Biophysical and computational characterization of vandetanib–lysozyme interaction

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Abstract

Interaction of an anticancer drug, vandetanib (VDB) with a ligand transporter, lysozyme (LYZ) was explored using multispectroscopic techniques, such as fluorescence, absorption and circular dichroism along with computational analysis. Fluorescence data and absorption results confirmed VDB–LYZ complexation. VDB-induced quenching was characterized as static quenching based on inverse correlation of $K_Q$ with temperature as well as $k_q$ values. The complex was characterized by the weak binding constant ($K_θ = 4.96−3.14 \times 10^3$ M$^{-1}$). Thermodynamic data ($\Delta S = +12.82$ J mol$^{-1}$ K$^{-1}$; $\Delta H = -16.73$ kJ mol$^{-1}$) of VDB–LYZ interaction revealed participation of hydrophobic and van der Waals forces along with hydrogen bonds in VDB–LYZ complexation. Microenvironmental perturbations around tryptophan and tyrosine residues as well as secondary and tertiary structural alterations in LYZ upon addition of VDB were evident from the 3-D fluorescence, far- and near-UV CD spectral analyses, respectively. Interestingly, addition of VDB to LYZ significantly increased protein’s thermostability. Molecular docking results suggested the location of VDB binding site near the LYZ active site while molecular dynamics simulation results suggested stability of VDB–LYZ complex. Presence of Mg$^{2+}$, Ba$^{2+}$ and Zn$^{2+}$ was found to interfere with VDB–LYZ interaction.

1. Introduction

VDB (Fig. 1) is a potent, orally active, selective inhibitor of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor 2 (VEGFR2) tyrosine kinases [1]. The FDA approved drug is being used in cancer therapy for metastatic medullary thyroid cancer [1,2]. Owing to the inhibition properties, VDB halts certain signal transduction pathways through VEGFR and EGFR tyrosine kinases that are responsible for tumour cell growth, and therefore prevents tumour growth [1,3].

Drug-protein interactions are vital in the transportation and delivery of small drug molecules to their specific target sites [4,5]. Such interaction increases drug’s in vivo half-life, improves its solubility, prolongs drug’s efficacy, defends drug’s elimination from the body and reduces drug’s toxicity [6,7]. Therefore, characterization of the drug binding to the protein is vital.

Serum albumin, lysozyme and α-1-acid glycoprotein are generally used as the model carrier proteins in various studies involving drug-protein interaction [8–11]. In a previous study [12], we have described the VDB–human serum albumin interaction in detail. However, binding of VDB to lysozyme has not been characterized yet. LYZ is a monomeric protein of 129 amino acids, which include six tryptophan (Trp) and three tyrosine (Tyr) residues while four disulfide bonds stabilize its structure [13]. Out of 6 Trp residues, 3 residues are located at the protein’s substrate binding site and can easily interact with a ligand [14]. Here, we report the characteristics of VDB–LYZ interaction using fluorescence, absorption and circular dichroism spectroscopic methods along with molecular docking and simulation techniques.

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme (purity ≥98%; Lot #061M1329V) was procured from Sigma-Aldrich (St. Louis, USA). Vandetanib (purity ≥98%; Batch #0454075–6) was the product of Cayman Chemical Company (Michigan, USA). All chemicals used in this study were analytical grade samples.

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2.2. Preparation of working solutions

Stock solution of lysozyme (LYZ) was made in sodium phosphate buffer (60 mM, pH 7.4) and its concentration was determined spectrophotometrically using $E_{280} = 38,940 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm [15]. Preparation of vandetanib (VDB) stock solution (1.0 mg ml$^{-1}$) was made in dimethyl sulphoxide (DMSO). VDB stock solution was further diluted in the above buffer to prepare the working solution. The final DMSO concentration in all experiments was $< 1\%$ (v/v). Such DMSO concentration has no effect on the overall conformation of LYZ [16]. All the experiments were carried out at 298 K unless otherwise stated.

2.3. Absorption spectra

Absorption spectral measurements were made on a double beam UV–Vis spectrophotometer (Perkin-Elmer Lambda 25), using a pair of 1 cm path length cuvettes. Absorption spectra of LYZ (4 μM) in the presence of increasing concentrations (0–100 μM with 10 μM intervals) of VDB were recorded in the wavelength range, 280–400 nm at 298 K. These spectra were used for inner filter effect correction.

In order to monitor VDB-induced changes in the absorption spectrum of LYZ, spectral measurements were made in the same way as described above by using a LYZ concentration of 15 μM and wavelength range of 230–360 nm. The absorption spectra of free VDB were also collected using similar VDB concentrations in the same wavelength range.

