Heme Protein Binding of Sulfonamide Compounds: A Correlation Study by Spectroscopic, Calorimetric, and Computational Methods
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ABSTRACT: Protein–ligand interaction studies are useful to determine the molecular mechanism of the binding phenomenon, leading to the establishment of the structure–function relationship. Here, we report the binding of well-known antibiotic sulfonamide drugs (sulfamethazine, SMZ; and sulfadiazine, SDZ) with heme protein myoglobin (Mb) using spectroscopic, calorimetric, $\zeta$ potential, and computational methods. Formation of a 1:1 complex between the ligand and Mb through well-defined equilibrium was observed. The binding constants obtained between Mb and SMZ/SDZ drugs were on the order of $10^4$ M$^{-1}$. SMZ with two additional methyl (–CH$_3$) substitutions has higher affinity than SDZ. Upon drug binding, a notable loss in the helicity (via circular dichroism) and perturbation of the three-dimensional (3D) protein structure (via infrared and synchronous fluorescence experiments) were observed. The binding also indicated the dominance of non-polyelectrolytic forces between the amino acid residues of the protein and the drugs. The ligand–protein binding distance signified high probability of energy transfer between them. Destabilization of the protein structure upon binding was evident from differential scanning calorimetry results and $\zeta$ potential analyses. Molecular docking presented the best probable binding sites of the drugs inside protein pockets. Thus, the present study explores the potential binding characteristics of two sulfonamide drugs (with different substitutions) with myoglobin, correlating the structural and energetic aspects.

INTRODUCTION
Sulfamethazine (SMZ) and sulfadiazine (SDZ) are sulfonamide antibiotics having extensive antibacterial activities. The structure of SNs contains $\text{SO}_2\text{NH}_2$ or $\text{SO}_2\text{NH}^-$ groups with distinct five- or six-membered heterocyclic rings; there are presently many derivatives under the same group of drugs, while some are newly emerging. These are used for the treatment of bacterial and fungal infections. SMZ and SDZ are also widely used in veterinary medicine to promote growth in animal feed and play major roles in livestock, aquaculture, and pharmaceutical industries. These, along with some of their metabolites, are among the most commonly used antibiotics that have been detected in wastewaters. Bacterial resistance and toxicity of these drugs can cause a genuinely negative impact on human well-being and the environment. Sulfamethazine (Figure 1A) and sulfadiazine (Figure 1B) are not well-disposed and have serious side effects that can cause prompt indisposition in humans, such as central nervous system disorders, urinary tract disorders, hemopoiesis, porphyria, and extreme touchiness responses. So, antibiotic drugs when found irregularly in the environment can be hazardous to our condition and to human health because of their toxic and cancer-causing properties. Therefore,
Myoglobin (Mb) is a cytoplasmic heme-containing oxygen-binding monomeric water-soluble protein in the muscle tissues of vertebrates responsible for the characteristic red color of the muscle tissue. Mb (Figure 1C) is a single-polypeptide globular heme protein consisting of ∼150 amino acid residues with a molecular mass of 16.5 kDa. The tertiary structure of Mb is composed of eight separate right-handed α-helices connected by short nonhelical regions.17,18 The helices provide a rigid structural framework to the heme pocket. Mb is a useful molecular biomarker for advanced assessment of ill health such as severe myocardial infarction, commonly known as a heart attack.19,20 When the muscle tissues are damaged, Mb moves to the bloodstream, which further increases the level of Mb, converting it into an undesired toxic molecule that may in turn lead to various serious cases like kidney failure.21 However, during the transient decrease in the blood O2 level, a high concentration of Mb allows organisms to hold their breath longer by supplying O2 to the organism. The main function of Mb succeeds only after receiving oxygen from red blood cells (hemoglobin (Hb)), transporting it to the mitochondria of the muscle tissues to produce energy.22,23 The previous study (hemoglobin (Hb)), transporting it to the mitochondria of the muscle tissues to produce energy.22,23 The previous study described further the binding of the drugs with heme protein Mb.24 There are several reports on the interaction study of Mb with antibiotic drugs such as ciprofloxacín, promazine, amoxicillin, aspirin, sodium penicillin, sodium cloxacillin, and sodium dicloxacillin.25−27 However, its interaction study with sulfonamide group drugs is not known yet; hence, this directed us to study the undiscovered heme protein−sulfonamide drug interaction.

