addition of oxaloacetic acid was 9.0). The aconitase activity (EC 4.2.1.3) was measured as the appearance of *cis*-aconitate registered at 240 nm [9] at 25°C in the assay mixture containing 90 mM Tris-Cl^-, pH 9.0, and 2 mM DL-isocitrate or 4 mM citrate. Before the aconitase assay, the

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protein was activated [10] as follows. Small samples of the protein stored in liquid nitrogen were thawed and dialyzed at 4°C for 4 h (1 change of the buffer) against 100 ml of 5 mM Hepes, pH 7.5, and 5 mM dithiothreitol. 0.5 mM $FeSO_4$ (final concentration) was added to 0.1 ml of the dialyzed protein solution under argon, and the mixture was incubated anaerobically at 0°C for 20 min. All the kinetic parameters for the aconitase reaction were measured for the activated protein.

The protein content was determined with Coomassie G-250 [11]. Acid-labile sulfur [12] and non-heme iron [13] contents were determined according to the published procedures. Oxaloacetate, Tris, Hepes, DL-isocitrate and fluorocitrate were from Sigma (USA); NAD·H and pig heart L-malate dehydrogenase were from Reanal (Hungary); *N*-ethylmaleimide was from BDH (England). Other chemicals were of the highest quality commercially available.

3. RESULTS AND DISCUSSION

Examination of some properties of the intramitochondrial soluble enzymes has led us to the conclusion that OAT-2 is remarkably similar to aconitase. The molecular masses of OAT-2 (80 kDa as revealed by SDS gel electrophoresis and 89 kDa as revealed by Sephacryl gel filtration [7]) and that of the mitochondrial aconitase (83 kDa [14]) are very close. Concentrated solutions of OAT-2 have an amber color and aconitase is known as an iron-sulfur protein [15]. Interestingly, aconitase catalyzes the chemical transformation of that part of the C_4 fragment of citrate which originated metabolically from oxaloacetate [16], and the *cis*-form of *enol*-oxaloacetate is a structural analog of isocitrate and *cis*-aconitate. It is also worthwhile mentioning that chemical events in the catalysis of hydration/dehydration and *keto-enol* tautomerization may be remarkably similar: both reactions evidently involve the proton abstraction step. Taken together, these points make a close comparison of OAT-2 and aconitase quite judicious.

Fig.1 demonstrates the spectral properties of OAT-2 which are clearly typical for several iron-sulfur proteins including aconitase [15]. The electrophoretically homogeneous active preparations of OAT-2 as prepared do not catalyze the aconitase reaction. However, when OAT-2 was treated under the conditions known to activate aconitase [10,17], the protein showed a high aconitase activity without any substantial loss of the oxaloacetate *keto-enol* tautomerase activity (fig.2). Significantly, both the aconitase and tautomerase activities of the activated protein are irreversibly inhibited by *N*-ethylmaleimide in a pseudo-first order reaction with the same apparent rate constant. It is relevant to recall that the participation of the sulfhydryl group(s) in the aconitase reaction has been documented [18]. It was shown in separate experiments that the saturation of OAT-2 with oxaloacetate completely protects the enzyme against the inactivation by *N*-ethylmaleimide.

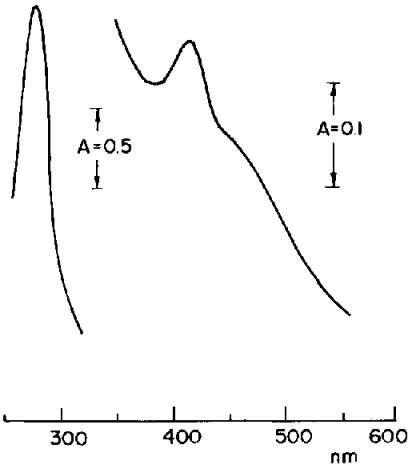

Fig.1. Optical spectrum of the 80 kDa oxaloacetate *keto-enol* tautomerase (2 mg of protein/ml in 75 mM Hepes, pH 7.5).

