Dynamic behavior and ligand binding properties of the wild type dCK and its characterized gemcitabine-resistant variant: a bioinformatics and computational study

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Research Article

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Abstract

In order to control the cell proliferation in various types of cancers, gemcitabine as the analogue of deoxycytidine is being used to perturb the DNA synthesis. The main challenge in using gemcitabine includes resistance of some patients to this compound due to the genetic mutations on deoxycytidine kinase (dCK) that are found in a fraction of human population. Here, we investigated the dynamics behavior and ligand binding properties of the wild type (WT) dCK and its characterized double mutant using a combination of the bioinformatics tools and molecular dynamics (MD) simulation studies. The crystal structure of the WT enzyme was trimmed by the MODELLER program and that of the double mutant was made by this program, and the structures were used as input for MD simulation. Root-mean-square deviation (RMSD) values of the WT and mutant enzyme in complex with both ligands, demonstrated that WT enzyme forms more stable complex with gemcitabine, as compared with its natural ligand (deoxycytidine). However, the stability of the double mutant-deoxycytidine complex is greater, when compared with gemcitabine. It was also found that Arg131 as a critical residue can affect the binding pattern of the enzyme to ligands in a manner that the WT enzyme can interact with both ligands, while, mutant enzyme cannot establish efficient interaction with gemcitabine, and shows high affinity to deoxycytidine. These data together indicate that gemcitabine can interact with the WT enzyme in a competitive manner, while double mutant can be considered as a resistant variant against gemcitabine.

Introduction

Gemcitabine (2', 2'-difluoro 2'deoxycytidine), an analogue of deoxycytidine (Commercial name: Gemzar), is a broad spectrum drug that affects a wide variety of cancers. In clinical medicine, it is ranked amongst top three drugs in cancer therapy [1]. It is used to treat a wide variety of cancers, including breast and pancreatic cancers, non-small-cell lung carcinoma, acute myeloid leukemia as well as the cancer of ovaries [2]. Compared with other drugs in cancer treatment, gemcitabine is actually less toxic, and it is being widely used for the treatment of cancers in children.

As a prodrug, gemcitabine is activated by several kinases. Upon entrance into the cell, it converted enzymatically to di- and tri-phosphorylated derivatives. These derivatives can perturb DNA synthesis by inhibition of ribonucleotide reductase enzymes within the cell, as well as incorporation into the DNA structure during replication [3, 4]. The effectiveness of gemcitabine as an anticancer drug is mainly mediated by deoxycytidine kinase (dCK) [5]. Accordingly, its action is dependent to the normal activity of dCK. However, the mechanism of the interaction between gemcitabine and dCK is controversial [5].

Human variant of dCK can phosphorylate natural ribonucleotides such as deoxycytidine, deoxyguanosine and deoxyadenosine that are needed for DNA synthesis. It is also an important enzyme involved in phosphorylation of the nucleoside analogues (containing modified base, sugar or both moieties) [6]. These analogues are being used as prodrug for treatment of cancers and some virus-related diseases.

For the majority of these compounds, phosphorylation reaction catalyzed by dCK is the rate limiting step of the whole process [7, 8].

This enzyme is continuously expressed during the cell cycle, and it is found in all tissues including normal and cancerous cells. It has been shown that the expression level of dCK have deterministic role in gemcitabine activity, and that the defective dCK enzymes are resistance to the nucleosides analogues [5, 9].

It was also shown that dCK-deoxycytidine complex has similar structure with the corresponding structure when deoxycytidine is substituted by gemcitabine. Hence, gemcitabine has been suggested as an effective competitive substrate for binding to the normal structure of dCK [7, 10].