Drug−protein binding interaction studies have been an important and insightful research topic in the last years in the biological, biomedical, and pharmaceutical sciences. Advance ment in the field of molecular biology and biochemistry research provides overview information on the functional and molecular effects of the individual protein.28 Protein−small molecule binding interaction studies are vital for understanding their biological processes at the molecular level affecting the absorption, disposition, cellular uptake, and activity of the drug in the cardiovascular framework.19,20 After drug−protein binding, just a couple of few unbound small molecules are able to pass through the membrane bar and be used in body metabolism. Thus, drug association with other compounds at the molecular level can determine its pharmacokinetics (absorption, distribution, metabolism, and elimination) and pharmacodynamics (effect) in the circulatory system.31 In this work, drug−protein binding of SMZ/SDZ and Mb is performed and elucidated through various biophysical tools, e.g., spectroscopic (UV−vis, fluorescence, circular dichroism (CD), and Fourier transform infrared), calorimetric (differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC)), · potential, and computational (density functional theory (DFT) and molecular docking) methods. The binding constants, stoichiometry values, and thermodynamic parameters have been elucidated in detail. Conformational changes of the 3D protein structure and probable binding sites of the ligands are also reported. Thus, the present study provides a comparative understanding of the interaction between the antibiotic drug SMZ/SDZ (which differ from each other in −CH3 substitutions) and the globular protein Mb at the molecular level. Thus, this research describes the variable binding affinity of sulfa molecules with different substitutions quantitatively. The structural effect of the organic molecule on its binding properties is hence collated.

RESULTS AND DISCUSSION

Absorption Spectral Study. The binding interaction was studied via UV−vis absorption spectroscopic experiment(s). UV−vis spectroscopy is an efficient and powerful method used for determining the complex formation in drug−protein interaction studies.32,33 The absorption spectra of Mb revealed two prominent peaks at 280 and 409 nm (Figure 2A,B). The weaker band in the near-UV zone with a maximum at 280 nm was due to the π−π* transition of the aromatic rings in the amino acid residues (for tyrosine and tryptophan (Trp)), and the latter most extreme wavelength at 409 nm was due to the heme or the porphyrin Soret band.34 The peak at 280 nm increased with increasing concentration of both drugs with a hyperchromic blue shift along the tryptophan and tyrosine residues. The increase in the peak was due to the dominant concentration of the drug (λmax of SMZ at 260 nm and λmax of SDZ at 255 nm), which is in agreement with or almost in the same range as that of the 280 nm peak arising from the aromatic rings in the amino acid residues. Upon titration, the spectra increased due to the concentration effect of the drug.

Figure 2. UV−vis absorption spectra of Mb (8 μM) treated with (A) SMZ (0−20 μM) and (B) SDZ (0−20 μM) titrated in 10 mM citrate phosphate buffer (pH 7.0).

http://pubs.acs.org/journal/acsodf
ACS Omega 2022, 7, 4932−4944
4933 https://doi.org/10.1021/acsomega.1c05554
ACS Omega 2022, 7, 4932−4944
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and shifted toward the absorbance maximum of the drug and hence toward a shorter wavelength, giving rise to a prominent blue shift. The Soret band region of Mb at 409 nm (inset peaks) showed a gradual decrease with increasing concentration of the drugs. Isosbestic points at 360 nm for SMZ—Mb and 370 nm for SDZ—Mb complexes obtained in the spectra indicate equilibrium between the free and bound drugs with Mb. The significant decrease in the Soret band region and isosbestic point in the spectra revealed binding and complex formation between the protein, Mb, and the drugs (SMZ, SDZ). Thus, the overall spectral analysis can be concluded as a state of penetration of the drugs into the protein molecule, resulting in complex formation restricting the mobility of the ligand. However, not many changes were observed in the Soret band region at 409 nm so the binding parameters were not evaluated from the UV—vis absorption experiments.