2.4. Fluorescence spectra

The protein’s fluorescence spectra were recorded on a spectrofluorometer (model: Jasco FP-6500) using a quartz cuvette of 1 cm path length, positioned in a thermostatically-controlled cell holder. The spectral measurements were made under the following conditions: protein concentration = 4 μM; excitation and emission bandwidths = 10 nm each; data pitch = 1 nm; scan speed = 500 nm min$^{-1}$; detector voltage = 240 V; excitation wavelength = 280 nm; emission wavelength range = 300–400 nm.

Three-dimensional (3-D) fluorescence spectra of free LYZ (4 μM) and its complexes with VDB were obtained using the excitation wavelength range, 220–350 nm (data pitch = 5 nm) and the emission wavelength range, 220–500 nm (data pitch = 1 nm). The scanning parameters were maintained same as described above for fluorescence measurements. The spectra were recorded in the absence and presence of VDB using VDB/LYZ molar ratios of 5:1 and 10:1.

2.5. Circular dichroism spectra

Circular dichroism (CD) spectra of the protein (LYZ) in the absence and upon addition of VDB were recorded on a spectropolarimeter (model: Jasco-815), equipped with a thermostatically-controlled, water-jacketed cell holder. These experiments were performed by measuring the CD spectra of LYZ and its complexes with VDB using VDB/LYZ molar ratios of 1:1 and 2:1 under nitrogen atmosphere. The following conditions were maintained throughout the experiment: scan speed = 100 nm min$^{-1}$; response time $= 0.5$ s; data pitch $= 1$ nm; number of scans $= 8$; $T = 298 K$; cuvette path length $= 1$ mm (far-UV), 10 mm (near-UV); protein concentration $= 4$ μM (far-UV) and 10 μM (near-UV); VDB concentration $= 4$ and 8 μM (far-UV), 10 and 20 μM (near-UV).

The measured ellipticity values were expressed as mean residue ellipticity (MRE) in deg·cm$^2$·dmol$^{-1}$ using the following equation:

$$\text{MRE} = \frac{\theta_{obs} \times \text{MRW}}{10 \times f \times C_p}$$

where $\theta_{obs}$ is the ellipticity in millidegree; MRW is the mean residue weight (molecular weight of the protein, 14,300 divided by the total number of amino acid residues, 129); $C_p$ is the protein concentration in mg ml$^{-1}$ and $f$ is the path length of the cuvette in mm [17].

Percentage contents of various secondary structures (α-helix, β-sheet, β-turn and unordered) present in LYZ in the absence and presence of VDB were obtained by analyzing the far-UV CD spectral data using the online SELCON3 program ( DichroWeb software) [18,19].

2.6. VDB binding studies

The interaction of VDB with LYZ was studied using titration according to the published procedure [20]. The reaction mixture (3 ml) was prepared by taking the fixed concentration of LYZ (4 μM) and increasing concentrations (10–100 μM with 10 μM intervals) of VDB in sodium phosphate buffer (60 mM, pH 7.4). The mixtures were incubated for 1 h at fixed temperature before fluorescence measurements. The titration experiments were performed at three different temperatures (288, 298 and 308 K).

The fluorescence data for VDB–LYZ interaction were treated for the inner filter effect correction according to the following equation [21]:

$$F_{cor} = F_{obs} 10^{A_{abs}+A_{em}}/2$$

where $F_{cor}$ and $F_{obs}$ indicate the corrected and the measured fluorescence intensity, respectively, while $A_{abs}$ and $A_{em}$ refer to the difference in the absorbance at the excitation wavelength (280 nm) and the emission wavelength (300–400 nm), respectively, upon addition of VDB to the protein.

The nature of quenching mechanism involved in VDB–LYZ interaction was elucidated by analyzing the fluorescence data using the Stern-Volmer equation [21]:

$$F_0/F = 1 + K_{SV} [Q] = 1 + k_q \tau_0 [Q]$$

where $F_0$ and $F$ are the values of the fluorescence intensity in the absence and upon addition of the ligand (VDB), respectively; $[Q]$ is the quencher concentration; $K_{SV}$ is the Stern-Volmer constant; $k_q$ is the bimolecular quenching rate constant and $\tau_0$ represents the fluorescence lifetime of the protein alone, taken as $1.8 \times 10^{-8}$ s for LYZ [22].

