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Biochemical studies of the enzyme *Mycobacterium tuberculosis shikimate kinase*

Leonardo A. Rosado[1,2], Luiz A. Basso[1] and Diógenes S. Santos[1,2]

[1]Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Instituto Nacional de Ciência e Tecnologia em Tuberculose (INCT-TB), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga 6681, Porto Alegre, RS 90619-900, Brazil.
[2]Programa de Pós-Graduação em Medicina e Ciências da Saúde, PUCRS, Av. Ipiranga 6681, Porto Alegre, RS 90619-900, Brazil.

Introduction

Tuberculosis is a worrisome disease due to its high infectious and mortality rates, reporting 9.2 million new cases and 1.7 million deaths each year (1). New resistant strains of the *Mycobacterium tuberculosis* are reported around the world; almost 500.000 cases of multi-drug resistance have been reported in 2006. The development of resistant strains was originated by an inappropriate use of the health system due the abandonment of the treatment. Rifampicin and isoniazid, discovered in 1966 and 1952, respectively, are the most powerful antibiotics used against *M. tuberculosis*; however, the new resistant strains and the high mortality rates are indicating a need of new drugs. The shikimate pathway (Ducati, R.G., 2007) is essential for the life of algae, higher plants, fungi, apicomplexan parasites and bacteria, including *M. tuberculosis.* In *M. tuberculosis* this pathway is responsible for the synthesis of aromatic amino acids, folates and ubiquinone; its essentiality was proved by disruption of the gene aroK. This pathway is formed by seven steps, catalyzed by a monofunctional enzymes encoded by separated genes. Phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) are the primary compounds of the pathway leading to the synthesis of chorismate, the final product (Hermann, K.M.,1999). Shikimate kinase (E.C 2.7.1.71) catalyses the fifth reaction of the pathway, converting adenosine triphosphate (ATP)

and shikimate to shikimate-3-phosphate and adenosine diphosphate by a phosphoryl transfer, inserting a phosphate in the carbon 3 of the shikimate ring. This enzyme belongs to the family of the nucleoside monophpsphate kinases (NMP). The NMP family is an important group of enzymes which catalyzes a reversible phosphoryl transfer from a nucleoside triphosphate to a specific nucleoside monophosphate, and the product of the reaction is subsequently phosphorylated, resulting in precursors of nucleic acids. *M. tuberculosis* constitutes a great problem to human health and the results of this work can be a great step to the development of agents against this pathogen.

Material and Methods

In the present work, experiments of binding were assayed with the intention of elucidating *M. tuberculosis* shikimate kinase (*Mt*SK) enzymatic mechanism. Fluorescence titration was carried out with shikimate and shikimate-3-phosphate, substrate and product, respectively, and equilibrium dialysis titration was carried out to determine the interaction of ATP and ADP, substrate and product, respectively.

Results and Discussion

The quenching in protein fluorescence upon shikimate and shikimate-3-phosphate binding to *Mt*SK monomer was monitored to determine the dissociation constant at equilibrium for *Mt*SK-shikimate and *Mt*SK-shikimate-3-phosphate binary complexe formation. A plot of shikimate concentration versus protein fluorescence variation upon binary complex formation shows an hyperbolic increase, and the data were accordingly fitted to equation y= yo + ax/b+x, yielding values of K_D = 124 ± 7 µM; a plot of shikimate-3-phosphate concentration versus protein fluorescence variation upon binary complex formation also shows an hyperbolic increase, and the data were accordingly fitted to equation the same equation, yielding values of K_D = 162 ± 37 µM. Due the great inner filter effect observed on the spectrofluorimetric assay, equilibrium dialysis and a spectrofluorimetric assay with an ATP analogue (ATP-Alexa Fluor 647) were carried out to determine a binding between the shikimate kinase monomer and ATP and ADP. The assays did not show a specific binding between the protein and the ligands. The data acquired indicates a Steady-State Ordered Bi Bi System mechanism (Segel., 1993), since without the presence of shikimate, ATP cannot bind

in the activity site, and without the presence of shikimate-3-phosphate, ADP cannot bind to the enzyme. Soaking and (co-)crystallization experiments realized by Hartmann et al. (Hartmann M.D., 2006) indicate a random sequence of substrate binding, a synergism between the substrates and a sequential mechanism to the release of the products, liberating ADP first and then shikimate-3-phosphate, suggesting two different routes of substrate binding; one of the proposed routes corroborates with the experimental data produced here, proposing a binding of shikimate, generating a conformational change enhancing the binding of ATP, which agrees with other works proposing a conformational change on the catalytic site without the presence of the substrate impairing the binding of ATP and its hydrolysis.

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