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Spectrofluorimetric and crystallographic studies of the effect of phosphorylation on thymidylate synthase

Project of the studies contemplated to lead to Ph.D. thesis

Thymidylate synthase (EC 2.1.1.45) catalyzes the C(5) methylation of 2'-deoxyuridylate (dUMP) in a concerted transfer and reduction of the one-carbon group (at the aldehyde oxidation level) of $N^{5,10}$-methylenetetrahydrofolate, and with concomitant production of dihydrofolate and thymidylate [1, 2]. As the sole *de novo* source of thymidylate synthesis in cells, it is a target in anticancer, antiviral, antifungal and antiprotozoan chemotherapy [3-7].

The essential step of thymidylate synthase catalysis involves the formation of a ternary substrate-enzyme-cofactor molecular complex. Following the $C_{(5)}$ hydrogen dissociates, as proton, from dUMP pyrimidine ring, with concommitant β-elimination of tetrahydrofolate, still remaining bound in the active center, a hydride is transferred from the pteridine $C_{(6)}$, resulting in reduction of the pyrimidine $C_{(5)}$ methylene group to methyl one. After some bond rearrangement, the complex splits into the product, thymidylate (dTMP), dihydrofolate and enzyme (Fig. 1; [reviewed in refs. 8-10]).

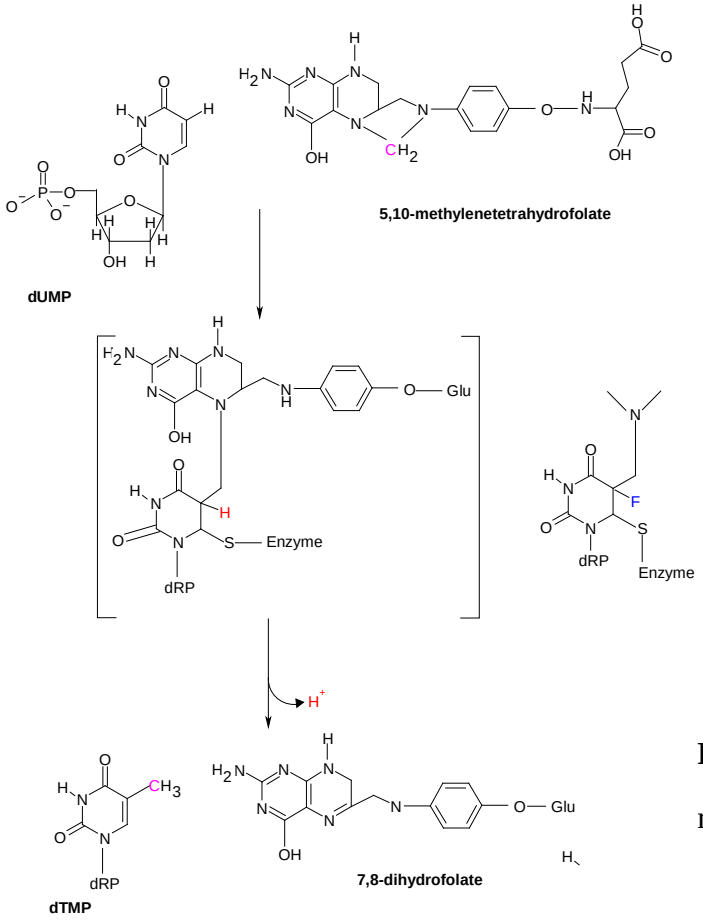

Fig. 1. Thymidylate synthase-catalyzed reaction.

The studies to be performed will be aimed at:

1. Spectrofluorimetric studies of the effect of phosphorylation on thymidylate synthase molecule and its interaction with ligands (steady-state and time resolved - stopped-flow experiments)
2. Crystallographic studies of the effect of phosphorylation on thymidylate synthase molecule and its interaction with ligands

Results of studies performed in the Laboratory of Comparative Enzymology (Pracownia Enzymologii Porównawczej, Instytut Biologii Doświadczalnej PAN) showed the presence of phosphorylated residues in recombinant human and mouse (also rat and *Trichinella spiralis*) thymidylate synthase preparations (stained with the Pro-Q® Diamond Phosphoprotein Gel Stain produced by Molecular Probes), expressed in bacterial cells [11]. The preparations underwent affinity chromatography separation, with the use of $Al(OH)_3$ column, yielding phosphorylated and non-phosphorylated protein fractions, the former constituting only 1 % of the total. Comparative studies of both forms revealed that each phosphorylated, compared with the corresponding non-phosphorylated enzyme form, shows 3-4-fold lower V_{max} value [11] and capacity to repress translation (catalyzed by rabbit reticulocyte preparation) of its own (as well as luciferase) mRNA (unpublished). Surprisingly, MS analyses failed to demonstrate the presence of phosphorylated amino acid residues in any of the fractions investigated. Only with the use of ^{31}P NMR analyses it was possible to demonstrate very clearly the presence of phosphorylated residues in the phosphorylated, and their absence in non-phosphorylated enzyme protein fractions. Moreover, a closer analysis of the ^{31}P NMR spectra (including testing their time-dependent changes following acidification) and comparison with those of synthesized phosphoramide derivatives of basic amino acids, and commercially available phospho-serine, phospho-threonine and phospho-lysine, revealed phosphorus to be involved in a phosphoramide (acid-labile) bond, pointing to modification of histidine residue(s) [*cf.* 12, 13]. Therefore the present project is aimed at determining influence of phosphorylation of amino acids other than hydroxy-amino acids, in particular basic amino acids, on the enzyme molecule and its interaction with ligands. Production and purification, and separation of phosphorylated and non-phosphorylated forms, of the recombinant mouse thymidylate synthase protein expressed in *E. coli*, previously demonstrated to show altered catalytic [11] and non-catalytic properties correlated with histidine phosphorylation (see above), will be done as previously described [11].

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