Values of the binding constant ($K_b$) for VDB–LYZ interaction were determined after analyzing the fluorescence data using the following equation [23]:

$$\log (F_0 - F)/F = n \log K_b - n \log [1/(K_b)] - (F_0 - F)/F_0$$

where different terms have their usual significance [20].

![Chemical structure of vandetanib. Inset shows the ball-and-stick model of vandetanib.](image)

**Fig. 1.** Chemical structure of vandetanib. Inset shows the ball-and-stick model of vandetanib.
To characterize the acting forces participated in the complex formation between VDB and LYZ, values of the enthalpy change ($\Delta H$) and the entropy change ($\Delta S$) were determined using the van’t Hoff equation:

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$  \(5\)

where $R$ is the gas constant (8.314 J mol$^{-1}$ K$^{-1}$) and $T$ is the absolute temperature (273 ± °C).

Value of the Gibbs free energy change ($\Delta G$) of the binding reaction was estimated by fitting $\Delta H$ and $\Delta S$ values into the following equation:

$$\Delta G = \Delta H - T\Delta S$$  \(6\)

### 2.7. Thermal stability

Thermal stability experiments were performed to check the possible influence of VDB binding on the protein’s thermostability. Fluorescence measurements of LYZ (4 μM) were carried out in the presence of VDB (VDB:LYZ molar ratios of 0:1 and 10:1) at different temperatures in the temperature range, 25–80 °C with regular increments of 5 °C. The solutions of LYZ and VDB–LYZ mixture were preincubated at 25 °C for 1 h, followed by 10 min incubation at each temperature in order to equilibrate the sample before spectral measurements. All other experimental conditions were kept same as described in Section 2.4.

### 2.8. Influence of metal ions on ligand–protein interaction

The effect of metal ions on the binding affinity of VDB towards the protein was probed by recording the fluorescence spectra of the VDB–LYZ mixture in the presence of $\text{K}^+$, $\text{Ca}^{2+}$, $\text{Mn}^{2+}$, $\text{Mg}^{2+}$, $\text{Cu}^{2+}$, $\text{Ba}^{2+}$ and $\text{Zn}^{2+}$. Titration experiments were performed in a similar way as described above in Section 2.6 using 4 μM LYZ. Metal ion–LYZ mixtures ([metal ion]:[LYZ] = 25:1) were preincubated for 1 h and the samples were further incubated with VBD for additional 1 h prior to fluorescence data acquisitions. All procedures were carried out at 298 K.

### 2.9. Molecular docking

The AutoDock 4.2 and AutoDockTools 1.5.6[24,25] were utilized to perform molecular docking (MD) analysis and visualization. The LYZ crystal structure (PDB ID: 1AKI) was obtained from the PDB at a resolution of 1.5 Å. Co-crystallized water molecules were removed prior to docking. Chemical structure of VDB was drawn, followed by geometry optimization and energy minimization using Avogadro software [26]. Torsional degrees of freedom were allowed in the ligand with 5 rotatable bonds. Grid box for docking simulation was parameterized to accommodate the entire protein with 126 × 126 × 126 grid points with a distance of 0.375 Å between them. The exploration of configurational space was made using Lamarckian genetic algorithm. For docking simulation, 100 rounds of calculation were executed, each with a maximum of 2,500,000 steps of energy evaluation to converge into the energy minimum. Cluster analysis of binding modes was performed with AutoDockTools 1.5.6 with a cutoff at 2.0 Å. The VDB–LYZ complex was visualized and prepared using UCSF Chimera 1.11[27].

