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博 士 学 位 论 文

交替氧化酶突变体 T179A 蛋白的原核表达、纯化、结晶以及电子顺磁共振研究

Prokaryotic Expression, Purification, Crystalization and Electron Paramagnetic Resonance of a Mutant T179A of Alternative Oxidase

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中文摘要

交替氧化酶（Alternative Oxidase，AOX），广泛存在于高等植物中，是植物线粒体内膜上呼吸链中抗氰呼吸途径的末端氧化酶。AOX参与植物多种生理代谢调节，例如调节呼吸代谢、抑制活性氧的形成、抵抗逆境以及调节细胞凋亡等。然而，有关AOX的调控机理尚不清楚，对其结构也不完全清楚，目前仅有其二级结构模型的两种假说：SUM模型和AN模型，而AOX三级结构研究一直没有结果，这阻碍了对AOX结构与功能的研究。

本研究利用DNA重组技术，克隆AOX基因及其第179位苏氨酸（T）突变为丙氨酸（A）的突变基因至原核表达载体pET-15b，运用原核表达系统BL21和FN102，经异丙基β-D-硫代半乳糖苷（IPTG）成功诱导表达并得到有活性的野生型AOX和突变型T179A蛋白。表达的蛋白完全结合在菌膜上，洗涤剂洗脱得到可溶蛋白。经Ni-NTA亲和层析柱纯化野生型和突变型蛋白，得到野生型和突变型的纯化蛋白。通过悬滴气相扩散法对突变型T179A蛋白结晶条件进行初筛。用电子顺磁共振（EPR）技术初步测定了交替氧化酶保守区域第179位苏氨酸（Thr）的生理功能。主要研究结果如下：

1. 野生型AOX蛋白基因及其突变体T179A蛋白基因在原核表达系统BL21和FN102中，经IPTG诱导表达后均得到结合在菌膜上的蛋白。采用超声破碎、低温超速离心的方法，制备得到菌膜；SDS-PAGE电泳和Western blotting检测蛋白的表达。1％DDM（十二烷基麦芽糖苷），低温洗脱得到野生型AOX和突变型T179A可溶蛋白。

2. 双向电泳(2-DE)分离BL21和FN102表达的野生型AOX和突变型T179A蛋白，确定蛋白的等电点，蛋白主要分布在pH 3-7范围内，以及pH 10处。（见结果与分析）

3. 镍-NTA(Ni-NTA)琼脂糖凝胶层析柱纯化野生型AOX和突变型T179A可溶蛋白，用SDS-PAGE检测纯化蛋白电泳纯度，在BL21表达系统中的蛋白电泳纯度＜10％，而在FN102中表达的纯化蛋白电泳纯度达到90-95％。说明大肠杆菌FN102本身的背景信息对交替氧化酶表达影响小于BL21，这在2-DE的凝胶图中也得到了证实。

4. 用BCA蛋白定量分析试剂盒分析了野生型AOX和突变型T179A蛋白浓度，分光光度法测定活性，发现突变型T179A蛋白的浓度和活性均高于野生型，证明突变型T179A蛋白较野生型AOX蛋白更适合做蛋白结晶研究。

5. 用Memsys^{TM} MD1-25 kit对突变型T179A纯化蛋白结晶条件进行初筛，在0.1mol L^{-1} NaHepes，0.1mol L^{-1} NaCl，0.1mol L^{-1} Li_2SO_4，30％PEG400，pH 7.5的结晶液中产生了板状结晶。在这种板状结晶条件基础上可对结晶条件进行优化，得到高质量的蛋白单晶体。在 0.1mol L^{-1} NaHepes，2.5mol L^{-1} $(NH_4)_2SO_4$，pH 7.5；0.1mol L^{-1} NaHepes，0.1mol L^{-1} NaCl，30％PEG400，pH 7.5；0.1mol L^{-1} Tris，0.1mol L^{-1} NaCl，0.1mol L^{-1} $MgCl_2$，30％PEG400，pH 8.5；0.1mol L^{-1} CAPSO，0.1mol L^{-1} NaCl，0.1mol L^{-1} Li_2SO_4，30％PEG400，pH 9.5 和 0.1mol L^{-1} CAPSO，0.1mol L^{-1} NaCl，0.1mol L^{-1} $MgCl_2$，30％PEG400，pH 9.5 的 5 种结晶缓冲液中均得到微晶体。这些微晶体可以作为晶种，在蛋白结晶中做晶核，有利于高质量单晶的形成。

