Structural and functional analyses of cytidine deaminase from *Mycobacterium tuberculosis* and mutagenesis studies

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**Introduction**

Nowadays tuberculosis (TB) remains a major cause of mortality caused for only one infections agent. This is especially true in developing countries, since 98 % of deaths caused by TB occur in developing areas. In addiction, the World Health Organization estimates that one-third of the population is currently infected with *M. tuberculosis* in a latent form and risked of developing the active disease [1]. Thus, there is an urgent necessity of studies on mycobacterial metabolism to identify promising targets for the development of new anti-TB agents and new effective vaccines to prevent TB. Cytidine deaminase (CDA) is an evolutionarily conserved enzyme of the pyrimidine salvage pathway and belongs to a family of enzymes that catalyze the irreversible hydrolytic deamination of cytidine and 2’-deoxycytidine to form uridine and 2’-deoxyuridine, respectively [2]. CDA plays an important role in the metabolism of a number of antitumoral and antiviral cytosine nucleoside analogues, leading to their pharmacological inactivation [3]; this fact has increased the interest in developing CDA inhibitors for therapeutic use and attempt to identify potential targets for rational drug and vaccine design.

**Methodology**

PCR amplification and cloning of the *cdd* coding sequence was performed using PCR and the cloning and expression vectors were pCR-Blunt and pET-23a(+), respectively. Nucleotide sequence of the cloned *M. tuberculosis cdd* gene was determined by automated DNA sequencing. The recombinant plasmid pET-23a(+)::*cdd* was transformed into
electrocompetent *E. coli* and the recombinant CDA from *Mycobacterium tuberculosis* (MtCDA) was purified using anion-exchange, gel filtration and hydrophobic interaction columns. Kinetics parameters and pH rate profiles were determined following the direct spectrophotometric assay described by Cohen and Woldfenden [4]. Crystals of MtCDA were obtained using the vapor-diffusion hanging-drop method at 273 K using 24-well tissue-culture paltes. The X-ray diffraction data for MtCDA crystal was colleted at a wavelength of 1.437 Å using synchrotron radiation source (Sation MX-1, LNLS, Campinas, Brazil) and a CCD detector (MAR CCD).

Mutagenesis was performed using a PCR-amplification technique followed by an overlapping-PCR step both using the Pfu DNA polymerase. The gene product was than ligated into the pCR-Blunt cloning vector and subcloned into the pET-23a (+) expression vector. The sequences of the mutant *cdd* genes have been verified to both confirm the insertion of the desired mutation and to ensure that no unwanted mutations were introduced by the PCR steps. The expression of the mutant proteins in the soluble fraction was tested in *E. coli* cells and the purification tests were performed according to described above for wild type CDA.

**Results and Discussion**

A probable CDA gene (*cdd, Rv3315c*) from *M. tuberculosis* H37Rv was cloned, sequenced, and the protein was over-expressed in *Escherichia coli* BL21(DE3) cells. Recombinant CDA was purified to homogeneity, with a specific activity value of 5.4 U mg\(^{-1}\) and a yield of 10% using a three-step purification protocol. Mass spectrometry analysis, N-terminal amino acid sequencing, and gel filtration chromatography confirmed the predicted molecular mass, identity, and oligomeric state of the *M. tuberculosis* CDA enzyme, which was estimated to be 52.99 kDa. Together with evidence from multiple sequence alignment data, we strongly suggest that *M. tuberculosis* CDA is a tetramer of identical subunits in solution. The enzyme exhibits apparent *K*\(_{m}\) and *V*\(_{max}\) values of 1004 ± 53 µM and 21 ± 1 U mg\(^{-1}\) for cytidine, and 1059 ± 64 µM and 15 ± 1 U mg\(^{-1}\) for 2’-deoxycytidine, respectively. A *k*\(_{cat}\) of 4.8 ± 0.1 s\(^{-1}\) for cytidine and 3.5 ± 0.1 s\(^{-1}\) for 2’-deoxycytidine was found. MtCDA was crystallized and crystal diffracted at 2.0 Å resolution. Analysis of the crystallographic structure indicated the presence of a Zn\(^{2+}\) coordinated by three conserved cysteines and the structure exhibits the canonical cytidine deaminase fold.
Our pH rate profile results suggested that the glutamate in the position 47 of plays a critical role in catalysis and/or substrate binding. Then, we have performed site-directed mutagenesis to prove this hypothesis. We have substituted the glutamate 47 residue for alanine (E47A), histidine (E47H), glutamine (E47Q) or aspartic acid (E47D) amino acids. The mutant \textit{cdd} genes were sequenced both to confirm the insertion of the desired mutation and to ensure that no unwanted mutation was introduced by the PCR steps. The expression of the E47Q and E47D mutant proteins in the soluble fraction was observed in \textit{E. coli} BL21(DE3), grown without isopropyl beta-d-thiogalactoside (IPTG) induction in LB medium at 37 °C for 6 h after the cultures reached an \( \text{OD}_{600} \) 0.4-0.6. The E47A mutant enzyme was observed in the soluble fraction in \textit{E. coli} BL21(NH) strain, grown with IPTG induction, in LB medium at 37 °C for 24 h after the cultures reach an \( \text{OD}_{600} \) 0.4-0.6. The expression tests for E47H enzyme and the protein purification experiments are currently underway in our laboratory and will provide enzymes in quantities necessary for kinetic studies, then paving the way for the assignment of the function of this residue in the catalytic mechanism of MtCDA.

This work will provide homogeneous MtCDA for further studies on the enzyme mechanism of action by, for instance, pre-steady-state kinetics and microcalorimetry. We will also try to determine the X-ray crystal structure of MtCDA-ligand binary complexes. Our results improved the understanding about the enzyme catalytic mechanism and may help in the rational production of attenuated mycobacterial samples that could be used as vaccines to prevent TB.

**References**