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Studying anti-oxidative properties of inclusion complexes of α-lipoic acid with γ-cyclodextrin in single living fission yeast by confocal Raman microspectroscopy

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ABSTRACT

α-lipoic acid (ALA) is an essential cofactor for many enzyme complexes in aerobic metabolism, especially in mitochondria of eukaryotic cells where respiration takes place. It also has excellent anti-oxidative properties. The acid has two stereo-isomers, R- and S- lipoic acid (R-LA and S-LA), but only the R-LA has biological significance and is exclusively produced in our body. A mutant strain of fission yeast, *Δdps1*, cannot synthesize coenzyme Q10, which is essential during yeast respiration, leading to oxidative stress. Therefore, it shows growth delay in the minimal medium. We studied anti-oxidant properties of ALA in its free form and their inclusion complexes with γ-cyclodextrin using this mutant yeast model. Both free forms R- and S-LA as well as 1:1 inclusion complexes with γ-cyclodextrin recovered growth of *Δdps1* depending on the concentration and form. However, it has no effect on the growth of wild type fission yeast strain at all. Raman microspectroscopy was employed to understand the anti-oxidant property at the molecular level. A sensitive Raman band at 1602 cm^{-1} was monitored with and without addition of ALAs. It was found that 0.5 mM and 1.0 mM concentrations of ALAs had similar effect in both free and inclusion forms. At 2.5 mM ALAs, free forms inhibited the growth while inclusion complexes helped in recovered. 5.0 mM ALA showed inhibitory effect irrespective of form. Our results suggest that the Raman band at 1602 cm^{-1} is a good measure of oxidative stress in fission yeast.

1. Introduction

The α-lipoic acid (ALA), 1,2-dithiolane-3-pentanoic acid, is a naturally occurring anti-oxidative reagent [1,2]. ALA is pale yellow solid at room temperature and is hard to dissolve in water. It generally localizes in mitochondria of cells and functions as a coenzyme for pyruvate dehydrogenase complex or α-ketoglutaric acid dehydrogenase complex to produce ATP [2]. The carbon atom C6 in α-LA is chiral and thus the molecule exists as two stereo-isomers, R-lipoic acid (R-LA) and S-lipoic acid (S-LA), as shown in Fig. 1A. Only R-LA is exclusively synthesized in our body. The amount of production in our body decreases with aging with a maximum production in our 20's [1].

ALA is also available in foods such as liver, spinach or broccoli. However, the amount is not large. Thus, the administration of ALA was first started in Germany in mid-1960s for patients with liver cirrhosis, diabetes mellitus and polyneuropathy where the conditions improved [3,4]. Such administration of ALA is widely done now as commercially available drug or as dietary supplements [1]. However, it is difficult to dissolve purely isolated R-LA or S-LA alone in water. The pure ALA is unstable and easily degraded in the presence of air, heat or light. The degraded ALA forms sticky and insoluble polymer which cannot be utilized by our body and so is ineffective [1]. For such reasons, relatively stable racemic mixture of ALAs is commonly used as supplements instead of pure R-LA or S-LA. However, the side effects of the mixture should be considered carefully. It was reported that hypoglycemic attack occurred resulting in shaking of limbs, palpitation and cold sweats when racemic mixture of ALAs was consumed [3]. The uptake of S-LA, which does not naturally exist in our body, is suspected to be the

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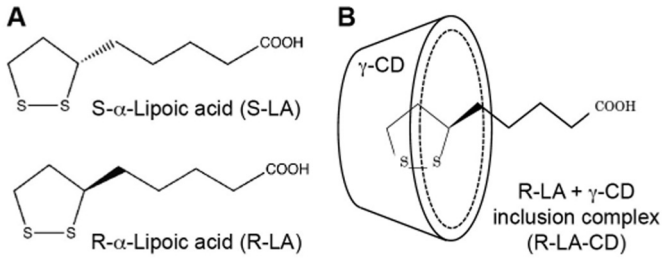

Fig. 1. ALA and its inclusion complex. (A) S- and R-Lipoic acid (S-LA and R-LA respectively) and (B) inclusion complex of R-lipoic acid with γ-cyclodextrin (R-LA-CD).

cause of such symptoms [2]. It has also been understood that uptake of the R-LA alone is more effective than its S-counterpart or their racemic mixture, when they were used for the diabetes prevention by promoting saccharo-metabolism [2]. Against such a background, pure R- or S-LA included by γ-cyclodextrin (γ-CD) in 1:1 M ratio (Fig. 1B) has been recently developed by Cyclochem Co. [1]. The γ-cyclodextrin (γ-CD) is constructed of eight α-glucopyranosyl units to form a truncated torus form with a hydrophobic internal cavity, in which oily guest compound is included [5]. The inclusion complexes of ALA with γ-CD (LA-CD) is significantly stable against air, heat and light and is evenly distributed in aqueous media, which is expected to result in excellent uptake into our body. One of the aims of this study is to investigate the effect of free forms of ALAs (R-LA and S-LA) and their inclusion complexes with γ-CD (R-LA-CD and S-LA-CD) and to understand the differences at the molecular level. For this purpose, we chose fission yeast, *Schizosaccharomyces pombe*, as a model microorganism. Fission yeast is a single celled organism and is one of the simplest eukaryotic models available. Indeed, a variety of mutant strains have already been established and well-studied, whose results can be correlated to higher organisms including humans [6]. We used two strains in this study, wild type (WT) and a mutant strain, *Δdps1*. The *Δdps1* lacks the ability to synthesize isoprenoid side chain (decaprenyl diphoshate) of coenzyme Q10 (CoQ10) [7]. Due to this defect, the *Δdps1* cannot synthesize CoQ10 which is essential for yeast respiration. One of the main characteristics of this mutant fission yeast strain is that it can still live without being able to respire because it can create ATP by fermentation.