On the other hand, representative cancers are shown to be resistance to drugs [11]. Clinical investigations have also demonstrated that some patients are resistance to gemcitabine -based treatment. Okazaki et al. has estimated that approximately 75% of patients of pancreatic cancer are resistance to gemcitabine [12]. Investigations of Bergmen et al. have shown that drug resistance feature in patients has multifactorial origin, and the mechanism of resistance is mainly related to the complexity of drug metabolism [13]. In line with these findings, it seems that future studies should pay special attention to the parameters that could predict resistance to gemcitabine. Ruiz van Haperen et al. have reported that resistance to gemcitabine in different cells is originated from the changes in the kinetics of the dCK activity due to the genetic modifications [14]. Keeping in mind that the reaction catalyzed by the dCK is rate-limiting step in the metabolism of gemcitabine, it is proposed that inactivation of dCK is the main mechanism for gemcitabine –resistance feature [14, 15]. By studying various cell lines, it is found that inactivating mutations as well as decreasing the expression level of dCK leads to gemcitabine-resistance property in cell lines. Sebastiani et al. have shown that the expression level of dCK decreases with increasing the age of patients, demonstrating that there is a positive correlation between the age and resistance to gemcitabine [16]. These findings together, indicate that the expression level and genetic mutations are two important factors in drug resistance feature [4]. The second factor is the consequence of the single nucleotide polymorphism (SNP) found in human population. Indeed, several SNPs in the exon as well as intron region of immature hnRNA of dCK have reported by various research groups. A correlation between the SNPs and the cancer mortality in patients that has been treated by gemcitabine was also stablished [17–19]. Regarding the gemcitabine resistance mechanisms, Kocabas et al. have reported that patients bearing a presentative SNP are producing a double mutant of deoxycytidine (Ile24Val and Pro122Ser), are significantly resisted against gemcitabine therapy. Further enzymatic assay showed that the k_m parameter of the mutant deoxycytidine was significantly different from WT.

The aim of the current work is bioinformatics analysis and computational study on the dCK and its characterized double mutant (Ile24Val and Pro122Ser) to find a molecular explanation to the mechanism of gemcitabine resistance in patients containing this variant of the enzyme.

Materials And Methods

Bioinformatics

The canonical sequence of dCK (P27707 (dCK_HUMAN) was obtained from Uniprot database [20] and then subjected to pairwise local sequence alignment against protein sequence databases using BLAST tool under the NCBI database [21] to find the homologous sequences from different organisms. Clustal Omega tool [22] was used to construct multiple sequence alignment file, and the result was used as input for ESpript server to graphical representation of data [23].

There are 47 X-ray resolved structure related to the human dCK provided in Uniprot. After careful searching in Protein Data Bank [24] the crystal structure of human dCK complexed with gemcitabine (PDB-ID: 1P62, resolution: 1.90 Å) was selected for further study. Using this structure as template, the structures of double mutant (Kocabas et al.) was constructed. Docking of ligands including gemcitabine and deoxycytidine into the core structure of the WT and mutant enzymes were also performed by the MODELLER program, and finally four structures including dCK-GEO, dCK-DCZ, mut-GEO and mut-DCZ were prepared.

Molecular Dynamics (MD) Simulation

The prepared structures were used as input for MD simulation. All simulations and analysis of the results were performed using GROMACS software package version 2018 [25] and CHARMM27 force field [26]. The structures were placed in a periodic truncated tetrahedron box that has 1Å distance from the structure in each side, and then solvated with water, TIP3 model [27]. Appropriate numbers of Na^+ and Cl^- ions were added into the system for neutralizing as well as mimicking the physiological condition. Pre-simulation steps include energy minimization (50000 cycles by the steepest descent algorithm) and equilibrations (NVT followed by NPT, each 100 ps duration, T = 300K). The main MD simulation was performed on each structure for 100 ns with time steps of 2 fs.

During the simulation production, coordinates were saved every 500 time steps and used for further structure calculation. Long-range electrostatic and non-bonded interactions were measured using the particle mesh Ewald (PME) method and periodic boundary conditions with a 10 Å cutoff, respectively [28]. The SHAKE algorithm [29] was used to fix covalent bonds involving hydrogen atoms. The root-mean-square deviations (RMSDs) and the root-mean-square fluctuations (RMSFs) of each system were analyzed using the GROMACS tools package.

It should be noted that the topology for the ligands were produced using CGenFF program [30] server based on the well optimized structures using Gaussian09 quantum chemistry program [31] using DFT method [32] under the B3LYP 6-311G* level of theory [33]. The quality of topologies met the appropriate criteria for simulation.