Fig. 2. (A) VDB-induced fluorescence quenching of LYZ. Spectrum 1 shows fluorescence spectrum of LYZ while spectra 2–11 refer to the fluorescence spectra, obtained in the presence of increasing VDB concentrations (10–100 μM with 10 μM intervals) in 60 mM sodium phosphate buffer, pH 7.4 at 298 K. The fluorescence spectrum of 4 μM VDB is marked as ‘a’. (B) The Stern-Volmer plots for the VDB–LYZ system at three different temperatures viz. 288, 298 and 308 K, obtained by treating the fluorescence quenching data according to Eq. (3). (C) The double logarithmic linear plots at three different temperatures viz. 288, 298 and 308 K, obtained by treating the fluorescence quenching data according to Eq. (4). Inset shows the van’t Hoff plot for VDB–LYZ system.
showed an emission maximum at 342 nm due to the presence of Trp residues [14]. Two of these residues (Trp62 and Trp108) are mainly responsible for the binding site and are vital for stabilizing the complex with the substrate. Trp residues, 3 residues are closely arranged at the protein’s substrate binding site, and concentrations are shown in Fig. 2A. The fluorescence spectra of LYZ in the absence and upon addition of increasing VDB concentrations (0–100 μM) were observed in the range (spectrum ‘a’ in Fig. 2A). The Stern-Volmer plots for the fluorescence results of VDB–LYZ interaction, obtained at 288, 298 and 308 K are shown in Fig. 2B. These plots exhibited good linearity with a correlation coefficient (r) of ≥0.994. Values of $K_{SV}$, as determined from the slope values of these plots are summarized in Table 1. Temperature dependence behavior of $K_{SV}$ distinguishes between dynamic and static quenching mechanisms. Whereas increase in temperature leads to faster diffusion of molecules thus producing an increase in collisional quenching, dissociation of weakly-bound complexes at increasing temperature may lead to the decrease in the static quenching [21]. Therefore, quenching constant shows direct correlation with temperature for collisional quenching while inverse correlation for static quenching. Reduction in the values of $K_{SV}$ with rising temperature (Table 1), as observed in VDB–LYZ system clearly characterized VDB-induced quenching of LYZ fluorescence as static quenching, hence suggesting VDB–LYZ complexation. Furthermore, calculated values of $k_q$ for VDB–LYZ system (2.72 × 10^{12} M^{-1} s^{-1} at 288 K, 2.22 × 10^{12} M^{-1} s^{-1} at 298 K and 1.65 × 10^{12} M^{-1} s^{-1} at 308 K) were much greater than the value of the maximum dynamic quenching constant (2.0 × 10^{10} M^{-1} s^{-1}) for different ligands’ interactions with proteins [36]. These results also opposed the involvement of dynamic quenching mechanism and suggested VDB–LYZ complex formation.

Table 1

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>$K_{SV}$ (M^{-1})</th>
<th>$K_f$ (M^{-1})</th>
<th>$\Delta S$ (J mol^{-1} K^{-1})</th>
<th>$\Delta H$ (kJ mol^{-1})</th>
<th>$\Delta G$ (kJ mol^{-1})</th>
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<td>288</td>
<td>(4.90 ± 0.20) × 10^{3}</td>
<td>(4.96 ± 0.15) × 10^{3}</td>
<td>+12.82</td>
<td>-16.73</td>
<td>-20.42</td>
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<tr>
<td>298</td>
<td>(4.00 ± 0.20) × 10^{3}</td>
<td>(4.15 ± 0.22) × 10^{3}</td>
<td>-</td>
<td>-</td>
<td>-20.55</td>
</tr>
<tr>
<td>308</td>
<td>(2.97 ± 0.06) × 10^{3}</td>
<td>(3.14 ± 0.11) × 10^{3}</td>
<td>-</td>
<td>-</td>
<td>-20.67</td>
</tr>
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2.10. Molecular dynamics

The execution of molecular dynamics (MD) simulations was performed with the GROMACS program [28] using the CHARMM36 force field [29]. The SwissParam webserver with Merck force field [30,31] was utilized to create the ligand’s topology and parameter files. The treatment of long-range electrostatic forces was made according to the CHARMM method while the SHAKE algorithm [32] was employed for constraining all bonds. The VDB–LYZ complex was bordered by the TIP3P aperiodic box [33] water molecules with a verge of 20 Å for different temperatures. Initially, the energy minimization was done for 10,000 steps, followed by the phases of equilibration for 1 ns as well as production for 10 ns. The simulations were performed at 300 K for Maxwell-Boltzmann distribution, with the fixed pressure (1.0 bar) throughout the equilibration phase. While the simulation time step was set to 10 ns, the coordinates were saved each 1 ps. The UCSF Chimera [27] and VMD [34] software packages were exploited for molecular visualization and analyses.

2.11. Statistical analysis and curve plotting

The experimental data of the VDB–LYZ interaction are expressed as the mean ± standard deviation (SD) by taking the average values of a minimum three individual experiments. The statistical data processing, curve fitting and smoothing were performed using the software, OriginPro 8.5 (OriginLab Corp., Northampton, USA).

