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6A, 18B/2000G296

Structural determination of *Alcaligenes faecalis* DA1 D-aminoacylase by Se-SAD

Shen-Jia Chen[1], Chih-Yung Hu[1], Shwu-Huey Liaw[123]*
[1]Institute of Biochemistry, [2]Department of Life Science, National Yang-Ming University, and
[3]Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan

Introduction

N-acyl-D-amino acid amidohydrolases (D-aminoacylases) catalyze the hydrolysis of *N*-acyl-D-amino acids to produce the corresponding D-amino acids, which are intermediates in the preparation of pesticides, bioactive peptides, and antibiotics. Several D-aminoacylases screened from microorganisms in various soils have been isolated, and characterized. The D-aminoacylases are zinc enzymes, and removal of the bound zinc ions abolishes completely the catalytic activity. Recently, the putative metal chelating ligands were identified based on structural prediction and mutational studies. D-aminoacylases may belong to the recently identified amidohydrolase superfamily, in which only the metal ligands, four histidines and one aspartate, are strictly conserved. To understand the structural details of the metal-assistant catalysis, we have obtained the first D-aminoacylase crystal from *A. faecalis* DA1, and solved the structure by using a SeMet SAD data set at 1.8 Å resolution.

Materials and methods

The 483-residue *A. faecalis* DA1 D-aminoacylase was expressed in *E. coli* M15 and isolated by four chromatographic procedures as described previously [1-2]. To express the SeMet protein, the recombinant expression vector, the pQE30 (Qiagen), was transformed into *E. coli* B834 (DE3). The crystals suitable for the x-ray diffraction analysis were grown in 25-30% polyethylene glycol 4000, 0.1 M Na citrate (pH 5.6), and 0.2 M ammonium acetate. Data were collected at 100 K by using an ADSC Quantum 4 CCD camera at beamlines 6A and 18B of the Photon Factory of the High Energy Accelerator Research Organization, and then processed with the program *MOSFLM*. The phase problem was solved by using the program *CNS*. After heavy-atom search and density modification, the electron-density map was submitted to the automatic *wARP* procedure. The initial atomic model was subsequently refined by *CNS* and *TURBO*.

Results and discussion

Crystals could be obtained from the recombinant protein purified by either four columns or only the Ni-NTA (Qiagen) column. However, both the Ni-NTA and HiTrap Q (Pharmacia) columns were required for higher Se-Met protein purity suitable for crystallization. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 60.2 Å, b = 76.6 Å, and c = 135.3 Å. There is one molecule in an asymmetric unit (V_M = 2.9 Å^3/Da), with a solvent content of 57%. A single SeMet SAD data set has been collected at the peak wavelength of 0.9787 Å. The data-collection statistics are summarized in Table 1. A virtually complete atomic model with 472 residues was obtained in an almost automatic manner by the sequential use of the programs *CNS* and *wARP*. The recombinant protein contains 14 methionines. Twelve Se peaks were identified, and the first two methionines in the vector were disordered (Fig. 1). Unexpectedly, the structure revealed a novel zinc-binding site in the amidohydrolase superfamily. Structural refinement based on a native data set at 1.5 Å resolution is still in progress, and a full description of the refined structure will be published elsewhere [3].

Table 1. Summary of data-collection statistics

Resolution (Å)	1.8 (1.9-1.8)
Total observations	360069
Unique reflect	56888
Completeness (%)	98.3 (94.4)
Anomalous completeness (%)	92.4 (80.7)
I/σ (I)	12.2
Multiplicity	
R_{merge} (%)	5.1 (15.7)
R_{anom} (%)	6.2 (11.0)

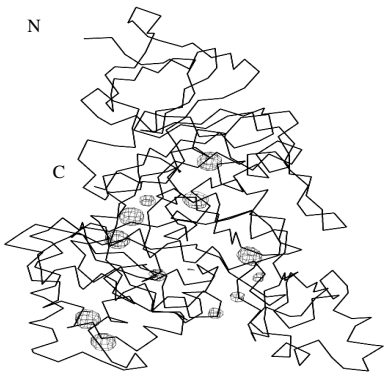

Fig. 1 The Cα trace of D-aminoacylase with an anomalous difference Fourier map contoured at 6 σ showing 12 Se peaks.

