The Effects of Hydration on Protein of Azurin using Coarse-Grained Method and The Free-Energy Analysis

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Abstract. Proteins play important roles in body metabolism. However, to reveal hydration effects, it is cost computing especially for all-atom calculation. Coarse-grained method is one of potential solution to reduce the calculation and computable in longer timescale. Furthermore, the protein of Azurin is interesting protein and potentially applicable to cancer medicine for the stability property reason. We investigate the effects of hydration on Azurin, the conformation and the stabilities. Furthermore, we analyze the free-energy of the conformation system to find the favorable structure using free energy perturbation (FEP) calculation. Our calculation results show that free energy value of azurin is -136.9 kJ/mol. It shows a good agreement with experimental results with relative error index remained at 0.07%.

1. Introduction
The hydration process is important for physical and biological properties of protein [1]. We investigate hydration effects and protein stability. Basically, the stability of protein can be revealed by the amount free energy of hydration such as the negative free energy value indicates the system is favorable. Azurin is interesting protein for potentially applied as anticancer therapeutic agents. It causes apoptosis without much negative effect in cancer patient when enter the human breast cancer cells [2]. Moreover, it can be found in bacterial species Pseudomonas aeruginosa and it is known as donor at electron transfer process [3]. Furthermore, blue copper ions in polypeptide chains contribute the stability of Azurin protein [3-5].

In this study we perform molecular dynamic (MD) simulation with various parameters consist of simulation parameters (force field), integrator parameters (time step, rigid bonds), constant temperature control, constant pressure control and coordinate structure. The characterization of Azurin is calculated by coarse-grained methods and the free energy is calculated by using NAMD 2.12-multicore software with MARTINI force field and pseudo atom.

2. Method
A coarse-grained (CG) describes model on smaller scale (fine details) for smoothed or averaged. It observes molecular level systems with longer time scale, while maintaining physical properties similar to more detailed scale (small) in the all-atom system. The group of atoms is mapping in cluster to CG site called beads.

A Hamiltonian of system can be described by the function of the general extent parameter, \( \lambda \), that connect between state \( a \) to state \( b \). The connection can be achieved by linear combination of Hamiltonian as can be found in reference [6]:
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\[
H(x, p_x; \lambda) = H_0(x, p_x) + \lambda H_b(x, p_x) + (\lambda - 1)H_a(x, p_x)
\]  

(1)

where \( H_a(x, p_x) \) describes the interaction of the group of atoms representing reference state, \( a \). \( H_b(x, p_x) \) characterizes interaction of the final state, \( b \). \( H_0(x, p_x) \) is the Hamiltonian describing those atoms that is not transforming during the MD simulation. \( \lambda \) is the initial Hamiltonian parameter and \( \lambda - 1 \) is the final Hamiltonian parameter, this parameter describes the function of energy and forces.

The coupling parameter \( \lambda_{LI} \) scales the Lennard-Jones interactions and \( \lambda_{elec} \) scales electrostatic interactions, so the actual value of \( \lambda \) can be provided by:

\[
V_{NB}(r_{ij}) = \lambda_{LI} \varepsilon_0 \left[ \frac{R_{ij}^{min}}{r_{ij}^6} \right]^6 - \frac{R_{ij}^{min}}{r_{ij}^2} + \delta(1 - \lambda_{LI}) - \lambda_{elec} \frac{q_i q_j}{\varepsilon_0 r_{ij}} \]

(2)

The free energy calculation does not change intermolecular bonded potentials. The interactions within the perturbed atoms in vacuum are scaled but in hydration state only non-bonded interactions are scaled. The free energy difference between initial and final state is expressed by:

\[
\Delta A_{a \rightarrow b} = -\frac{1}{\beta} \ln \langle \exp \left[ -\beta \left( H_b(x, p_x) - H_a(x, p_x) \right) \right] \rangle_a
\]

(3)