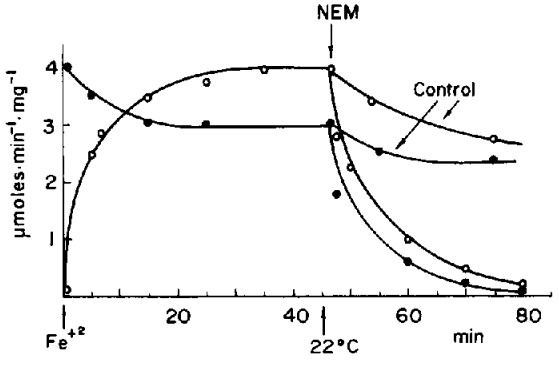

Fig.2. The tautomerase and aconitase activities of 80 kDa oxaloacetate *keto-enol* tautomerase. 0.3 ml of OAT-2 (2 mg/ml in 5 mM Hepes, pH 7.2, containing 1 mg/ml of bovine serum albumin) was saturated with argon at 0°C for 20 min. 0.5 mM $FeSO_4$ (final concentration) was added at zero time to an anaerobic solution and incubation continued. The proper amounts of the samples were withdrawn during incubation and the oxaloacetate *keto-enol* tautomerase (●) and aconitase (○) activities were assayed. The sample was then rapidly heated up to 22°C (indicated by arrow), 0.5 mM *N*-ethylmaleimide was added and incubation and assays were continued. No *N*-ethylmaleimide was added to the control sample.

Table 1

A comparison of the high molecular mass oxaloacetate *keto-enol* tautomerase and aconitase

Kinetic properties	Oxaloacetate *keto-enol* tautomerase [7]	Aconitase[a]	
		DL-Isocitrate → *cis*-aconitase	Citrate → *cis*-aconitase
V_{max} (μmol · min^{-1} · mg^{-1})	20[b]	10	6.5
K_m (μM)	220 (*enol*-oxaloacetate)[b]	73	190
Inhibitors			
DL-Isocitrate	K_i = 25 μM	-	-
Pyrophosphate	20 μM causes 50% inhibition at 50 μM *enol*-oxaloacetate	2 mM causes 50% inhibition at 120 μM DL-isocitrate	2 mM causes 50% inhibition at 150 μM citrate
Fluorocitrate	0.2 mM causes complete inhibition		
NEM	irreversible inhibition; k = 160 min^{-1} · M^{-1} at 22°C, pH 7.2		
Other properties			
Molecular mass	80–89 kDa[b] single polypeptide	80–83 kDa [14] single polypeptide	
Iron content (atoms/mol)	1.5	1.02 [10]; 2.9 [14]	
Acid-labile sulfur (mol/mol)	1.9	3.9 [14]	

[a] Measured after activation (see section 2) at 15°C, pH 9.0, 90 mM Tris-Cl$^-$
[b] Data taken from [7]

Some kinetic and other properties of OAT-2 and aconitase are listed in table 1. The data presented leave no doubt that the high-molecular-mass OAT-2 and aconitase are identical.

The functional significance of the enzyme-catalyzed oxaloacetate tautomerization in the mitochondrial matrix, namely, the rapid ketonization of *enol*-oxaloacetate produced by the malate dehydrogenase activity of succinate dehydrogenase has been suggested [6]. The findings documented in this report raise the possibility of the aconitase (active or inactive) involvement in the regulation of succinate dehydrogenase. On the other hand, the oxaloacetate tautomerase activity of aconitase discovered may just be an artificial side reaction catalyzed by the enzyme. If the last statement is correct, the unique role of oxaloacetate *keto-enol* tautomerase in the mitochondrial matrix is fulfilled by the recently described lower-molecular-mass (37 kDa) protein [7].

The oxaloacetate tautomerase activity of inactive aconitase may shed some light on the reaction mechanism of hydration/dehydration catalyzed by the enzyme [16,19–21]. The discovery of 3Fe-4S → 4Fe-4S transformation responsible for aconitase activation [22] raised the question of the functional role of the iron-sulfur core in the catalytic mechanism. The hypothesis on the participation of the unique Fe_a atom of activated aconitase in the coordination of the citrate or isocitrate OH-group [19,20] has been put forward, which is definitely different from the 'iron-wheel' mechanism [16] proposed earlier. Our data show that inactive aconitase, which contains a modified inactive iron-sulfur cluster, is still capable of binding and transforming *enol*-oxaloacetate. Since *keto-enol* tautomerization is known to involve the proton abstraction step [1–4], we believe that the proposed aconitase mechanism which includes the coordination of the substrate by the Fe_a atom with a subsequent liberation of the deprotonated cysteinyl residue accepting proton from the substrate [19,20] needs some modifications.

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