
Summary and Final Remarks

The main objective of my PhD studies was to contribute to the understanding of enzymes and their mechanisms. To this end, I applied several computational techniques to the analysis of two different enzymes: DfrB dihydrofolate reductase (DHFR) and methyltetrahydrofolate:corrinoid/ iron-sulfur protein methyltransferase (MeTr).

DfrB DHFR, a bacterial enzyme that confers resistance to the drug trimethoprim, has no sequence or structural homology with the chromosomal DHFR despite both catalysing the reduction of dihydrofolate (DHF) using NADPH as a cofactor. Experimental approaches have failed to provide a structure for the reactive ternary complex DfrB DHFR•DHF•NADPH and, therefore, I combined different computational techniques to generate a model of the structure and test its feasibility.

Two different docking programs (AutoDock and FlexX) were employed to construct the initial model of the ternary complex. As more than one docked structure was in agreement with experimental findings, the feasibility of selected complexes was further analysed using molecular dynamic (MD) simulations. It was found that the ligands dihydrofolate and NADPH show unusual mobility within the active site: the reacting rings adopt a stable stack conformation at the centre of the pore, where there is little if any water access, while the charged tails extend towards the openings, adopting multiple conformations in a solvent-rich environment.

Prompted by these initial findings, a bioinformatic analysis was carried out to understand the possible origin of this peculiar enzyme. The DfrB DHFR family of proteins was found to have no homologues, and to be encoded exclusively as gene cassettes within integrons. Most gene cassettes have an obscure origin, with codon usage and base composition suggesting several sources including eukaryotes, bacteria and viruses. Integrons are thought to be ancient bacterial structures, natural cloning and expression systems likely to have an important

role in bacterial evolution and adaptability. DfrB DHFR is also an atypical enzyme from a structural point of view, consisting of a homotetrameric structure with a single central active-site pore. Each monomer presents an SH3-fold domain; this is a eukaryotic auxiliary domain involved in signalling pathways and protein-protein interactions, but never found before as the unique domain of a protein, let alone as the catalytic one.

All in all, mechanistic, structural and genetic analyses suggest that the DfrB family does not conform to the group of proteins commonly known as enzymes. DfrB DHFR seems to be a ‘minimalist’ catalyst, a protein recently recruited to fulfil a novel function in response to antibacterial drug pressure. This could be just the ‘tip of the iceberg’; continuous challenges to bacterial communities by new antibiotics, chemical pollutants, or other environmental factors may trigger the emergence of novel catalysts with unprecedented functions, currently dormant within integrons.

The second enzyme I studied was methyltetrahydrofolate:corrinoid/iron-sulfur protein methyltransferase (MeTr). This enzyme catalyses the transfer of a methyl group from CH₃THF to the cobalt centre of a corrinoid/iron-sulfur protein (C/Fe-SP). Given the difficulty of the reaction, it has been suggested that the tertiary amine must be activated possibly by protonation at the N5 position. Despite the overall catalytic and structural similarity between MeTr and the cobalt-dependent methionine synthase (MetH), experimental studies on both systems have led to different, if not contradictory, activation/protonation mechanisms. While CH₃THF binding to MeTr has been said to increase the p*K_a* of N5 and lead to proton uptake, binding to MetH was suggested to have the opposite result, decrease of the p*K_a* and no net proton exchange. Therefore, in order to explain these seemingly opposing conclusions, I used several of computational tools to study the effect of the protein environment on the protonation behaviour of CH₃THF.

Initially, the ability of QM calculations to predict both protonation patterns and p*K_a*s of a series of folate analogues in water was tested. Encouraged by the consistency of these first results with experimental data, I proceeded to study the effect of the protein environment on the protonation properties of CH₃THF.

A simplified representation of the active site was modelled by including the sidechains of selected residues in QM calculations. The protonation state of the tetrahydro pterin ring of CH₃THF, as well as that of two active-site aspartic residues, was analysed. These fragment calculations suggested that the preferred protonation position on the pterin ring is N5, as observed in water, regardless of the protonation state of the active-site Asp75 or Asp160 residues. However, the protonation state of these two residues was observed to affect the pK_a of N5. When both residues were ionised, the pK_a of N5 was predicted to increase by ~ 3 units, and this was reduced to half when Asp160 was protonated. On the other hand, when Asp75 was protonated the pK_a of N5 decreased in 0.4 units compared with its value in solution. Therefore, proton uptake upon CH₃THF binding would be expected to occur only if Asp75 was ionised. As the pK_a s of aspartic acids D75 and D160 are not known, no further inferences can be made at this point.

In a complementary approach, and with the objective of incorporating a more realistic representation of the protein environment, ONIOM calculations were carried out. The final energies suggested that the aspartic acid residues D75 and D160 are likely to be protonated before the ligand. However, the specific pK_a s at which these events may occur are uncertain.

Finally, a series of free energy calculations were done to compare the relative stability of different protonated forms of CH₃THF in both water and within the protein environment. These studies showed that the relative interaction energies of different protonated form of the ligand within the protein environment are similar to those observed in water. The calculation of relative binding energies did not lead to meaningful conclusions, because of the magnitude of the energies and the associated errors.

In order to explain the apparently contradictory activation mechanism of CH₃THF within MeTr and MetH further studies to establish the protonation state and pK_a of active-site aspartic residues are necessary. The calculations done so far suggest that either proposal, increasing of the pK_a or decreasing the pK_a of CH₃THF upon protein binding, could be possible depending on the protonation state of active-site aspartates.

During my PhD studies I have applied a series of computational tool to the study of two enzymes, DfrB DHFR and MeTr. I hope that the results I have presented highlight the versatility and importance of computational biology in the analysis of enzymes and their mechanisms. These approaches not only complement traditional experimental studies, but also allow us to obtain an atomic explanation of the phenomena measured empirically. With constantly increasing computer power, and the development of new and more accurate algorithms, the importance of computational approaches in the study of complex biological systems, such as enzymes, is expected only to increase in the foreseeable future.

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