References

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* shliaw@ym.edu.tw

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Preliminary study of X-ray sensitizer for cancer-specific therapy

Masao TANIHARA*, Geki FUJII, Kazumi KAJIWARA, Atsuhiro SAITO
NAIST, Ikoma, Nara 630-0101, Japan

Introduction

To develop a cancer-specific radiotherapy, we have proposed a novel method that introduces a heavy atom, such as Nb or Ba, into cancer cells and induces cell death with active oxygen species produced by Auger process after X-ray irradiation. We previously prepared the Nb-containing cationic liposome and transfected L-929 cells with the liposomes, however, the liposomes aggregated on the cell surface and no lethal effect was shown by X-ray irradiation [1]. Here we report that the new liposomes could introduce Nb atoms into the cells, electroporation could also introduce Nb atoms into the cells, and active oxygen species were produced by X-ray irradiation on Nb aqueous solution.

Experiments and Results

Preparation of Nb-containing liposomes

$Nb(OEt)_5$ was diluted with water in the presence of diisopropylethylamine and the aqueous solution of desired Nb concentration was obtained. Cationic liposomes composed of DOTAP:DOPC:DOPE=1:3:1 [2] aggregated on the cell surface. Therefore we prepared new cationic liposomes composed of DOTAP:DOPE=1:1 [3]. When we transfected HeLa cells with the old liposomes including pGFP [4], the cells showed no green fluorescence, however, about 30% of the cells transfected with the new liposomes including pGFP showed fluorescence of GFP. The results show that the newly formed liposomes can indeed introduce included molecules into the cells. Therefore, Nb-containing liposomes having the new phospholipid composition were prepared and transfected to HeLa cells. Incorporated Nb atoms measured by MIP-MS (Hitachi, P-6000) were about 8×10^7 atoms/cell at 1×10^{-3} M Nb solution and about 5×10^8 atoms/cell at 1×10^{-2} M Nb solution, respectively.

Introduction of Nb atoms by electroporation

HeLa cells were suspended in PBS containing 1×10^{-5} M or 1×10^{-4} M Nb atoms. The cell suspension was charged with 960 μF and 300 V electric pulse using GenePulser (BIO-RAD). Viability of the cells after electroporation was more than 95% in any conditions, and incorporated Nb atoms were about 5×10^8 atoms/cell at 1×10^{-5} M Nb solution and about 3×10^9 atoms/cell at 1×10^{-4} M Nb solution, respectively. Electroporation may introduce a larger number of Nb atoms into the cells than the method using the cationic liposomes.

X-ray irradiation of the cancer cells containing Nb atoms

Our previous work revealed that the aqueous solution of Nb compounds showed sharp K-edge near 19 keV in the X-ray absorption spectrum. After including Nb atoms in HeLa cells with the cationic liposomes or electroporation, the cells were irradiated by 2 Gy of monochromatic X-ray beam from BL-27B with the energy just above or below K-edge of Nb atoms. However, no lethal effect was caused by X-ray irradiation.

Active oxygen species in the cells induced by X-ray irradiation

The Nb PBS solution (1×10^{-3} M or 1×10^{-2} M) was irradiated using X-ray fluorescence analyzer (Horiba, MESA-500W). Concentration of active oxygen species in the irradiated solutions was measured as the method reported previously [1]. Only the 1×10^{-2} M Nb solution showed significant increase of active oxygen species after the X-ray irradiation. Our previous study using H_2O_2 showed that the similar concentaration of active oxygen species could induce significant cell death [1]. These results mean that Nb atoms as high as 1×10^{-2} M in the cell may produce active oxygen species and may promote cell death by X-ray irradiation.

Conclusion

We selected Nb atom as an X-ray sensitizer, and up to 10^9 Nb atoms were incorporated to the cells with newly prepared cationic liposomes or electroporation. However, there was no significant increase of cell death after X-ray irradiation at a dose of 2 Gy. We could detect active oxygen species only at 1×10^{-2} M of Nb solution by X-ray irradiation. These results show that Nb atom is not so efficient to produce active oxygen species enough to promote cell death by X-ray irradiation. Heavier atom, such as Ba, which has higher efficiency both to absorb X-ray and to produce active oxygen species, may be needed for development of a useful cancer-specific therapy.

References

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*mtanihar@ms.aist-nara.ac.jp