Here, \( \beta^{-1} = k_B T \), where \( k_B \) is the Boltzmann constant, \( T \) is the temperature. \( H_a(x, p_x) \) and \( H_b(x, p_x) \) are the Hamiltonians describing states \( a \) and \( b \), respectively. \( \langle \ldots \rangle_a \) denotes an ensemble average over configurations representative of the initial, reference state, \( a \). Transformation between two thermodynamic states is replaced by a series of transformations between non-physical, intermediate states along a well-delineated pathway that connects \( a \) to \( b \). This pathway is characterized by the general extent parameter, \( \lambda \), it cause the Hamiltonian and, hence, the free energy, a continuous function of this parameter between \( a \) and \( b \):

\[
\Delta A_{a \rightarrow b} = -\frac{1}{\beta} \sum_{i=1}^{N} \ln \langle \exp \left[ -\beta \left( H(x, p_x; \lambda_{i+1}) - H(x, p_x; \lambda_i) \right) \right] \rangle
\]

(4)

Here, \( N \) stands for the number of intermediate stages.

In this study, we use NAMD 2.12-multicore program package for calculating the free energy hydration in coarse-grained scale, to determine the native state of protein. The hydration system of azurin is visualized by VMD 1.9.3. The initial structure coordinates of azurin obtain from protein data bank prepare by VMD 1.9.3 through coarse-grained builder menu with Residue-Based Coarse Graining (RBCG) method by NAMD. The schematic diagram of molecular dynamic simulation for calculating the free energy hydration of Azurin using coarse-grained can be shown in Figure 1.

X-ray crystal structure of oxidized AZ derived from the Protein Data Bank (PDB) (4AZU). AZ structure has 128 residues and is composed of two layers of sheet-\( \beta \) with eight \( \beta \)-strands arranged in topology. Copper ions are bound between two \( \beta \)-sheets bi-pyramidal coordinated trigonal geometry to five atoms, three equators (Nd from His46, N of His117, and SC of Cys112) and two axial (S of Met121 and carbonyl groups of Gly45) atom. Amino acids copper protrudes at His117 through the surface of the protein and is surrounded by a group of hydrophobic residues (Met13, Leu49, Val43, Met44, Met64, Phe114, Pro115, Gly116, Ala119 and Leu120), and this area is known as hydrophobic patch.

The initial state of the system is prepared by using X-ray structure at 2.7 Å resolution. In this study, three subunit proteins, moisture and nitrate removed to get just one protein molecule (930 protein molecules with one molecule blue copper ion), this structure obtained from protein data banks (1AZU) as the initial configuration. Azurin then modeled in CG structure to reduce the number of molecules to
269 molecules with the addition of 1746 water molecules (water sphere) and neutralized by the addition of 5 molecules of Na\(^+\) and 2 molecules of Cl\(^-\). The number of atoms changes to 2022 atoms. At the end of the addition of water put anti-freeze molecules (AF) in the form BP\(_4\) as much as 10% of the total water molecules. This is because when the all-atom models turned into a coarse-grained model the freezing point of water increases. This model represents a system with a salt concentration of 0.1 mol / L.

The initial state of the copper ion surrounded by three amino acids azurin planar (His46, His117, Cys112) and two axial amino acids (Met121 and Gly45). Coulomb potential with cutoff 9 Å and 15 Å. Time step integration time of minimization is 40 fs and time step integration time of equilibration is 20 fs with NPT ensemble (no change in temperature and pressure, wherein the temperature of 310 K to follow the body temperature and standard pressure 1 atm).

The final state azurin is modeled in CG structure so as to reduce the number of molecules to 269 molecules with the addition of as many as 1746 molecules of water molecules (water sphere) and neutralized by the addition of 5 molecules of Na\(^+\) and Cl\(^-\) molecules. The number of atoms changes to 2022 atoms. This model represents a system with a salt concentration of 0.1 mol / L.

### 3. Results and Discussion

Figure 2.a. illustrates the structure of the all-atom 1 azurin protein molecule composed of 930 protein atoms and one atom from blue copper ion. Then, in figure 2.b., all-atom structure of azurin reduced to coarse-grained structure. Figure 2.c. show every four heavy atom represent an interaction center.