Although the mutant strain can live in nutritionally rich medium, there is a delay in growth in minimal medium due to intolerance against oxidative stress in the absence of CoQ10, a strong anti-oxidative agent. Therefore, this model system (*Δdps1*), lacking the naturally occurring intracellular anti-oxidative defense mechanism, is suitable to study the effect of external addition of different anti-oxidative agents by monitoring the recovery in yeast cells' growth, especially when compared to its wild type [7–9]. Thus, in this study, we have investigated the effect of free ALAs and their inclusion complexes with γ-CD on *Δdps1* yeast strain at the molecular level by confocal Raman microspectroscopy.

2. Materials and Methods

2.1. α-lipoic Acids

The free R- and S-lipoic acids (R-LA and S-LA) and their 1:1 inclusion complexes with γ-CD (R-LA-CD and S-LA-CD) were prepared by Cyclochem Co. which were used without further purification. Others were all chemical grade reagents.

2.2. Reagents for Growth of Fission Yeasts

Standard yeast culture media were used as described [7,10]. Fission yeast (*Schizosaccharomyces pombe*) strains were grown in complete YES medium (0.5% yeast extract, 3% glucose, 225 mg/L each of adenine, leucine, uracil, histidine, and lysine hydrochloride) or in minimal PM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts). The appropriate auxotrophic supplements were added as necessary (75 mg/L of leucine or uracil).

2.3. Yeast Strains

Two strains of fission yeast were used. 1) PR110 (wild type, WT) and 2) a mutant strain LJ1030 (*Δdps1*), which cannot synthesize CoQ10. The genotypes of these strains were h^+, *leu1–32, ura4-D18* for W.T. and h^+, *leu1–32, ura4-D18, dps1::kanMx6* for *Δdps1*, respectively [11].

2.4. Yeast Culture and Growth Curve Measurements

Fission yeast were cultured by two successive steps, pre-culture and main culture as described as follows.

2.4.1. Pre-culture

To control the cell numbers of fission yeast to an equal order, a pre-cultivation was performed prior to the main cultivation. Cells of fission yeast were plated on complete medium and were incubated at 30 °C for two or three days. Then a single colony was taken and inoculated in a 10 ml of YES medium and was incubated with shaking at 30 °C for 20 h at which time cell number was ~1×10^7 cells/ml.

2.4.2. Main Culture

Cell numbers were measured by cell counter (Sysmex, Kobe, Japan). The cells were diluted into PMLU medium at 1×10^5 cells/ml. Free R-LA, S-LA and their inclusion complexes R-LA-CD and S-LA-CD at various concentrations (0.5 mM, 1 mM, 2.5 mM and 5 mM) were added in the medium followed by the adjustment of the volume of the PMLU medium at 10 ml. Then the yeast strains were cultured in an incubator for three days until the fission yeast cells reached to the stationary phase. The cell numbers of fission yeast were measured within the culture time to obtain a growth curve by plotting culture time vs log (cell number).

2.5. Raman Microspectroscopy

The Raman spectra of fission yeast strains were obtained in PMLU medium. PMLU medium includes minimal necessary nutritious components. It is transparent in visible light region to give good Raman spectra.

2.5.1. Sample Preparation

Fission yeast cells were pre-cultured in YES medium for 15 h. The cultured cells were moved to PMLU medium after adjusting the cell numbers at about 1.0×10^6 cells/ml. 50 μl of this medium was taken on a glass bottom dish and transferred to microscope for Raman spectral measurements.

2.5.2. Raman Spectroscopic Measurements

Raman spectra and imaging of yeast cells were performed using a laboratory-made confocal Raman microscope with a He—Ne laser emitting 632.8 nm as an excitation source. The laser output is coupled to an Olympus inverted microscope (IX70) in which a high NA objective lens (100×, NA = 1.3) was used to tightly focus the light onto the sample. Raman scattering was collected in backscattering geometry using the same objective lens, passed through an edge filter and a pinhole before being introduced into an imaging spectrometer (Chromex, 250IS) and detected by a liquid nitrogen cooled CCD detector (Princeton Instruments, Spec-10). For Raman imaging measurements, sample was moved in X and Y using a piezo stage (Physik Instrumente). Average laser power of 2.1 mW was used at the sample point. An exposure of 1 s/point with a step size of 0.6 μm/point was used for Raman imaging experiments. The system was controlled with LabVIEW software (National Instruments) and data were analyzed using IGOR Pro

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