

論 文

アフリカツメガエル *Xenopus laevis*
分岐鎖 α -ケト酸脱水素酵素複合体の
デカルボキシラーゼ成分の性質

Properties of the Decarboxylase Component of *Xenopus laevis*
Branched-chain α -Keto Acid Dehydrogenase Complex

小 野 和 夫
Kazuo Ono

キーワード：アフリカツメガエル、ミトコンドリア、分岐鎖 α -ケト酸脱水素酵素複合体、デカルボキシラーゼ、サブユニット

Keywords: *Xenopus laevis*, mitochondria, branched-chain α -keto acid dehydrogenase complex, decarboxylase component, subunit

SUMMARY

Branched-chain α -keto acid dehydrogenase complex (BCKADC) contains decarboxylase (E1), dihydrolipoyl transacylase, and dihydrolipoyl dehydrogenase as catalytic components. To characterize BCKADC of amphibian, mitochondrial extract of *Xenopus laevis* liver was subjected to immunoblotting analysis using rat anti E1 antibodies. As a result, three immunoreactive proteins were detected; the molecular mass of proteins was estimated to be 36, 37 and 46 kDa. Incubation of liver mitochondria from *Xenopus laevis* prior to solubilization of E1 resulted in no significant change in their appearance and amount. These proteins were also detected in heart of *Xenopus laevis* as well as liver of the amphibian. Sucrose density gradient centrifugation under the condition, which brings dissociation of BCKADC to each component enzyme, revealed that the three proteins of *Xenopus laevis* moved together through the gradient. The density that three proteins settled corresponded well with that of rat E1 did. Present results obtained here showed that E1 of *Xenopus laevis* contains three proteins at least, and the 47 kDa protein corresponds to E1 α subunit in comparison with molecular mass of E1 α of other species.

INTRODUCTION

Branched-chain α -keto acid dehydrogenase complex (BCKADC) functions as a rate limiting enzyme of branched-chain amino acids such as leucine, isoleucine and valine^{1,2,3,4)}. Mammalian BCKADC is constituted by three catalytic components of decarboxylase (E1), dihydrolipoylacyl-transferase (E2)

and dihydrolipoamide dehydrogenase (E3)^{4,5,6}. The multi enzyme complex is composed of 12 molecules of E1, 6 molecules of E3 and E2 core, which consists of 24-mer of E2 molecules. The E1 and E3 are bound noncovalently to the E2 core^{5,6,7}. The E2 subunit of mammals contains three structural domains, a lipoyl-bearing domain in the amino-terminal portion, an E1/E3-binding domain and an inner-core domain in the carboxyterminal portion, linked by two interdomain segments (linkers)^{6,7}. In addition, mammalian BCKADC contains regulatory enzymes, E1 specific kinase and phosphorylated E1 phosphatase^{3,8,9}.

BCKADC is also found in non-mammalian vertebrates such as avian¹⁰ and fish^{11,12,13}. It had been already reported that the similarity in the primary structure of chicken E1 α and E1 β to human are 93.3 % and 95.9 % , respectively¹⁰. E1 from these species worked interchangeably with rat E1 or bovine E1 as decarboxylase component of BCKADC^{10,13}.

We had also reported that E2 subunits of chicken and rainbow trout are comparable to mammalian E2 in regard to the primary structure and function^{10,13}. However additional residues in the linkers of E2 leading to reduced E1 binding capacity and reduced activity of the overall reaction catalyzed by chicken and rainbow trout BCKADC^{10,13}. As to E3, its amino acid sequence and function are conserved very well among animal species^{14,15}.

These studies suggest that both structure and functions of E1, E2 and E3 are conserved well among animal species. On the other hand, we had detected unexpectedly three proteins by immunoblotting with mitochondrial extract of an amphibian, *Xenopus laevis*, liver using anti rat E1 IgG. E1 from mammals, chicken and rainbow trout consist of α and β subunit^{10,13} in tetramer of $\alpha 2 \beta 2$ ^{16,17}.

In this study, the relationship between E1 molecule and three antigens to the anti rat E1 was investigated to assign three proteins to exact subunit(s) of E1 of *Xenopus laevis*. Interaction between these three proteins and E2 core was also examined using sucrose density gradient centrifugation.

MATERIALS AND METHODS

Materials

Male *Xenopus laevis* was obtained from a domestic farm. Aprotinin and leupeptin were from Boehringer (Mannheim, Germany) and Peptide institute (Osaka, Japan), respectively. All other chemicals were the highest grade commercially available. Rat E1, E2, E3, and chicken E2 were prepared as described previously^{10, 18}. Polyclonal anti-rat E1 and rat E2 antibodies were raised in rabbits with purified rat E1 and rat E2, respectively, as described¹⁹.