Results And Discussion

Description of the mutation

As mentioned above in introduction section, it has been shown that some SNPs within human population result in occurrence of resistance against cancer therapy. In a special case, Kocabas et al. have reported that patients containing a representative double mutant of dCK show significant resistance to gemcitabine in corresponding chemotherapy. To investigate the position of the aforementioned mutations on the sequence and structure of the enzyme, bioinformatics analyses were performed on the sequence and structure of the WT and mutant enzyme. At first, Multiple sequence alignment between the WT enzyme and selected similar sequences from other organisms as well as the target sequence for this study as reported by Kocabas et al. was performed. As shown in Fig. 1 by blue arrow, positions 24 and 122 in the target sequence are occupied by Valine (V) and Serine (S), respectively. While these positions in other sequences have high degree of conservation having Isoleucine (I) and Proline (P), respectively. Observing highly degree of conservation in these positions indicates that they have important structural and/or functional roles in dCK. Accordingly, in this study, we have tried to investigate the effects of residue substitution in these positions on the ligand binding properties and the dynamics of the protein structure.

To find the location of residual substitution on the tertiary structure of the dCK, the structural models of the double mutant were constructed in various forms in which docking of ligands were done on both WT and mutant proteins. The sites of mutations as well as the position of the ligands are shown in Fig. 2. In current work, the WT and double mutant dCKs are compared in conditions, where they complexed with deoxycytidine (DCZ) or gemcitabine (GEO). Figure 2A and B shows the whole tertiary structure of the WT protein in complex with deoxycytidine. As can be seen, the sites of mutations are far from the position of the ligands. These structures were used as input for the MD simulation studies.

MD simulation

Figure 3A and B, show the variations in the values of the RMSD for the enzyme variants to compare their dynamics behavior in the presence of both ligands. According to data of Fig. 3A, the WT enzyme forms more stable complex with gemcitabine, indicating higher affinity of the enzyme toward gemcitabine, as compared with DCZ. Interestingly, as can be seen from Fig. 3B, in the case of the mutant enzyme, the DCZ-bearing complex is more stable when compared with the respective complex formed with the gemcitabine, demonstrating that the double mutant prefers to form complex with DCZ rather than gemcitabine. These data together are in good agreement with the experimental observations on the drug resistance feature of the double mutant dCK against gemcitabine treatment [6].

In order to evaluate the effects of mutations and ligand type on the flexibility of different regions of the protein structure, their RMSF values were calculated, as provided in Fig. 4.

Examining of the data of Fig. 4 shows that the local dynamics near the positions of mutations in double mutant enzyme is the same as observed for the WT dCK. However, some differences in the dynamics of the other parts of the structure far from the positions of the mutations can be observed as shown by arrow in Fig. 4 showing the distal spatial effects of the mutations on the dynamics behavior of the

enzyme. However, it is worth to mention that the observed differences are mainly related to the loop regions as well as the N-terminal side of the structure. Accordingly, it can be said that the changes in the pattern of the residual interactions with the ligands leads to the variation in the stability of the Enzyme-ligand complexes as reported in the RMSD results of Fig. 3. Hence, the ligand binding properties of the equilibrated structures were compared by the LigPlot program and the results are shown in Fig. 5.

Comparing the interaction of the WT enzyme with DCZ and gemcitabine ligands shows that the overall orientation of the ligands in the core structure of the enzyme is approximately the same. However, detailed pattern of the interactions shows some differences in the interacting residues that have been reported as the critical residues for interaction with gemcitabine [7]. More importantly, as shown by black arrows in upper panel of Fig. 5, Arg131 which stablishes a hydrophobic interaction with DCZ in the WT enzyme can provide various numbers of interactions of the electrostatics and hydrogen bond types with gemcitabine. It is also revealed that the strengths of the interaction of Phe140 and Gln100 is greater in dCK-GEO complex as compared with that in dCK-DCZ complex. However, a reverse condition is observed for residues Tyr89 and Glu200 toward the stability of the dCK-DCZ complex. Also, the hydrophobic interaction of residues Leu85 and Ile35 in WT-DCZ complex are lost in the respective gemcitabine-bearing complex. These data together indicate that the WT enzyme is able to form complex with both ligands in a competitive manner. The RMSD data demonstrates that this competition is favored to the formation of dCK-GEO complex, indicating the importance of the interaction of gemcitabine with Arg131.

From Fig. 5 it can be seen that the double mutant enzyme has more affinity to interacts with DCZ, as compared with gemcitabine that is used as drug. The low affinity of the mutant to the gemcitabine is repeatedly observed in several MD simulations in which the final complex does not show significant interaction between the mutant enzyme and gemcitabine.

