OP-IV-03

Modeling the Enzymatic Reaction in N-Acetylglutamate Synthase: Role of GNAT-Conserved Structural Elements in Catalysis and Protein Stabilization

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N-Acetylglutamate synthase (NAGS, EC 2.3.1.1) is an enzyme from the large family of Gcn5-related N-acetyltransferases that catalyzes the transfer of an acetyl group from acetyl-CoA to glutamate to form N-acetylglutamate (NAG). The enzyme is found in all eukaryotes and some prokaryotes and belongs to the arginine biosynthesis pathway. As live evolved, NAG drastically changed its role from a simple intermediate in a linear pathway for arginine synthesis to an obligatory activator of carbamoyl phosphate synthase I, which catalyzes the rate-limiting step of the urea cycle. This change was also reflected in the quaternary structure of the corresponding NAGS. Meanwhile the catalytic mechanism of NAGS still needs to be refined. Therefore, both the refinement of the mechanism and the establishment of its role in the evolution of NAGS are of interest.

In this work the understanding of acetyl-CoA binding sites in NAGS of *Neisseria gonorrhoeae* (PDB ID: 3B8G) was further improved. The acetyl-CoA recognition motif (Gln364-Glu365-Gly366-Gly367-Tyr368-Gly369), which is conserved in GNAT-superfamily, was revealed to be insufficient to retain the substrate. Hydrogen bonds between acetyl-CoA and basic amino acids (Arg134, Arg151 *и* Lys152) from amino acid kinase domain of adjacent monomer turn out to be stronger, thus building another essential acetyl-CoA binding site.

We question the role of Glu353 as general base for glutamate amino group deprotonation, which was widely attributed to this GNAT-conserved residue in literature. Glu353 forms a salt bridge with Arg416, thus becoming ineffective proton acceptor. Similar salt bridges are found in all bacteria-like NAGS with determined crystal structure. We argue, that Glu353, establishing the salt bridge, acts as the stabilizer of the tertiary structure of the protein and, moreover, as the stabilizer of oxyanion hole conformation. The decrease in enzymatic activity of E353D and E353A mutants of NAGS is explained within the framework of the suggested model.

We determined free binding energies of glutamate with protonated and non-protonated amino group to NAGS and showed, that the protein has greater binding-affinity for the non-protonated form. Together with the absence of the suitable proton acceptor in the active site this implies, that the deprotonation of glutamate amino group happens before the substrate reaches the catalytic site. A surface binding site of glutamate was found at the edge of the tunnel, leading inside the protein to its active site. It consists of Ser326, Glu327 *и* Thr345, and we assume, that the deprotonation could be carried out there.

We suggest, that the enzymatic reaction proceeds trough three steps. Throughout the first step enzyme-substrate complex is formed: substrates approach each other along the

C(acetyl-CoA) –N(glutamate) line. The second step progresses through proton transfer from the amino group of glutamate to its α-carboxyl group, which is associated with the cleavage of C-S bond. This proton is then transferred to acetyl-CoA anion along the H-bond network The negatively charged intermediates of the reaction are stabilized in the oxyanion hole, formed by Cys356 and Leu357.

We discovered, that in the course of NAGS evolution the chemical nature of its active site changes radically. However, the geometry of the oxyanion hole remains the same. This suggests, that the active site of the protein has evolved in such a way as to preserve its geometry rather than amino acid composition and it is possible to attribute the mechanism, established for NAGS from Neisseria gonorrhoeae, to NAGS from more highly organized organisms.

The acetyl-CoA binding sites of NAGS and the mutant proteins were studied via molecular dynamic simulation with the applied CHARMM36 force field. The molecular mechanism of the reaction was revealed using the QM/MM approach.

Acknowledgement: This work was supported by the Russian Science Foundation (project #23-13-00011). We acknowledge the use of supercomputer resources of the Joint Supercomputer Center of the Russian Academy of Sciences and the equipment of the shared research facilities of HPC computing resources at Lomonosov Moscow State University.