Preparation of mitochondria and mitochondrial extract

Xenopus laevis liver mitochondria were prepared basically according to the method of Roche and Cate²⁰. The mitochondria were suspended in a minimum volume of 20 mM potassium phosphate buffer (KPB) (pH6.5) containing 2 μ g/ml of leupeptin, 2 μ g/ml of aprotinin, 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM benzamidine. The suspension was frozen in an Erlenmeyer flask in dry ice/isopropanol bath and stored at -80°C until use. The frozen mitochondria were thawed in cold running water, diluted three times the original volume with 20 mM KPB (pH 6.5) and homogenized with a motor-driven Potter-Elvehjem homogenizer. The suspension was made up in 50 mM NaCl, 0.1 mM

phenylmethylsulfonyl fluoride, 0.5 mM benzamide, 1 μ g/ml leupeptin and 1 μ g/ml aprotinin, followed by centrifugation at 32,000 *g* for 30 min at 4°C. The supernatant was diluted with 20 mM KPB(pH 7.5) to make a concentration of 9 mg protein per ml, and made up in 0.2 mM thiamine pyrophosphate (TPP), 1%(w/v) bovine serum albumin (BSA) and 10 mM MgCl₂. The solution was brought to pH 7.5 by adding 10 M KOH drop by drop with stirring.

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to Laemmli²¹⁾ with 10% SDS-polyacrylamide gel, and proteins were electro blotted onto polyvinylidene difluoride membrane (Nihon Millipore Ltd, Yonezawa, Japan). Western blot analysis was performed as described by Harlow and Lane²²⁾, using goat anti-rabbit IgG conjugated alkaline phosphatase (ZYMED, San Francisco, CA, USA).

Sucrose density gradient centrifugation

The mitochondrial extract was incubated for 30 minutes at 4°C in the presence of 1M NaCl followed by layering onto a ultracentrifugation tube (1.5 cm in diameter x 9.6 cm long) containing 11.5 ml of a linear sucrose density gradient from 0 to 20%(w/v) in KPB(pH7.5) containing 5mM DTT and 1M NaCl^{10,18)}. The centrifugation was performed at 110,000 *g* in RPS40T rotor by Hitachi SCP 85H centrifuge for 17 hours. After the centrifugation, the gradient was divided into 23 fractions in equal volume (0.5ml) from the top. E1 in each fraction was detected by immunoblotting. E2 in the extract from rat liver mitochondria was detected by anti-rat E2 IgG.

Protein determination

The amount of protein was measured by bicinchoninic acid method²³⁾, using BCA Protein Assay Reagent Kit (PIERCE, Rockford, IL, USA) with BSA as a standard.

RESULTS

Immunoblotting of BCKADC E1 of mitochondria from *Xenopus laevis* liver

To evaluate whether the primary structure of *Xenopus laevis* E1 is distinguished immunologically from other vertebrates, immunoblotting of the E1 was conducted using anti rat E1 IgG. For comparison of the composition and molecular mass of E1 subunit, liver mitochondria extracts of *Xenopus laevis*, rat, chicken and rainbow trout were subjected to the analysis. The result was shown in Fig. 1. Except *Xenopus laevis*, clearly two

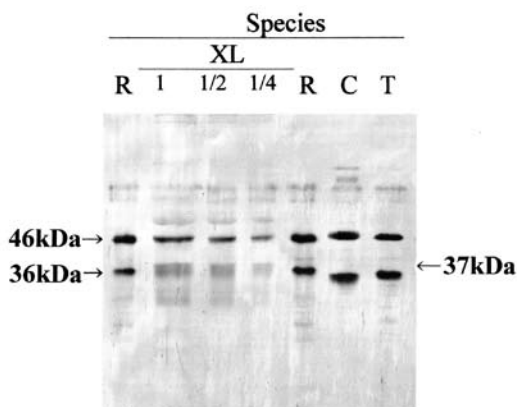


Fig.1. Immunoblotting analysis of E1 component of *Xenopus Laevis*.

Mitochondrial extract of liver from *Xenopus laevis* was prepared as described in the section of "Materials and Methods". The extract was electrophoresed on 10% polyacrylamide gel, followed by electro-transferring to polyvinylidene difluoride membranes. The resolved subunits were probed with anti-rat E1 antibodies. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG. Lanes of R, C and T are purified rat BCKADH (2 μ g), partial purified chicken BCKADH (14 μ g) and purified rainbow trout BCKADH (3.6 μ g), respectively. Lanes of XL1, XL1/2 and XL1/4 are 125, 62.5 and 31.3 μ g protein of mitochondrial extract from *Xenopus laevis* liver, respectively.

immunoreactive bands of 46 kDa and 36 kDa were detected.

In the mitochondrial extract of liver from *Xenopus laevis*, one more immunoreactive smear band was detected in addition of 46 kDa and 36 kDa proteins (Fig. 1). The molecular mass of the protein on the blot was estimated to be approximately 37kDa by comparison of relative mobility of the α (46 kDa) and β (36 kDa) subunits of rat E1^{4,10,13}.