These data are obtained from a computational study on a single enzyme molecule that is complexed with a single ligand. In real cellular conditions, however there is a population of the enzyme molecule encountering with numerous number of ligands. Accordingly, the computational data in current study should be extended to the statistical paradigm, where there are statistically significant numbers of the enzyme and ligand molecules. In this regard, the WT enzyme has slightly more affinity to gemcitabine that is comparable with its natural ligand, indicating that both complexes including dCK-DCZ and dCK-GEO can be formed with the equilibrium constant favored toward the dCK-GEO. Accordingly, this process is dependent on the relative concentration of ligands. In the case of the mutant enzyme, there is no significant competition between ligands to interact with the double mutant enzyme, and the majority of the mutant enzyme molecules form dCK-DCZ complex, demonstrating that it is drug resistance even in conditions, where high concentration of gemcitabine is present in the environment. Therefore, it is predicted that the mutant enzyme prefers to interact with DCZ, when both ligands are present in the environment.

Conclusion

From the current data, it can be concluded that the WT enzyme can competitively adopt both DCZ and gemcitabine ligands into its core structure that results in observing the relative pharmaceutical effects of the gemcitabine based on the concentration of the drug. However, the double mutant enzyme can be considered as a drug resistance variant due to the very low affinity to the gemcitabine.

Declarations

Competing interests

We emphasize that the contents of this manuscript have neither been published nor submitted elsewhere. All authors are aware of this submission and there is no conflict of interest (financial or others) upon data presented. This Research is not involving Human Participants and/or Animals. The authors have equal contribution to this work.