3. Results and discussion

3.1. Fluorescence quenching mechanism and binding characteristics of VDB–LYZ complex

Ligand binding to the protein may alter the microenvironment around the fluorophores (Trp and Tyr residues), thus leading to quenching in its fluorescence intensity. Various mechanisms, i.e., ground-state complex formation, excited state reactions, molecular rearrangements, energy transfer and dynamic quenching process have been suggested to be responsible for such phenomenon [21]. Fluorescence spectra of LYZ in the absence and upon addition of increasing VDB concentrations are shown in Fig. 2A. The fluorescence spectrum of LYZ showed an emission maximum at 342 nm due to the presence of Trp residues. LYZ consists of 6 Trp, 3 Tyr and 3 Phe residues [13]. Among the 6 Trp residues, 3 residues are closely arranged at the protein's substrate binding site and are vital for stabilizing the complex with the substrate [14]. Two of these residues (Trp62 and Trp108) are mainly responsible for the LYZ fluorescence [35]. The fluorescence intensity of LYZ gradually decreased by the addition of increasing concentrations of VDB, showing ~34% reduction in the fluorescence intensity as well as 3 nm red shift in the emission maximum at the highest concentration used. Such changes in the fluorescence characteristics might result from the increased microenvironmental polarity around Trp and Tyr residues [21], suggesting VDB–LYZ complex formation. It may be noted that uncorrected fluorescence spectra of LYZ in the absence and upon addition of increasing concentrations of VDB (supplementary Fig. 1) were devoid of the second peak, which emerged at 326 nm only at higher VDB concentrations after correcting the inner filter effect (Fig. 2A). Free VDB did not produce any fluorescence signal within this wavelength range (spectrum ‘a’ in Fig. 2A).

The Stern-Volmer plots for the fluorescence results of VDB–LYZ interaction, obtained at 288, 298 and 308 K are shown in Fig. 2B. These plots exhibited good linearity with a correlation coefficient (r) of ≥0.994. Values of $K_{SV}$, as determined from the slope values of these plots are summarized in Table 1. Temperature dependence behavior of $K_{SV}$ distinguishes between dynamic and static quenching mechanisms. Whereas increase in temperature leads to faster diffusion of molecules thus producing an increase in collisional quenching, dissociation of weakly-bound complexes at increasing temperature may lead to the decrease in the static quenching [21]. Therefore, quenching constant shows direct correlation with temperature for collisional quenching while inverse correlation for static quenching. Reduction in the values of $K_{SV}$ with rising temperature (Table 1), as observed in VDB–LYZ system clearly characterized VDB-induced quenching of LYZ fluorescence as static quenching, hence suggesting VDB–LYZ complexation. Furthermore, calculated values of $k_q$ for VDB–LYZ system (2.72 × 10^{12} M^{-1} s^{-1} at 288 K, 2.22 × 10^{12} M^{-1} s^{-1} at 298 K and 1.65 × 10^{12} M^{-1} s^{-1} at 308 K) were much greater than the value of the maximum dynamic quenching constant (2.0 × 10^{10} M^{-1} s^{-1}) for different ligands’ interactions with proteins [36]. These results also opposed the involvement of dynamic quenching mechanism and suggested VDB–LYZ complex formation.

Values of $K_f$ for VDB–protein system, as acquired from the double logarithmic plots (Fig. 2C) at three different temperatures are included in Table 1. $K_f$ values were found to lie in the range, 4.96–3.14 × 10^{3} M^{-1}, indicating weak binding affinity between VDB and LYZ. Several ligands have been shown to bind proteins with a binding affinity in the order of 10^{3} [37–39]. Decrease in the $K_f$ value with rising temperature may arise due to loss of stability of the VDB–LYZ complex at higher temperature.

![Fig. 3. UV–vis absorption spectra of LYZ (15 μM) in the presence (bottom to top) of increasing concentrations (0–100 μM with 10 μM intervals) of VDB, obtained in 60 mM sodium phosphate buffer, pH 7.4 at 298 K.](image-url)
3.2. Binding forces involved

Knowledge of the thermodynamic parameters (ΔH and ΔS) for ligand–protein interaction is crucial for predicting nature of the acting forces participated in the complex formation. Values of ΔH and ΔS for VDB–protein system were attained from the van’t Hoff plot (depicted as inset of Fig. 2C), while substitution of these values into Eq. (6) yielded the values of ΔG at different temperatures. Table 1 shows the values of ΔH, ΔS and ΔG for VDB–LYZ system. Sign and magnitude of the thermodynamic parameters characterize various forces believed to participate in the association process of a ligand with the protein [40]. The negative value of ΔH (−16.73 kJ mol⁻¹) for VDB–LYZ interaction characterized the binding reaction as an exothermic process and suggested participation of van der Waals forces as well as hydrogen bonds in the VDB–LYZ complexation [40]. On the other hand, the positive ΔS value (12.82 J mol⁻¹ K⁻¹) was suggestive of the contribution of hydrophobic as well as ionic forces [40]. However, ionic interactions are usually accompanied by a small value (close to zero) of ΔH [40]. Furthermore, VDB seems less likely to participate in ionic interactions in the absence of any charge in the molecule. Besides, it is not reasonable to account single intermolecular binding force for drug–protein interaction [40]. Several drug–protein interaction reports have been published with similar conclusions about the involvement of intermolecular forces based on negative ΔH and positive ΔS values [9,14,41]. Therefore, hydrophobic and van der Waals forces as well as hydrogen bonds are supposed to play major role in VDB–LYZ interaction.