Effect of the incubation of *Xenopus laevis* mitochondria on the pattern of immunoblotting

E1 molecule has been reported so far consists of α and β subunits in $\alpha 2\beta 2$ ^{16,17}. If it is true of *Xenopus laevis*, it is uncertain how three proteins with molecular mass of 36, 37 and 46 kDa correspond to the α and β subunits of *Xenopus laevis* E1. One possible explanation is that proteolytic cleavage of E1 α of 46 kDa by mitochondrial protease²⁴ produced 37 kDa protein. Therefore, to ascertain whether endogenous mitochondrial protease action involves or not, mitochondria of liver from *Xenopus laevis* were incubated at 25°C for 2 hours. The result indicates clearly that the incubation caused no significant change of the apparent molecular mass and quantitative ratio of the three immunoreactive proteins as shown in Fig. 2.

Immunoblotting analysis of kidney and heart of *Xenopus laevis*

Relatively high BCKADC activity was found in kidney and heart of several animal species^{4,10,13}. Thus, immunoblotting analysis was also conducted using kidney and heart of *Xenopus laevis* to confirm the profile of immunoblot obtained with liver is applicable to these organs. As to kidney and heart of rat, two major immunoreactive proteins with molecular mass of 46 kDa and 36 kDa were detected by anti rat E1 IgG (Fig. 3). In case

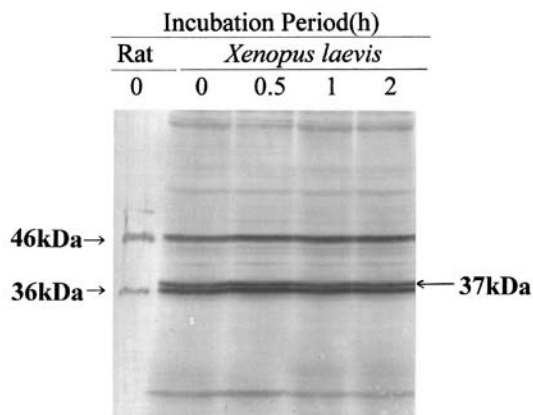


Fig.2. Effect of incubation of mitochondria from liver of *Xenopus laevis* on immunoblotting pattern with anti rat E1 antibodies.

Mitochondria from *Xenopus laevis* liver were incubated at 25°C for 2hours. For immunoblotting analysis, an aliquots of mitochondria were taken at intervals as indicated, and then the sample was solubilized in the presence of the following protease inhibitors: 0.1mM PMSF, 0.1mM benzamidine, 0.3mM aprotinin, and leupeptin 1mg/ml. Immunoblotting of the extract conducted as described in "Materials and Methods".

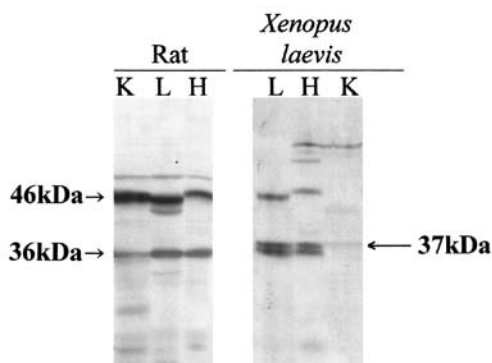


Fig.3. Immunoblotting analysis of E1 component of BCKADC from kidney and heart from *Xenopus laevis*.

Homogenate from liver, heart and kidney (250 μ g protein each) were subjected to SDS-PAGE with 10% polyacrylamide gel, followed by electro-transferring to polyvinylidene difluoride membranes. The resolved subunits were probed with anti-rat E1 antibodies. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG as described in "Materials and Methods". H, K and L indicate heart, kidney and liver, respectively.

of heart from *Xenopus laevis*, it contained three immunoreactive proteins of 36, 37 and 46 kDa as well as liver. Kidney showed very weak signal corresponding to 37 kDa and 46 kDa. These observation indicate that there are at last three proteins, which are recognized as antigens to rat E1 antibodies, in liver and heart from *Xenopus laevis*.

