Kinetic Studies of Histidinol Dehydrogenase from
Mycobacterium tuberculosis

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Introduction

The aetiological agent of tuberculosis (TB), Mycobacterium tuberculosis, was responsible for approximately 1.8 million deaths in the year of 2007. The increasing prevalence of TB, the emergence of multidrug-resistant and extensively drug-resistant strains of the tubercle bacillus, and the devastating effect of co-infection with the human immunodeficiency virus have led to an urgent need for the development of new and more efficient antimycobacterial drugs. This context leads us to the search of potential molecular targets for rational drug design. Embedded in this idea, it comes into sight the histidine biosynthetic pathway that is present in bacteria, archaeabacteria, lower eukaryotes and plants, but is absent in mammals.

The histidine biosynthetic pathway has been studied in detail in Salmonella typhimurium and Escherichia coli. There are ten enzymatic reactions carried out by eight genes in the unbranched pathway that include several complex and unusual reactions, and form a critical link between amino acid and purine biosynthesis. The final reaction, first described enzymatically in Arthrobacter histidinovorans and E. coli, and in yeast, is catalyzed by histidinol dehydrogenase (HisD) [L-histidinol:NAD oxidoreductase (EC 1.1.1.23)]. HisD is a bifunctional enzyme that catalyzes the NAD+- and Zn2+-dependent conversion of L-histidinol to L-histidine through an L-histidinaldehyde intermediate, with the concomitant reduction of 2 moles of NAD+.

The inability of M. tuberculosis histidine auxotrophs to survive single-amino-acid starvation, the identification of genes required for mycobacterial growth, and the essentiality
of hisD gene product (Histidinol Dehydrogenase or MtHDH) for M. tuberculosis survival suggest that MtHDH is a promising target for antitubercular agent development. Not surprisingly, MtHDH has been ranked among the top 50 targets by the TDR Targets database. However, it has not been shown yet whether the hisD gene codes for a histidinol dehydrogenase activity as predicted by in silico analysis of M. tuberculosis genome sequence.

In the previous event, amplification, cloning, overexpression, purification and preliminary kinetic data were presented along with a tridimensional model constructed by molecular homology modeling. Presently, we describe further kinetic studies, including MtHDH inactivation by chelating agents, its activation by divalent cations, pH rate profiles and initial velocity that allowed a first insight into enzyme catalytic mechanism.

Materials and Methods

Histidinol dehydrogenase assay

Histidinol dehydrogenase catalyzes the sequential NAD+-dependent oxidations of L-histidinol to L-histidinaldehyde and then to L-histidine. The enzymatic activity was assayed in the forward direction by continuously monitoring the increase in absorbance at 340 nm due to the conversion of NAD+ to NADH, in 50 mM Pipes pH 7.2.

Inactivation by Chelating Agents

Homogeneous MtHDH was treated with EDTA (0.1, 1 and 10 mM) and 1,10-Phenantroline (1, 2 and 5 mM) and its activity was measured in different time periods to evaluate a time dependent inactivation due to metal sequestration. Buffers used in these assays were rendered metal free by passing them through chelex resin (BioRad).

Divalent Cation Activation

MtHDH was partially inactivated with 1,10-Phenatroline 5 mM for 30 min and then diluted 10-fold. A set of divalent cations (Ca²⁺, Cd²⁺, Co²⁺, Cu²⁺, Mg²⁺, Mn²⁺, Ni²⁺ and Zn²⁺) were added to the assay in varying concentrations (1, 10, 20 or 40 mM) to assess the capability of these metals to reestablish enzyme activity, proving MtHDH as a metalloenzyme.

pH Rate Profiles

To determine the dependence of the kinetic parameters on pH, initial velocities were measured in the presence of varying concentrations of one substrate and a saturating level of the other in a buffer mixture over the following pH values: 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. Amino acid residues involved in either catalysis or substrate binding were proposed.
based on apparent acid and base dissociation constant for ionization groups obtained by pH-rate profiles assays.

**Initial Velocity**

To determine the true steady-state kinetic parameters, initial velocity studies were carried out at varying concentrations of one substrate and several fixed-varied concentrations of the co-substrate.

**Results and Discussion**

Histidinol Dehydrogenase has been described as a Zn$^{2+}$ metalloenzyme for several organisms, from plant to bacteria. The metal bound to metalloenzymes usually displays important roles in catalysis or substrate biding, and its removal either inactivates the enzyme or causes substantial loss of its activity. Though EDTA is a well-known chelating agent, it was not capable of causing any effect on MtHDH activity in concentrations up to 10 mM over 45 min of contact with the enzyme. Similar behavior was observed for *S. typhimurium* Histidinol Dehydrogenase. Differently, 1,10-Phenantroline proved to affect enzyme activity in a time dependant fashion for 1 and 2 mM concentrations but to 5 mM the enzyme exhibited a 90% loss in its activity after 3 min. This procedure allowed us to study MtHDH reactivation by divalent cations. After being partially inactivated by treatment with 1,10-Phenatroline, the enzyme was assayed in the presence of different divalent cations to assess which of them were capable of activating the enzyme. Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ activated the enzyme in different extents, being Mn$^{2+}$ the cation that exhibited the best activation profile. Cd$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ seemed to inhibit the enzyme very strongly since it showed no activity. Cu$^{2+}$ readily precipitated when mixed with the other substrates and buffer and could not be tested.

Enzyme activity increased in basic pH with its maximum at pH 9.0. A molecular model previously constructed by us was helpful in assigning the pKa obtained by pH rate profiles analysis to the amino acids involved in catalysis / substrate biding.

Lineweaver-Burk plots of data generated in initial velocity experiment indicated a ping-pong mechanism, to be confirmed with further experiments, currently underway.

**Conclusion**

The data presented in this work are the first reports of kinetic data concerning MtHDH, and helped to have a glimpse about the enzyme kinetic mechanism. Completely unveiling MtHDH kinetic and chemical mechanism is the first step to propose a specific inhibitor that could lead to a potential new effective drug for TB treatment.