PH.D. DISSERTATION

Mammalian Serine Racemase as a Pharmaceutical Target

Jana Jirásková

Supervisor: Jan Konvalinka

Department of Biochemistry
Faculty of Science
Charles University

Institute of Organic Chemistry and Biochemistry
Gilead Sciences & IOCB Research Centre
Academy of Sciences of the Czech Republic

Praha 2010

Introduction

Serine racemase is a pyridoxal-5'-phosphate (PLP) -dependent enzyme that is responsible for D-serine production. D-serine is a neurotransmitter that acts, together with L-glutamate, as agonist of ionotropic *N*-methyl-D-aspartate (NMDA) receptors, which are important for neuronal tissue signalization. Recent serine racemase knock-out mouse studies revealed that SR produces approximately 90% of brain D-serine.

SR was first isolated from a pool of rat brains about a decade ago. Its orthologs are present in mammals as well as plants and yeast. Mammalian SRs share high sequence identity, about 90%. Mouse and human SRs are similar enzymatically, suggesting that mouse SR and mouse model are suitable to shed light on human SR.

SR forms homodimers in solution and has a molecular weight of approximately 37 kDa per monomer. In addition to pyridoxal-5'-phosphate, the enzyme requires divalent cations such as Ca^{2+} or Mg^{2+}, nucleotides such as ATP, and reducing agents for full activity. The mammalian enzyme is most active at 37 °C and slightly alkaline pH. In addition to its racemization activity, SR also acts as a β-eliminase (or serine dehydratase), converting L-serine, and to a lesser extent D-serine, to pyruvate and ammonia.

Recently a 3D structure of the rat and human SRs has been solved. The overall shape as well as the active site resembles that of yeast SR and other PLP-enzymes of the same fold (type II). PLP-dependent enzymes are divided into subclases based on structural similarities. Serine racemase belongs to Fold type II family named also after serine/threonine dehydratase, the typical member of the family. The 3D structure provided valuable insight into the enzyme's putative catalytic mechanism.

Further study of SR is warranted based on SR's role in a variety of disease states connected with aberrant NMDA receptor regulation. Excitotoxic D-serine levels have been implicated in neuropathologies including Alzheimer's disease and amyotrophic lateral sclerosis, while low levels of D-serine are associated with symptoms of schizophrenia. Recent studies have shown that amyloid β-peptide-mediated neurotoxicity is significantly reduced in SR knock-out mice, providing compelling evidence for the potential of SR inhibitors as therapeutic agents. While high affinity NMDA receptor blockers are often employed to treat conditions related to the pathway dysfunction, the use of such drugs can be compromised by undesirable side effects. SR inhibitors may offer a novel and potentially highly specific approach for attenuation of NMDA receptor-mediated glutamate excitotoxicity and for further study of the pathway.

Aims of the project

- Recombinant expression and purification of mouse serine racemase.
- Enzymatic characterization of the enzyme.
- Development of methods for activity reaction products detection.
- Identification of potent, specific SR inhibitors.
- Experimental determination of the SR structure

Results

Active recombinant mouse serine racemase was produced in *E. coli*, and the purified enzyme was kinetically characterized using an HPLC-based activity assay. The kinetic parameters revealed that mSR is able to catalyze LS racemization and β-elimination and DS racemzation, but not DS β-elimination. Surprisingly, the catalytic efficiency of LS β-elimination, the supposed side reaction, is slightly higher than the main racemization reaction. Serine racemase converts other substrates more efficiently than serine, for example L-serine-*O*-sulfate, L-*threo*-3-hydroxyaspartate, and L-Cl-β-alanine. These substrates undergo β-elimination but not racemization. Threonine is the only other known amino acid that can be racemized (L-*allo*-threonine to D-threonine, L-threonine to D-*allo*-threonine and *vice versa*) as well as β-eliminated by SR. L-*erythro*-3-hydroxyaspartate, another 3-hydroxyaspartate isomer, remains the most potent competitive SR inhibitor identified to date. Various small dicarboxylic acids, such as malonic, dihydroxyfumaric, and maleic acid, are also competitive inhibitors. The smallest molecule to competitively inhibit SR is glycine. Though its affinity is lower, its inhibition may be physiologically relevant.

Since the majority of known SR inhibitors are naturally abundant small molecules, we conducted screening experiments in hopes of identifying novel structures capable of SR inhibition. The screening revealed hydroxamic acids as potent SR inhibitors. More detailed studies showed that the mechanism of SR inhibition by hydroxamic acids is not straightforward. Some of the inhibitory hydroxamic acids acted by irreversibly modifying the pyridoxal-5'-phosphate (PLP) cofactor, while others did not. In addition to identifying the novel ability of selected hydroxamic acids to modify PLP, we also characterized a purely competitive, potent hydroxamic acid SR inhibitor, L-aspartic acid β-hydroxamate.

During our work on the SR inhibition we developed a less time consuming and less laborious capillary electrophoresis assay for DS detection. The method employed in-capillary derivatization with the *ortho*-phtaldialdehyde and 2-mercaptoethanol as the SH-group donor. The resolution was maintained by background electrolyte containing 2-hydroxypropyl-γ-cyclodextrin as a chiral selector. The results of the CE-based technique were comparable to the usually used HPLC-based technique.

While numerous attempts were made to solve the SR structure experimentally, this aim of the project has not been realized. Recently, the X-ray structure of human SR with malonate in the active site was released. We therefore set out to analyze the binding of inhibitors, such as L-*erythro*-3-hydroxyaspartate, and the ATP/Mg^{2+} complex using docking. The results are included in a review on serine racemase inhibition that also includes our observations of inhibition by citrate or various malonic acid analogs.

Publications included in the thesis:

Stříšovský, K.; Jirásková, J.; Mikulová, A.; Rulíšek, L.; Konvalinka, J., Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxylate substrate and inhibitors and analysis of the beta-eliminase activity. *Biochemistry*. 2005. 44(39), 13091-100.

Koval, D.; Jirásková J.; Stříšovský, K.; Konvalinka, J.; Kašička, V., Capillary electrophoresis method for determination of D-serine and its application for monitoring of serine racemase activity. *Electrophoresis*. 2006. 27(13), 2558-66.

Hoffman, H.E.; Jirásková, J.; Cígler, P.; Šanda, M.; Schraml, J.; Konvalinka, J., Hydroxamic acids as a novel family of serine racemase inhibitors: mechanistic analysis reveals different modes of interaction with the pyridoxal-5'-phosphate cofactor. *J Med Chem.* 2009. 52(19), 6032-6041.

Jirásková, J.; Ettrich, R.; Vorlová, B.; Hoffman, H.E.; Lepšík, M.; Jansa, P.; Konvalinka, J., Inhibition of Human Serine Racemase, an Emerging Target for Medicinal Chemistry. *Curr. Drug Targets* (submitted, May 2010)