Author Contribution

Y. Rahmati: Performing computational runs.

K. Khalifeh: Data analysis and writhing the manuscript.

E. Heshmati: Research conception and writhing the manuscript.

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Figures

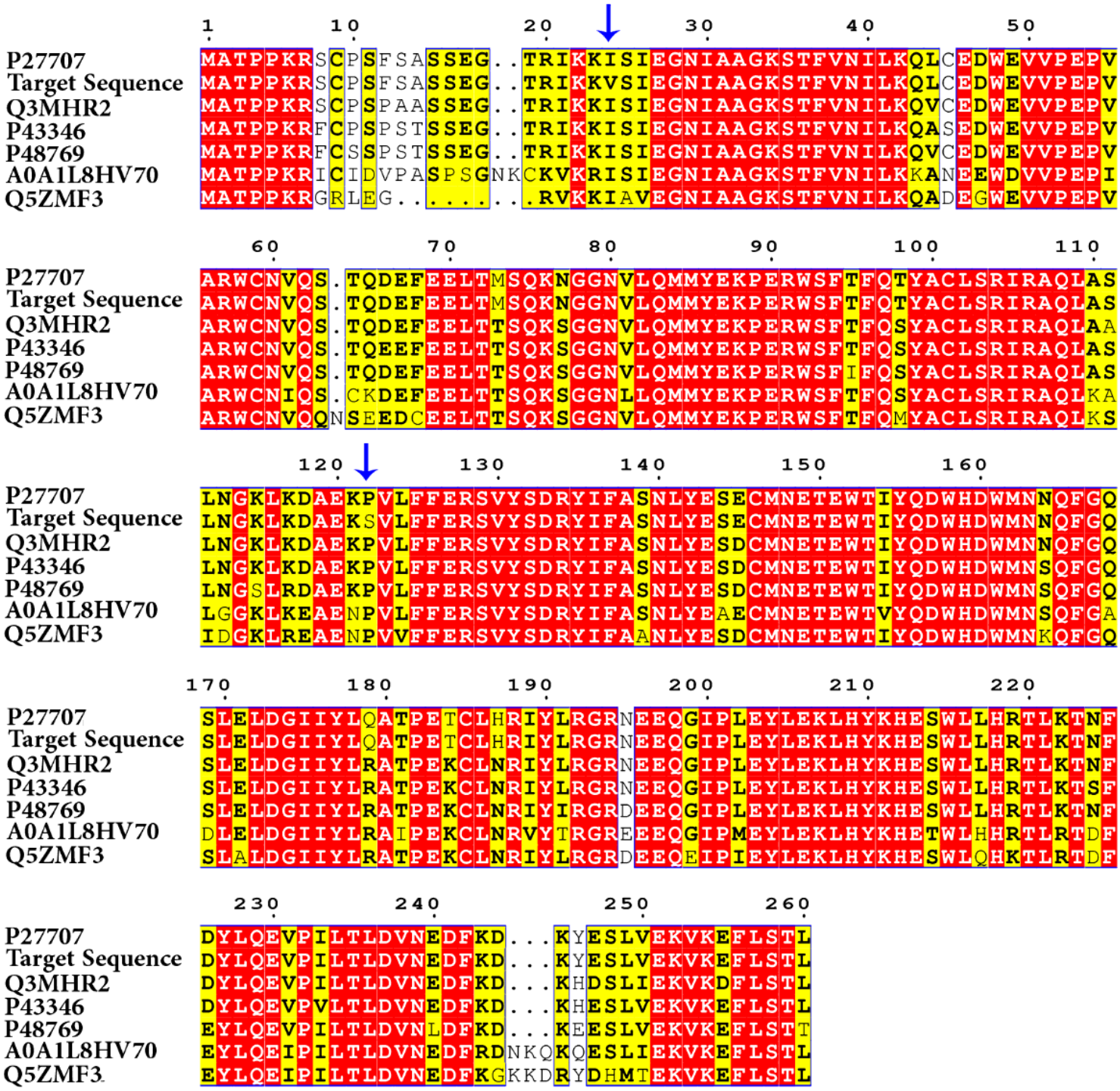

Figure 1

Sequence analysis. Multiple sequence alignment between the sequences of dCK s from different organisms performed by the Clustal Omega followed by the ESPript program. The protein with the ID number P27707 refers to the canonical sequence of the human dCK. The target sequence is based on the reports on the existence of a double mutant found in human population. Other ID numbers from top to bottom refers to the dCK variants from *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Xenopus laevis* and *Gallus gallus*, respectively.