Sucrose density gradient centrifugation of mitochondrial extract of liver from *Xenopus laevis*

It is still unclear which of the three immunoreactive proteins function as E1 subunit(s) of E1? Sucrose density gradient centrifugation was carried out to answer this query. E1 component of BCKADH binds noncovalently to E1 binding domain on E2 molecule through E1 β subunit²⁵⁾, accordingly if the three proteins, which were detected by anti-rat E1 IgG, correspond to subunits of E1 of *Xenopus laevis*, the immunoreactive proteins should move together through the gradient independent on individual molecular mass under the experimental condition. Sucrose density gradient centrifugation was conducted after incubation of the extract of liver mitochondria with 1M NaCl for dissociation of BCKADC¹⁸⁾. Molecular mass of Rat E1 and E2 core are estimated to be 160 kDa and 2,000 kDa based on composition and molecular mass of subunits, respectively⁴⁾. As expected from the estimation, rat E1 was detected in fractions containing lower concentration of sucrose than that of fractions E2 moved (Fig. 4). Under the same experimental conditions, the three immunoreactive proteins of liver of *Xenopus laevis* were detected in the fractions corresponding to the fractions in which rat E1 α and E1 β were detected (Fig. 4).

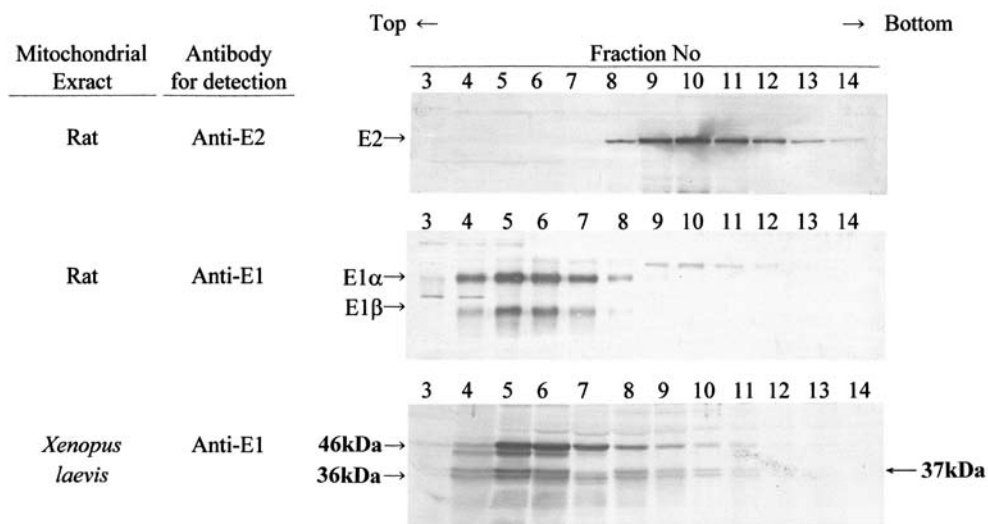


Fig.4. Sucrose density gradient centrifugation under the condition of resolution of E1 from E2 core.

Mitochondrial extract (3 mg of protein) from liver of *Xenopus laevis* was incubated at 4 °C for 30 min with 1M NaCl. The sample was subjected to sucrose gradient centrifugation, followed by fractionation in order from the top, as described in Materials and Methods. The numbers in each panel correspond to the fraction number.

DISCUSSION

Three proteins with molecular mass of 36, 37 and 46 kDa were detected by immunoblotting of mitochondrial extract of *Xenopus laevis* liver using rat anti E1 antibodies (Fig. 1). The relative amount of three proteins were not influenced by incubation of mitochondria at 25°C for 2 hours before extraction from mitochondria (Fig. 2). These results demonstrate that the appearance of three proteins on the blot was not caused by proteolysis of intact E1 subunit during solubilization from mitochondria. This thought is also supported by the result of which three proteins were detected in heart of *Xenopus laevis* (Fig. 3).

Sucrose density gradient centrifugation showed that there was no significant difference on the sedimentation profile among three proteins of 36, 37 and 46 kDa in the presence of 1M NaCl (Fig. 4). Moreover the ratios in amount of 37 kDa to 46 kDa and 36 kDa to 46 kDa were constant through the above mentioned fractions (Fig. 4). It is noteworthy that fractions to which these proteins moved correspond to fractions rat E1 moved. These results suggest that all the immunoreactive proteins of 36, 37, 46 kDa in *Xenopus laevis* behaved together, not individually. During the centrifugation, each species of macromolecule in the original solution moves through the gradient at a rate that is mainly dictated by its mass or size^{10,18}. Since molecular mass of rat E1 was estimated to be 160 kDa approximately^{4,10}, the above observation may provide a possible explanation of three proteins associating each other, and constituting E1 molecule of *Xenopus laevis*.

Taking into consideration obtained results from present study, it is concluded that *Xenopus laevis* has three proteins, which react with anti rat E1 IgG. Based on the limited information on primary structure of E1 of nonmammalian vertebrates^{10,11,13}, it can be speculated that the protein with molecular mass of 46 kDa is almost likely to be E1 α subunit. As to E1 β subunit, it is hard to distinguish between 36 and 37 kDa proteins except the difference in the molecular mass. Which of proteins correspond to functional E1 β subunit? To clarify these queries, cDNA cloning of E1 subunit(s) of *Xenopus laevis* is essential.

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