

Abstract

The medicine achievements and development helped us to treat or eradicate diseases that were not curable years ago. The unstoppable evolution of medicine prolongs and ameliorates humans lifespan. With this undisputed advantage, however comes another challenge, cancer. In high-income populations this type of disease is one of the main players in the death rate. However, evolving treatment types, based on the constant growth of the knowledge related to the basis of the carcinogenesis, help us to treat or prevent the development to the next stages of the cancer. Among multiple treatment methods are transfections of the tumour cells with a "target protein" and designing more effective small molecule inhibitors which relates to the subject of this dissertation, namely mechanisms of interaction with inhibitors of two enzymes (Purine Nucleoside Phosphorylase and Thymidylate synthase) that play an important role in the DNA recycling and synthesis. Purine Nucleoside Phosphorylase executes purine base salvage *via* a reversible phosphorolysis of the (deoxy)ribonucleosides, leading to the glycosidic bond (between a purine base and sugar) cleavage, with phosphate acting as the second substrate and the reaction product, free purine ring, available to be used in DNA synthesis. Thymidylate Synthase, on the other hand, catalyses the conversion of dUMP to dTMP that is incorporated into DNA.

The present Ph.D dissertation demonstrates studies aimed at (i) analysis of the spectroscopic properties of two molecular targets in chemotherapy, *E.coli* Purine Nucleoside Phosphorylase (PNPr) and *M. musculus* Thymidylate synthase (mTS), and their mutants, and (ii) spectroscopic characterization of interactions of the above enzymes and their mutants with chosen ligands, formycin A (with PNP enzymes) and dUMP, FdUMP and N⁴-OH-dCMP (with mTS enzymes). In more details, regarding *E.coli* Purine Nucleoside Phosphorylase, the author wanted to determine (i) the role of Phe159 and Tyr160 in the interactions with nucleosides (formycin A was used as a nucleoside representative) and (ii) the role of the π - π stacking between formycin A-Phe159-Tyr160. Moreover, the author wanted to verify the hypothesis that Tyr160 is the only excitation energy donor to the formycin A. Additionally, a secondary goal was a spectral characterisation of the *E.coli* Purine Nucleoside Phosphorylase chosen mutants: PNPF159Y (PNPY), PNPF159A (PNPA), PNPY160F (PNPF) and PNPY160S (PNPS).

Goals related to the second subject of this dissertation, *M. musculus* Thymidylate Synthase, included (i) testing the hypothesis of uncoupling of two component reactions apparently involved in the TS-catalyzed reaction, resulting from a slow-binding

inhibition by N⁴-OH-dCMP, (ii) evaluation of the electrostatic potential role in the interactions between mTS and nucleotides and last but not least, (iii) spectroscopic characterization of mTS and its two mutants: H190A and W103G.

***E.coli* Purine Nucleoside Phosphorylase**

Results obtained for PNPr, its mutants and their complexes with formycin A (FA) provide an evidence of Phe159 and Tyr160 being amino acids residues essential for an efficient FA binding. All used methods, absorption and fluorescence spectroscopy, dissociation constants, FRET efficiency calculations and the study of impact of FA on the fluorescence decay parameters revealed that the lack of Phe159 (PNPA mutant) results in the loss of PNP ability to efficiently bind the nucleoside. The results testified rather to a collisional quenching of the PNPA fluorescence proving the lack or very weak FA binding to the active site of the PNPA enzyme.

Surprising results were obtained for the PNPY mutant (PNPF159Y, Phe159 replaced with Tyr) that were not only related to the interactions with formycin A but also to spectroscopic properties of the investigated mutant. Investigations showed the formation of the tyrosinate anion (Tyr) and its excited state (Tyr*), that influenced absorption and emission spectra, fluorescence decay parameters (presence of an additional decay component) and caused substantial diminution of FRET between PNPY and FA (relative to PNPr). Overall results, although showed only a small increase of the FA emission intensity in the complex with PNPY (relative to PNPr), testified that this observation is caused by the presence of the Tyr* and possibly perpendicular orientation between transition moments of the donor (Tyr159/Tyr160) and acceptor (FA). It was proved that PNPY strongly binds FA to the active site (in the presence of phosphate even more efficiently than PNPr), showing that the PNPY mutant is a very promising target for future investigations. Moreover, the presented results showed that Tyr160 plays a crucial role in the effective FA binding to the active site of PNP. Mutants deprived of this amino acid (PNPY160F and PNPY160S) were not able to form stable complexes with formycin A. Both, absorption and fluorescence difference spectra did not present any tendency towards signs of static interactions, suggesting rather collisional exchange of the fluorescence energy. To summarize, results obtained for *E.coli* Purine Nucleoside Phosphorylase strongly suggest the importance of the two specific pairs of the aromatic amino acids: Phe159- Tyr160 or Tyr159-Tyr160 simultaneously indicating a crucial role of the π - π stacking in the formycin A binding.