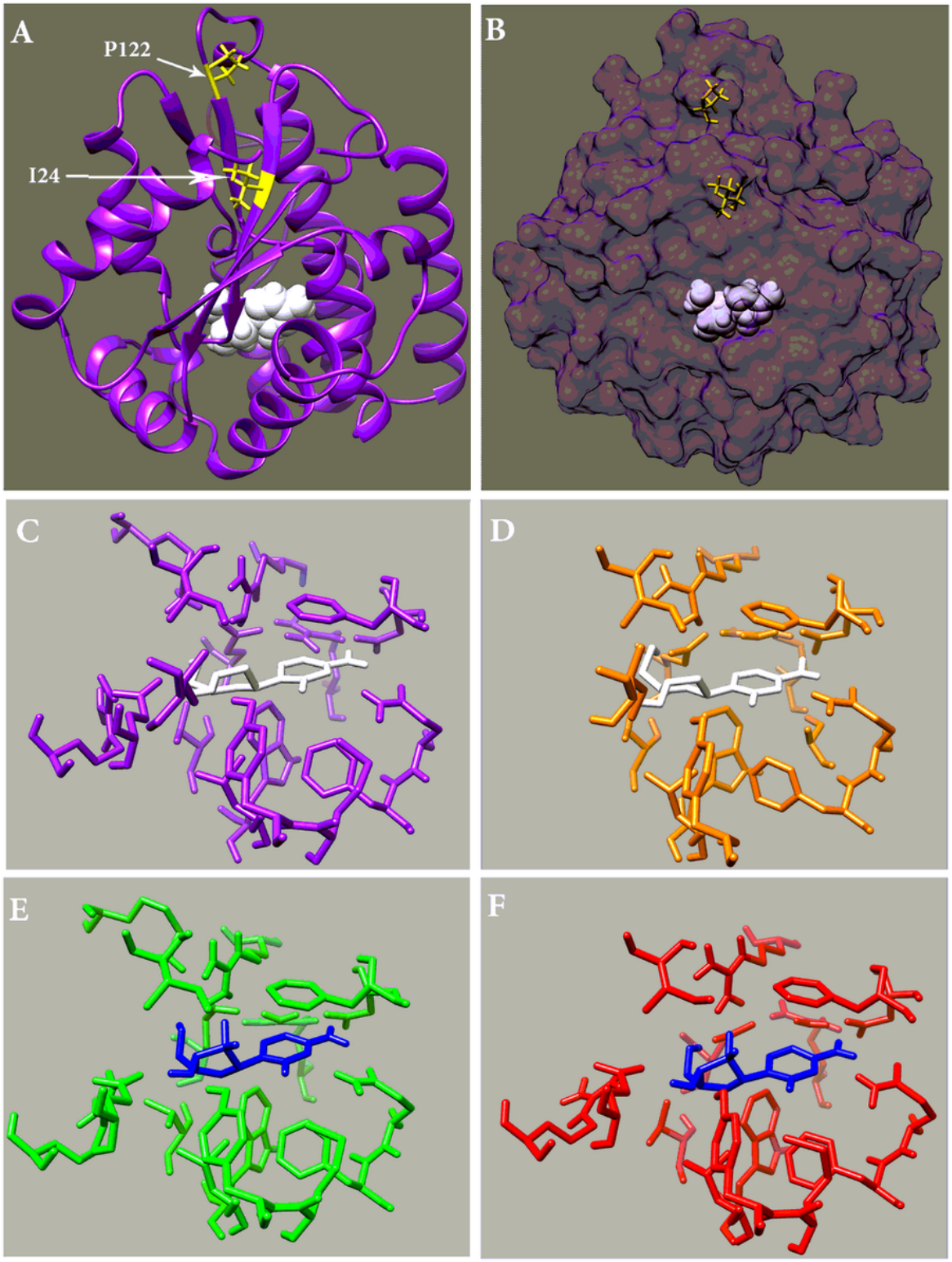

Figure 2

Tertiary structure of protein variants. (A) Ribbon representation of the tertiary structure of the WT dCK, where the sites of mutations are shown by yellow color, and the ligand by white sphere. (B) Surface representation of the tertiary structure of the WT dCK. (C-F) Interacting residues in the core structure of WT (C and E) and double mutant (D and F) with ligands. Deoxycytidine and gemcitabine are shown in white and blue, respectively.

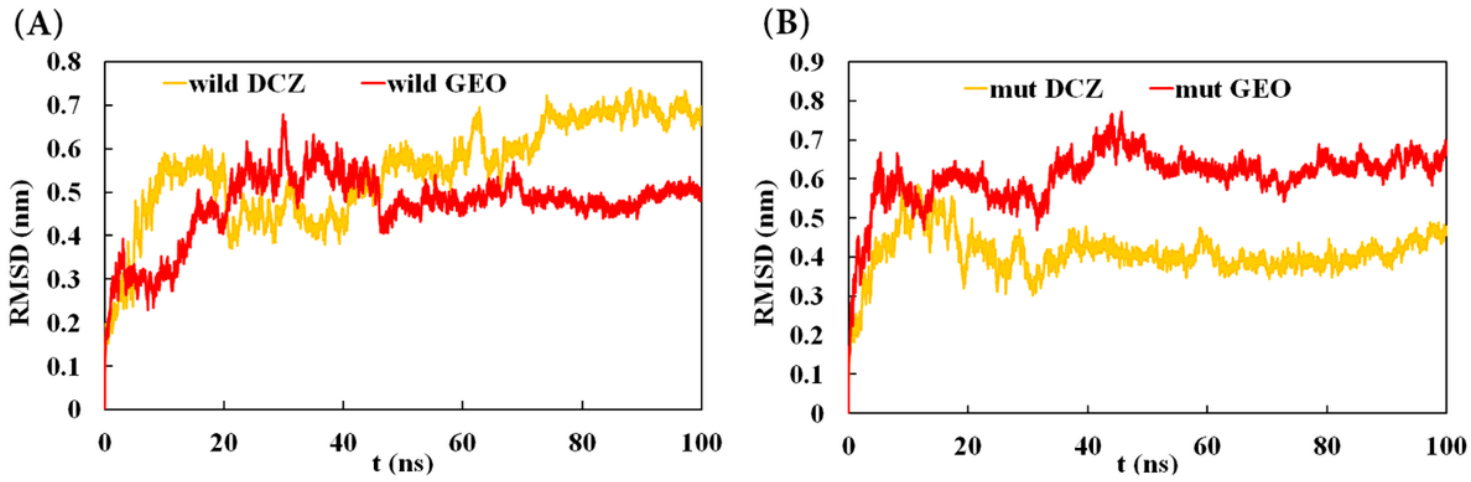

Figure 3

The values of the RMSD for the (A) WT dCK and (B) double mutant. For simplicity data are separately reported base on the dynamics behavior of individual protein variants in the presence of both ligands.