Figure 3 shows the beads light blue indicates water molecules, with a dark blue bead as anti-freeze molecules (AF) in the form BP\(_4\). Anti-freeze molecules included in the simulation box because the current structure was changed into structure AA, CG freezing point of water increases water can freeze at room temperature 310 K, which is the temperature used in the simulation. Red bead is Na\(^+\) ions that amount five atoms and green beads is Cl\(^-\) ions that amount two atoms. The additional function of ions completes the lack of electrons in each of amino acid residues so the system is in neutral. Neutral state
reduces interaction outside of the protein and the water caused the conformation of the protein in water is not affected by other interactions.

![Image](image1)

**Figure 2.** (a) Before the structure change into CG model (b) Transparent beads show four heavy atoms that represent one interaction center (c) Light blue bead show water atom; ice blue bead show anti freeze molecule (BP$_4$); red beads show Na$^+$ ions; green beads show Cl$^-$ ions; protein of azurin show in the middle of water box simulation, every residue of amino acid show in different color.

The simulation show that there are some amino acids that actually is not hydrophobic out from the water such as asparagine, aspartic acid, lysine, and threonine. This is due to the scheme of conformational azurin of 10 ns still move to find the stable native state. The interaction between the charge of water, BP$_4$ and NaCl can also cause it. Some of the amino acids of azurin also out from the water in this state such as histidine and methionine. Methionine is known to have hydrophobic properties while histidine residue with the mutant has a positive. It is different with other amino acids (glycine and cysteine), which is an amino acid uncharged polar. The conformation processes of two amino acids are in water simulation box.

At the end of the state 1000 ns simulation show azurin protein forming a favorable conformation and overall state of the protein in the water. This indicates azurin achieve a more stable state conformation in water, it is shown by the end position conformation.
In this study, we perform free energy perturbation simulations with molecular dynamics using MARTINI CG force field [8]. Figure 4 shows the free energy value of azurin. The straight line shows free energy of hydration and dotted line show free energy of vacuum. The net solvation free energy change $\Delta\Delta G$ for protein of azurin is the difference between free energy of hydration value and free energy of vacuum value. We find the net solvation free energy change of azurin, which shows in Figure 4, is -136.9 kJ/mol.

**Figure 3.** (a) Equilibration simulation of azurin protein in simulation time 0 ns (initial state); (b) Equilibration simulation of azurin protein in simulation time 10 ns; (c) Equilibration simulation of azurin protein in simulation time 100 ns; (d) Equilibration simulation of azurin protein in simulation time 1000 ns.

**Figure 4.** The free energy of azurin, straight line shows free energy of hydration and dotted line show free energy of vacuum.
The validity of FEP calculation compared with the value from a previous study, which is using three different force fields, AMBER, CVFF and CFF for validating the result. The difference between FEP calculation and another method to find free energy protein of azurin is shown in table 1.

**Table 1** The comparison of free energy value using FEP method and another method.

| Azurin     | AMBER  | CVFF  | CFF  | Experiment | FEP (This research) |
|------------|--------|-------|------|------------|---------------------|
| Native     | -200.0 ± 1.6 | -278 ± 12 | -127.0 ± 1 | -137.0 ± 3 | -136.9 ± 3 |
| Unfolded   | -      | -     | -91.0 ± 1.2 | -99.3 ± 2.0 | -       |

4. Conclusion

Coarse-grained method provides alternative methods to ensure all-atom simulation calculations for larger systems such as proteins and can reduce the calculation. We have investigated the effects of hydration on Azurin, the conformation and the stabilities and we have analyzed the free energy of the conformation system to find the favorable structure using free energy perturbation (FEP) calculation. Our calculation results show that the calculation of free energy perturbation values obtained free energy of -136.9 kJ / mol. It shows a good agreement with experimental result with relative error index remained at 0.07%.

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