**Steady-State Fluorescence Spectral Study.** Fluorescence spectroscopy is an important and useful technique for determining drug—protein interaction and providing information about drug association to proteins. Various binding mechanism details on the binding site, binding affinity, and structural and conformational adaptations can be studied from fluorescence spectrophotometry studies. For the binding study of Mb with SMZ and SDZ, the excitation wavelength was examined at 295 nm, which selectively excites the emission maxima at 337 nm. The intrinsic fluorescence is mainly due to tryosine (Tyr103, Tyr143, Tyr151) and tryptophan (Trp7, Trp14) residues present in Mb, which are subtle for the change in its microenvironment. Both Trp and Tyr excite at 280 nm wavelength; however, among all of the fluorophore amino acid residues in the protein, Trp remains the dominant intrinsic fluorophore amino acid residues. So, to excite the Trp residue specifically, the protein was excited at 295 nm, which absorbs 5 times more than the Tyr residue at 280 nm wavelength. So, to excite the Trp residue specifically, the protein was excited at 295 nm, which absorbs 5 times more than the Tyr residue at 280 nm wavelength. The decrease in both the fluorescence intensities in the absence and presence of the quencher (SMZ or SDZ) here. The values obtained from both the quenching data were found to be 2.4 × 10^5 M^-1 for the SMZ—Mb complex and 1.9 × 10^4 M^-1 for the SDZ—Mb complex, respectively. The quenching constant for the SMZ—Mb complex was found to be comparatively higher as compared to that for the SDZ—Mb complex. To further support the fluorimetric titration data, the following calorimetric experiments have been carried out.

**pH-Dependent Fluorescence Study.** Ionicizable amino acid residues in protein play a major role in the function, stability, and solubility of a protein determined by their net charges, which influence the binding of proteins to other small molecules. The stability of a protein depending on its pH condition is controlled by a change in the ionization state of the protein residues. Therefore, the nature of interaction or binding between the ligand and the protein may depend on the pH of the medium. The influence of pH on the binding of SMZ/SDZ with Mb was investigated using steady-state fluorescence studies by exciting the protein at 295 nm in the presence of the SMZ or SDZ drug at different pH values—5.0, 6.0, 7.0, and 8.0, respectively; the pH ranges selected were slightly above and below the pl of the protein. The data obtained from Stern—Volmer plots are presented in Table 1.

**Figure 3.** Fluorescence emission spectra of Mb (8 μM) treated with (A) SMZ (0—57.6 μM; slit 10) and (B) SDZ (0—57.6 μM; slit 10). Buffer = 10 mM citrate phosphate buffer, pH 7.0.

| Drug—Protein Complex | pH | Kq × 10^4 (M^-1) | Ks × 10^4 (M^-1) | n |
|----------------------|----|-----------------|-----------------|---|
| SMZ—Mb              | 5.0| 5.1             | 7.2             | 0.98|
|                      | 6.0| 4.0             | 4.6             | 1.00|
|                      | 7.0| 3.1             | 3.0             | 1.07|
|                      | 8.0| 2.4             | 2.2             | 0.95|
| SDZ—Mb              | 5.0| 2.8             | 5.6             | 0.89|
|                      | 6.0| 2.3             | 3.3             | 1.05|
|                      | 7.0| 2.0             | 2.5             | 1.04|
|                      | 8.0| 1.5             | 1               | 0.94|

The decrease in both the Kq and Ks values with an increase in pH showed no shift in the maximum wavelength of the protein for SDZ; however, in the case of SMZ, they showed a slight red shift. The importance of pH-dependent studies lies in hydrogen ion equilibrium; the increase or decrease in pH changes the protonation state of the protein, leading to a change in the conformation of the protein, altering its pK_a value. The main factor leading to the decrease in the binding affinity with an increase in pH lies in the electrostatic interactions, which decrease with an increase in pH. The electrostatic forces acting on the binding contribute to the stability and function of proteins, which decrease with an increase in the pH and are reflected in the pK_a value of an ionizable group of amino acid residues. The higher values

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(\(K_a\) and \(K_v\)) obtained for SMZ–Mb binding than those for SDZ–Mb binding are due to the better binding interaction, which is in accordance with the other experimental data.

Salt-Dependent Fluorescence Study. The ionic strength-dependent fluorescence analysis was performed to determine the nature of molecular forces associated with the binding process (Figure S1). SMZ and SDZ drugs are uncharged molecules, while protein Mb acquires a total net negative charge on its surface varying from values near to zero, \(-0.3\) to up to \(-7.5\). The charge on the protein may vary depending on the ionic strength of the medium; the negative charge is higher when the ionic strength of the medium is low.52