Fig. 4. Three-dimensional fluorescence spectra and corresponding contour maps of LYZ (4 μM) (A and A′), [VDB]:[LYZ] (5:1) (B and B′), [VDB]:[LYZ] (10:1) (C and C′) and VDB (40 μM) (D and D′), obtained in 60 mM sodium phosphate buffer, pH 7.4 at 298 K.
complexes, obtained at 298 K, pH 7.4. (B) are also included.

3.2. Absorption spectral analysis

Absorption spectral method has been used as an additional probe to examine the ligand–protein complex formation. The absorption spectra of LYZ in the absence and with addition of increasing VDB concentrations are presented in Fig. 3. These spectra were collected after subtracting the absorption contributions of the free VDB (Supplementary Fig. 2B) from the absorption contributions of VDB–LYZ complexes (Supplementary Fig. 2A). Appearance of an absorption maximum at 281 nm (spectrum 1) was due to the presence of 6 Trp residues in LYZ [13]. Significant hyperchromic effect produced by the addition of VDB (spectra 2–11) clearly indicated changes in the microenvironment of the chromophores as a result of VDB binding to LYZ. These results further supported the complex formation between VDB and LYZ.

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3.4. Three-dimensional fluorescence spectral analysis

The 3-D fluorescence spectra are generally used to monitor microenvironmental perturbations around protein fluorophores (Trp and Tyr residues) upon addition of ligand. Fig. 4 shows the 3-D fluorescence spectra along with their contour maps of protein (LYZ) in free form (A and A’), its complexes with VDB at molar ratios of 5:1 = [VDB]:[LYZ] (B and B’) and 10:1 = [VDB]:[LYZ] (C and C’) as well as free VDB (D and D’). As shown in Fig. 4D and D’, 3-D fluorescence spectra of free VDB did not produce any fluorescence signal. Spectral properties of these spectra are summarized in Table 2. The Rayleigh scattering peak (peak ‘a’) and the second-order scattering peak (peak ‘b’) are commonly observed with the protein [42–44]. The two main peaks, namely, peak ‘1’ ($\lambda_{ex} = 280$ nm) and peak ‘2’ ($\lambda_{ex} = 230$ nm) are due to the fluorescence behavior of aromatic (Trp and Tyr) residues of LYZ. Addition of VDB to LYZ solutions ([VDB]:[LYZ] = 5:1) produced significant reduction in the intensity value and red shift in the emission maximum of both peaks ‘1’ (~28%, 6 nm) and ‘2’ (~83%, 6 nm). Increasing the concentration of VDB ([VDB]:[LYZ] = 10:1) further quenched the fluorescence intensity of both peaks, showing decrease of ~49% for peak ‘1’ and ~89% for peak ‘2’. Such changes in the fluorescence properties clearly reflected altered microenvironment around aromatic fluorophores due to VDB–LYZ complex formation.

3.5. Circular dichroism spectral analysis

The possible effect of a ligand binding on secondary and tertiary structures of the protein can be studied by monitoring the variations in the CD spectral signals in the far- and near-UV regions, respectively. The far- and near-UV CD spectra of the protein and VDB–protein mixtures ([VDB]:[LYZ] = 1:1 and 2:1) as well as free VDB are shown in Fig. 5. Free VDB did not produce any spectral signal in the far-UV and near-UV regions. The far-UV CD spectrum of free LYZ showed two negative bands at 208 and 222 nm (Fig. 5A) due to the presence of the $\alpha$-helix structure in LYZ [45]. The two main peaks, namely, peak ‘1’ ($\lambda_{ex} = 280$ nm) and peak ‘2’ ($\lambda_{ex} = 230$ nm) are due to the fluorescence behavior of aromatic (Trp and Tyr) residues of LYZ. Addition of VDB to LYZ solutions ([VDB]:[LYZ] = 5:1) produced significant reduction in the intensity value and red shift in the emission maximum of both peaks ‘1’ (~28%, 6 nm) and ‘2’ (~83%, 6 nm). Increasing the concentration of VDB ([VDB]:[LYZ] = 10:1) further quenched the fluorescence intensity of both peaks, showing decrease of ~49% for peak ‘1’ and ~89% for peak ‘2’. Such changes in the fluorescence properties clearly reflected altered microenvironment around aromatic fluorophores due to VDB–LYZ complex formation.