***M. musculus* thymidylate synthase**

Thymidylate synthase catalyzes N⁵,10-methylene-tetrahydrofolate (mTHF)-dependent methylation of dUMP C(5), producing 5,7-dihydrofolate (DHF) and thymidylate, with the total reaction involving apparently two component reactions: transfer of the mTHF methylene group and its reduction at the cost of tetrahydrofolate (THF). Previously described crystallographic structure ([Dowiercia et al. Pteridines 24, 93-98, 2013](#)) suggested N⁴-OH-dCMP, a slow-binding thymidylate synthase inhibitor, to cause an ap-

parent "uncoupling" of the two component reactions, resulting in an irreversible enzyme self-inactivation due to the inhibitor C(5) reduction and covalent binding of C(6) to the active center cysteine residue. The latter prompted a hypothesis on the enzyme's capacity to use THF as a cofactor reducing the dUMP pyrimidine ring C(5) in the absence of the 5-methylene group. In order to test this hypothesis, the present study concentrated on learning whether the time-dependent enzyme inhibition by N⁴-hydroxy-dCMP is accompanied by parallel THF to DHF oxidation reflected by the increase of the absorption at $\lambda = 340$ nm.

Spectrophotometric results verified the mTHF-dependent N⁴-OH-dCMP inhibition to be accompanied by DHF production that was time- and temperature-dependent and thus TS-catalyzed, as deduced previously based on the crystal structure (PDB ID: 4EZ8). Moreover, observation of the mixtures of mTS with THF (an intermediate of the full mTS reaction) and N⁴-OH-dCMP or FdUMP (a strong TS inhibitor and active form of several chemotherapeutic drugs) also testified to the time-, temperature- and TS activity-dependent production of DHF, which is in accord with the hypothesis of the uncoupling of the mTS-catalyzed component reactions resulting from the mechanism-based N⁴-OH-dCMP inhibition.

Further studies of the mechanism of TS inhibition by N⁴-OH-dCMP involved two mTS mutant proteins, H190A and W103G. They were designed based on the TS crystallographic structure involving the inhibitor bound in the active center. Each of the mutated amino-acid residues (H190 and W103) were selected as capable of hydrogen-bonding with the N⁴-OH substituent. Repeating the above mentioned spectrophotometric studies testing DHF production in the course of mTHF/THF-dependent reaction of each TS mutant with N⁴-OH-dCMP/FdUMP confirmed the above presented findings.

Results obtained during the spectral analysis of all of the enzymes (mTS, H190A and W103G) revealed an interesting observation. Tryptophan at position 103 has very unique spectral properties that influence the emission and fluorescence decay parameters of the whole mTS molecule. The elimination of this residue in the W103G mutant of mTS causes the emission maximum shift of 16 nm towards the shorter wavelengths. Multi-dimensional fluorescence with PARAFAC analysis confirmed that emission of the mTS is composed of two components (one in the short wavelength and the second in the long wavelength part of the emission spectrum). While for mTS and H190A the ratio between these two components is rather identical (domination of the long-wavelength component), in the W103G mutant we observed that elimination of Trp103 causes a substantial change in this ratio in favour of the short-wavelength component. This indicated that the second component of the emission is mainly composed of the Trp103 fluorescence.

Additional multi-wavelength time-resolved fluorescence measurements of all three enzymes alone and with dUMP (substrate), FdUMP and N⁴-OH-dCMP confirmed the

above results and testified to a probable very weak collisional interactions between W103G and all of the ligands. This indicated a legitimacy of using time-resolved fluorescence in verification of the mTS interactions. Furthermore, calculations of electrostatic potential (E.P.) around tryptophan residues in mTS, showed a big negative difference in the E.P. in the benzene → pyrrole direction of the Trp103 residue, approx. 3-4 times larger than for other Trp residues in mTS (except Trp75), confirming Trp103 to play an important role in the mTS mechanism. As Trp103 closes the active site of the mTS, suggesting that the aspect of E.P. and its properties should be further investigated.

The presented doctoral dissertation shows that its objectives were successfully accomplished. The author showed the importance of the aromatic amino acid pair at the positions 159 and 160 of PNPr in the interactions of this enzyme with formycin A and proved the uncoupling of the TS reaction steps simultaneously proving that the utilization of emission spectroscopy methods is useful in the performed investigations. Obtained results may be useful in the improvement of the target chemotherapy. Additionally, the author showed a promising path for future analysis that uses the electrostatic potential calculations.