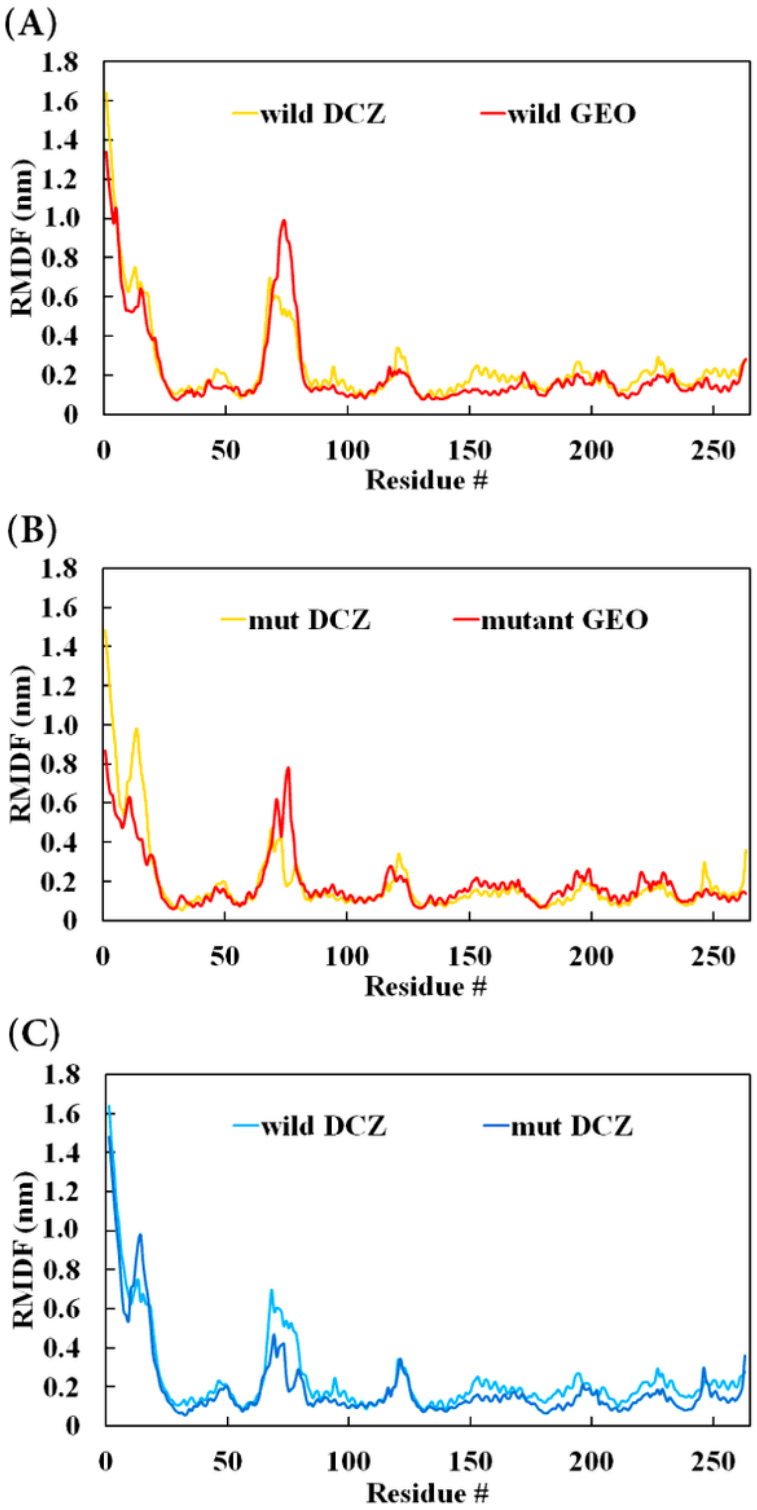

Figure 4

The values of the RMSF for the structures. (A) and (B) show the WT and double mutant, respectively; where, data of each protein variant is provided with both ligands. (C) different protein variants are shown with the natural ligand.