![Fig. 5. Far-UV (A) and near-UV (B) CD spectra of LYZ in the absence and the presence of VDB in 1:1 and 2:1 [VDB]:[LYZ] molar ratios, obtained in 60 mM sodium phosphate buffer, pH 7.4 at 298 K. LYZ concentration was 4 μM and 10 μM for the far-UV and the near-UV CD spectral measurements, respectively. Far-UV and near-UV CD spectra of free VDB (8 μM) (A) and (20 μM) (B) are also included.](image-url)
the unordered structure. Such loss in the percentage contents of secondary structures can be ascribed to the breakage of hydrogen bonds, known to stabilize various secondary structures [46]. These results clearly indicated VDB-induced changes in the secondary structures of the protein.

Similarly, near-UV CD spectrum of LYZ, characterized by the presence of two negative bands at 261 and 269 nm along with two positive signals at 283 and 289 nm (Fig. 5B) also showed changes in the magnitude of the spectral signals in the presence of VDB. These results suggested tertiary structural alteration of LYZ, which seems possible upon VDB binding.

3.6. Thermal stabilization of LYZ

Since many ligands have been shown to increase proteins’ thermal stability upon binding [9,47,48], LYZ thermal stability was checked in the presence of VDB. Fig. 6 displays temperature-induced changes in the fluorescence intensity at 342 nm (Fl342 nm) of LYZ in the absence as well as upon addition of VDB. Values of the relative Fl342 nm at different temperatures were calculated by taking the fluorescence intensity at 342 nm of LYZ or VDB–LYZ system at 25 °C as 100. Lesser loss in the fluorescence intensity was marked (Fig. 6) with addition of VDB at all temperatures compared to the decrease observed in its absence. Quantitatively, ~70% reduction in the protein’s fluorescence intensity was observed without VDB at 80 °C against ~57% decrease observed with VDB–LYZ mixture. Improvement in the fluorescence intensity of VDB–protein mixture at higher temperature suggested protection against thermal destabilization of the protein upon VDB addition. Higher temperatures might be needed to destabilize the noncovalent forces involved in the VDB–LYZ complex formation as well as maintaining the three-dimensional structure of the protein. Thermal denaturation of LYZ has been shown to start at ~60 °C [49,50]. Lesser decrease in the fluorescence intensity at 342 nm shown by VDB–LYZ mixture compared to LYZ alone in the temperature range, 45–60 °C clearly suggested VDB-induced thermal stabilization of LYZ. Even beyond 60 °C where LYZ denaturation starts, difference between the fluorescence intensity at 342 nm shown by VDB–LYZ mixture and LYZ alone was found to be more marked, suggesting VDB-induced protection of LYZ against thermal denaturation. These findings clearly revealed increased thermostability of LYZ upon VDB–LYZ interaction.

Table 3

<table>
<thead>
<tr>
<th>System</th>
<th>α-Helix (%)</th>
<th>β-Sheet (%)</th>
<th>β-Turn (%)</th>
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<td>24.0</td>
<td>20.7</td>
<td>30.1</td>
</tr>
<tr>
<td>[VDB]:[LYZ] = 1:1</td>
<td>22.5</td>
<td>23.1</td>
<td>20.2</td>
<td>32.6</td>
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<td>23.6</td>
<td>18.6</td>
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Fig. 6. Diagram showing the effect of temperature on the fluorescence intensity of LYZ (4 μM) at 342 nm in the absence and the presence of 40 μM VDB, obtained in 60 mM sodium phosphate buffer, pH 7.4 in the temperature range, 25–80 °C with 5 °C intervals.

Fig. 7. (A) Cluster analysis of the docking results of VDB–LYZ system. A total of 100 runs were performed for the binding analysis. (B) Predicted binding orientation of the lowest docking energy conformation of VDB (rendered in sticks) to LYZ. The enlarged view depicts the structural details of the interaction between VDB and LYZ, showing a hydrogen bond (red line) between Trp-63 residue (rendered in yellow stick) and VDB.
3.7. Molecular docking analysis

To predict the most favored binding mode of ligand (VDB) to its binding site on LYZ, molecular docking analysis was performed. The ligand was set to be flexible with 5 torsional degrees of freedom as a score of the rotatable bonds. VDB was allowed to explore different docked conformations by encompassing the entire LYZ in the docking grid space. The estimated free energy of binding was computed in each of the 150 runs based on enthalpic terms, which include electrostatic interactions, hydrogen bonds formation, hydrophobic interactions etc. Subsequently, binding modes were grouped according to the root mean square deviation (RMSD) with 2.0 Å cutoff, followed by ranking of clusters according to the mean free energy of binding.