6. 为了辅助蛋白晶体的X-射线衍射实验揭示AOX结构与功能的关系，对野生型AOX和突变型T179A蛋白做了垂直模式的电子顺磁共振（EPR）研究，野生型AOX蛋白没有检测到EPR信号，而突变型T179A蛋白在g=2.0047处具有EPR信号，g=2.0047是醌类结合的特征。所以这表明AOX的第179位氨基酸Thr具有结合醌的特性。这对AOX结构与功能的研究是一个新发现，可能具有重要意义。

关键词：交替氧化酶；蛋白结晶；电子顺磁共振

Abstract

Alternative Oxidase(AOX), widely existing in higher plants, is the terminal oxidase in the cyanide-resistant respiration pathway in plant mitochondria. AOX can regulate many physical metabolizable pathways in plant, for example regulation of respiration, restraining formation of reactive oxygen species, resistence of discipline and regulation of programmed cell death and so on. However, its regulating mechanism in vivo and structure are unknown. By now, there are only two hypotheses of AOX's 2-dimensional structure: SUM model and AN model, and its 3-dimensional structure isn't reported, which has been a barrier for the research of relationship between structure and function of AOX.

The genes of wildtype AOX and mutant T179A were inserted into the plasmid of pET-15b, using the technique of DNA recombinant. The proteins of wildtype AOX and mutant T179A were expressed in the *E.coli* cells of BL21 and FN102 by the inducement of isoprophylthio-β-D-galactoside(IPTG). The recombinant proteins were almost exclusively expressed in the membrane. The soluble protein was obtained by eluting the membrane with detergent. The wildtype AOX and mutant T179A soluble proteins were purified by affinity chromatography column containing Ni-NTA. The crystallization conditions of mutant T179A were primarily screened by the hanging drop vapor diffusion method. The function of AOX 179th threonine was detected by electron paramagnetic resonance(EPR). The main results are as follows:

1. The genes of wildtype AOX and mutant T179A were expressed in *E. coli* BL21 and FN102 cells, and induced by IPTG. The *E.coli* membrane was prepared with sonication and super-centrifuge at 4℃. The expressed proteins were identified by sodium dodecyl sulphate-polyacrylamide gelelectrophoresis(SDS-PAGE) and Western blotting. The soluble proteins of wildtype AOX and mutant T179A were obtained using 1% n-Dodecyl-β-D-Mal Toside (DDM) at 4℃.

2. The wildtype AOX and mutant T179A proteins from BL21 and FN102 cells were separated by 2-dimesional electrophoresis. The isoelectric points (pI) were 3-7

and 10.

3. The soluble proteins of wildtype AOX and mutant T179A were purified by affinity chromatography column containing Ni-NTA. The SDS-PAGE electrophoresis purities of purified protein from BL21 less than 10%, but from FN102 cells were up to 90-95%. It were suggested FN102 cells were more suitable for expressing wildtype AOX and mutant T179A proteins, which was also confirmed by 2-DE.

4. The concentration of wildtype AOX and mutant T179A proteins was assayed by the BCA protein assay kit. The activity of isolated proteins was measured by spectrophotometric method. The concentration and activity of mutant T179A were higher than the wildtype AOX's. So the mutant T179A is more applicable for isolated AOX protein, which will facility later crystallization research.