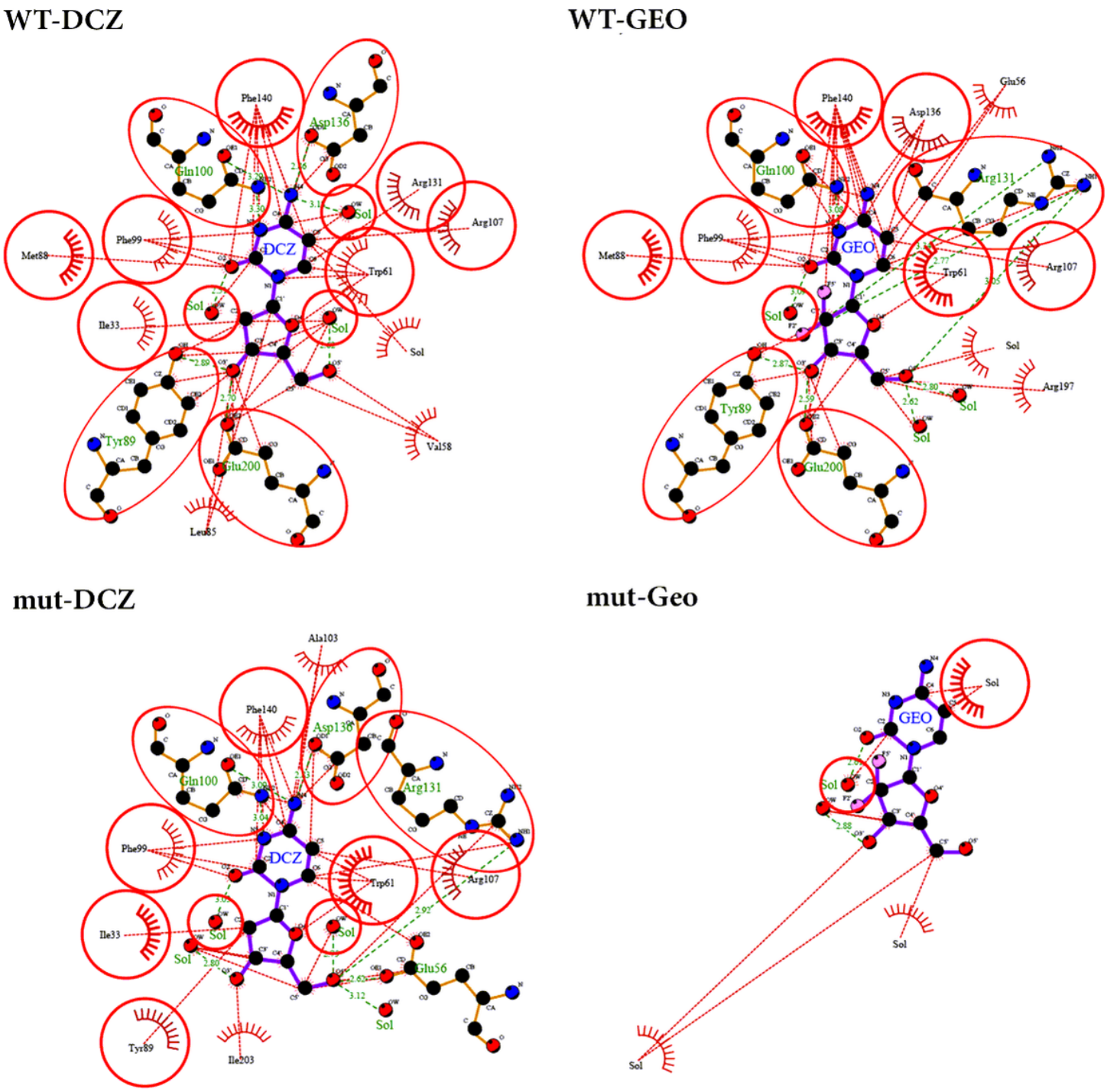

Figure 5

The pattern of protein-ligand interaction. The interacting residues with ligands as well as the solvent (sol) molecules in the core structure of the enzyme are shown. The picture is provided by the LigPlot program using the simulated pdb structures as input.