The most energetically stable binding mode was selected with an assessed free binding energy of $-32.4$ kJ mol$^{-1}$. It shared a high conformational similarity with 13 other predicted modes in the cluster with the lowest mean binding energy (Fig. 7A). Notably, this ensemble of binding modes seems to occur in the active site of LYZ. The binding locus of VDB was identified to be located along with the long fissure between two domains of LYZ (Fig. 7B). Interestingly, the active site of LYZ is also presented in the deep cleft with Glu35 and Asp52 residues being the key active site residues[9]. The interacting residues of LYZ, defined within a radius of 4 Å from central position of VDB include Glu35, Asp52, Gln57, Asn59, Trp62, Trp63, Ile98, Asp101, Gly102, Asn103, Gly104, Ala107 and Val109 (Fig. 7B). Specifically, the quinazoline ring was in close proximity of three tryptophan residues (Trp62, Trp63 and Trp108), Ile98 and Val109. A H-bond was also predicted between the OH group of VDB and NH group of tryptophan side chain (Trp63). The bound VDB also showed hydrophobic interactions with several hydrophobic residues in the cleft (Fig. 7B). Therefore, molecular docking results revealed contribution of hydrophobic forces and hydrogen bonds in stabilizing the ligand–protein complex, which were also supported by the thermodynamic results as discussed in Section 3.2.

3.8. Molecular dynamics simulations

Molecular dynamics (MD) simulations of ligand–protein complex were made to study conformational stabilization of the docked (VDB–LYZ) complex. A comparison of RMSD values between the free form of LYZ (red) and the VDB–LYZ complex (green) (Fig. 8A) indicated the influence of the ligand binding on the flexibility of LYZ during the

<table>
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<tr>
<td>K$^+$</td>
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Fig. 8. Analysis of MD simulation data. (A) RMSD plots of LYZ (red), VDB (black) and VDB–LYZ complex (green) against simulation time. (B) Time evolution of the total energy of VDB–LYZ system. (C) Superimposition of the initial (black) and final (purple) structures of the VDB–LYZ complex obtained during a 10 ns MD simulation. The zoomed-in view shows the 3-D structures of the VDB at its initial and final conformations.
simulation phase. Treatment of MD data revealed that the entire energy of VDB–LYZ system was closely uniform following the minimization process (Fig. 8B). The RMSD values of the superimposed initial (t = 0 ns) as well as final (t = 10 ns) structures of the VDB–LYZ system (Fig. 8C) was 1.0 Å. Overall, molecular dynamics simulation showed that the VDB–LYZ complex was stable up to 10 ns simulation time. In accordance to an earlier report [9], we suggested that hydrogen bond and hydrophobic inter-actions are the main intermolecular forces that stabilize the VDB–LYZ complex.

3.9. Interference of metal ions in VDB–LYZ interaction

Various metal ions distributed in human plasma may interfere the ligand–protein interaction. Accordingly, the effect of $K^+$, $Mg^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$ and $Ba^{2+}$ on the binding constant of VDB–LYZ interaction was explored. Values of the $K_b$ for the ligand–protein interaction in the presence of these ions are summarized in Table 4. No significant variation in the binding constant was marked with the addition of $K^+$, $Ca^{2+}$, $Mn^{2+}$ and $Cu^{2+}$, suggesting non-interfering nature of these metal ions. On the other hand, presence of $Mg^{2+}$, $Zn^{2+}$ and $Ba^{2+}$ in the incubation mixture affected the binding constant, showing a significant decrease in the $K_b$ value. Such affects might influence the half-life of VDB in biological fluids.

4. Conclusions

Both fluorescence quenching and UV–Vis absorption spectral results confirmed the VDB–LYZ complexation. Hydrophobic and van der Waals forces as well as hydrogen bonds seem to stabilize the VDB–LYZ interaction. While binding of VDB to LYZ affected the microenvironment around protein fluorophores and produced secondary and tertiary conformational changes in LYZ, but improved protein’s thermal stability. VDB binding seems to occur near active site of LYZ, as suggested by molecular docking results.

Acknowledgements

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Appendix A. Supplementary data

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References