5. The crystal of mutant T179A grew with Memsys^{TM} MD1-25 kit. The tabular crystals grew up in the buffer of 0.1mol L^{-1} NaHepes, 0.1mol L^{-1} NaCl, 0.1mol L^{-1} Li_2SO_4, 30% PEG400, pH 7.5. On the basis of the buffer of tabular crystal, the crystal condition could be optimized and the perfect single crystal would come into being. And the microcrystals were obtained in the buffers of 0.1mol L^{-1} NaHepes, 2.5mol L^{-1} $(NH_4)_2SO_4$, pH 7.5; 0.1mol L^{-1}NaHepes, 0.1mol L^{-1} NaCl, 30% PEG400, pH 7.5; 0.1mol L^{-1} Tris, 0.1mol L^{-1}NaCl, 0.1mol L^{-1} $MgCl_2$, 30% PEG400, pH 8.5; 0.1mol L^{-1} CAPSO, 0.1mol L^{-1} NaCl, 0.1mol L^{-1} Li_2SO_4, 30% PEG400, pH 9.5 and 0.1mol L^{-1} CAPSO, 0.1mol L^{-1} NaCl, 0.1mol L^{-1} $MgCl_2$, 30% PEG400, pH 9.5. The microcrystals could be used as crystal seeding and as the nucleus during crystallization, which is beneficial to the formation of crystal.

6. For assisting X-ray diffraction to elucidate the relationship of the structure and function of the AOX, the electron paramagnetic resonance (EPR) was done with the wildtype AOX and mutant T179A protein. The EPR signal was measured in the perpendicular mode. There was no EPR signal in wildtype AOX protein. The mutant T179A protein had EPR signal at g=2.0047. As the signal g=2.0047 means its characteristic of a semiubiquinone binding site, the 179th amino acid threonine has the feature of bound semiubiquinone. It is a new development and it could be a

important signification in the research of alternative oxidase's struncture and function.

Key Words: Alternative Oxidase, Protein Crystallization, Electron Paramagnetic Resonance

缩略词中英文对照表

缩略词	英文	中文
A	Alanine	丙氨酸
AA	Antibiotics A	抗菌素 A
Ab	Absorbance	吸光值
ALA	5-Aminolevulinic acid hydrochloride	5一氨基乙酰丙酸盐盐酸
AOA	Alternative Oxidase Antibody	交替氧化酶抗体
Amp	Ampicillin	氨苄青霉素
AOX	Alternative Oxidase	交替氧化酶
BB	Bromphenol blue	溴酚蓝
BSA	Bovine serum albumin	牛血清白蛋白
BCA	Bicinchoninic acid	二辛可宁酸
Cb	Carbencillin	羧苄青霉素
DDM	n-Dodecyl-β-D-Mal Toside	十二烷基麦芽糖苷
ddH_2O	Deionized distilled water	去离子蒸馏水
2-DE	2-Dimensional electrophoresis	双向电泳
DNA	Deoyribonucleic acid	脱氧核糖核酸
DQ	Duroquinone	四甲基对苯二醌
DQH_2	Duroquinol	四甲基对苯二酚
DT	Dithionite	连二亚硫酸盐
DTT	Dithiothreitol	二硫苏糖醇
EPR	Electron paramagnetic resonance	电子顺磁共振
Hr	Hour	小时
IFE	Isoelectric focusing electrophoresis	等电聚焦电泳
IPG	Immobilized pH gradients	预制 pH 胶条
IPTG	Isopropyl-β-D-thiogalactopyranosid	异丙基 β-D-硫代半乳糖苷
Kan	Kanamycin	卡那霉素
kDa	KiloDalton	千道尔顿

L	Liter	升
Min	Minute	分钟
Mr	Marker	分子量标记
OD	Optical density	光密度
OG	n-Octyl gallate	没食子酸正辛酯
PCD	Procedured cell death	细胞程序性死亡
pI	Isoelectric point	等电点
PMS	Phenazine methosulphate	吩嗪-甲氨-硫酸盐
PMSF	Phenylmethysulfonyl Fluoride	苯甲基磺酰氟
ROS	Reactive oxygen species	活性氧
rpm	Revolution per minute	每分钟转数
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis	十二烷基硫酸钠－聚丙烯酰胺凝胶电泳
T	Threonine	苏氨酸
TAO	Trypanosome alternative oxidase	锥形虫交替氧化酶
TEMED	N, N, N’,N’-Tetramethylethylenediamine	N, N, N’,N’-四甲基乙二胺
Tris	Tris hydroxymethyl aminomethane	三羟甲基氨基甲烷
Tween-20	Polyoxyethylene-sorbitan monolaurate	吐温 20
V	Voltage	伏特