From polyelectrolytic theory, counterion release is related to the slope of the best-fit line in the log\(K_a\) vs log[Na\(^+\)] plot (Figure 6A,B) by the following reaction53,54

\[
\Delta N(\text{ion}) = \left( \frac{\partial \log K_a}{\partial \log [\text{Na}^+]_{(T,P)}} \right)_{(T,P)} = -Z\Psi
\]

where \(\Delta N(\text{ion})\) is the number of ions released upon binding of SMZ/SDZ–Mb, \(Z\) is the apparent charge, and \(\Psi\) is the fraction of [Na\(^+\)] bound per Mb. This provided the estimation that there is an electrostatic force included in the binding reaction of SMZ or SDZ with Mb. The low values obtained from the slope suggested that the counterions released from the binding, which suggested weak electrostatic interaction between the protein and the drugs. From the data presented in Table 2, it was found that the apparent binding constant constant decreased with an increase in ionic strength (10, 20, and 50 mM), suggesting destabilization of SMZ–Mb and SDZ–Mb complexes with high sodium ion concentrations. Thus, the binding strength was dependent on the quantity of salt present in the mechanism. However, the number of binding sites “\(n\)” did not vary but remained close to unity in a 1:1 complexation ratio at all of the salt conditions. The \(\Delta G^0\) was partitioned between electrostatic (\(\Delta G_{pe}^0\)) and non-electrostatic (\(\Delta G_{npe}^0\)) components (Figure 4A,B). The polyelectrolytic/electrostatic (\(\Delta G_{pe}^0\)) contributions were further calculated from the following equation

\[
\Delta G_{pe}^0 = -Z\Psi RT \ln([\text{Na}^+]_{(T,P)})
\]

At 10, 20, and 50 mM salt concentrations, the polyelectrolytic (\(\Delta G_{pe}^0\)) components were found to be \(-1.30, -1.11,\) and \(-0.84\) kcal/mol for SMZ and \(-1.08, -0.92,\) and \(-0.70\) kcal/mol for SDZ, which are 21.3, 18.5, and 14.8\% and 18.5, 16.1, and 12.8\% of the total \(\Delta G^0\) for SMZ–Mb and SDZ–Mb complexes, respectively. The higher values for \(\Delta G_{pe}^0\) than \(\Delta G_{npe}^0\) show that the non-polyelectrolytic interactions like hydrophobic, van der Waals, H-bonding, etc. dominate the binding process between the amino acids of the protein and the drugs and remain almost invariant at all salt concentrations.

Fluorescence Resonance Energy Transfer (FRET) Study. The energy transfer between Mb and SMZ or SDZ was determined using the FRET technique. FRET is a physical phenomenon whereby the donor fluorophore in its excitation state emits energy nonradiatively to the acceptor molecule, causing the acceptor fluorophore to emit its characteristic fluorescence.55 Energy transfer takes place when the emission spectrum (donor) overlaps the (acceptor) absorption band (Figure 5). FRET is highly delicate to the distance of the transition dipole between the donor and acceptor molecules, which is within the 2–8 nm range. This technique is dependent on the reciprocal of the sixth power of the distance between Mb and SMZ/SDZ (\(r\)) and the Förster radius (\(R_0\)), which is the energy transfer distance at 50% efficacy. The intrinsic fluorescence of Mb is caused by two Trp residues (Trp14 and Trp7), located in the \(\alpha\)-helix A, one of which (Trp7) is exposed to the solvent, while the other (Trp14) is in the hydrophobic matrix of the protein.56–58 The interaction of Mb with SMZ/SDZ was studied by selectively quenching the intrinsic fluorescence of the protein [tryptophan (Trp) residues] at 295 nm wavelength.

The energy transfer between two molecules can be calculated from the following equation59

\[
E = 1 - \frac{F}{F_o} = \frac{R_0^6}{R_0^6 - r^6}
\]

\(R_0\) was further calculated from the following equation

\[
R_0 = 8.8 \times 10^{-25}kTn^{-4} \varphi J
\]

Table 2. Salt-Dependent Parameters Obtained for SMZ–Mb and SDZ–Mb Binding from Spectrofluorimetric Studies at 298 K

| complex   | [Na\(^+\)] (mM) | apparent binding constant (\(K_a \times 10^4\), M\(^{-1}\)) | \(n\) | \(\Delta G^0\) (kcal/mol) | \(\Delta G_{pe}^0\) (kcal/mol) | \(\Delta G_{npe}^0\) (kcal/mol) | \(Z\Psi\) |
|-----------|----------------|-----------------------------------------------------------|------|--------------------------|-------------------------------|-------------------------------|-------|
| SMZ–Mb    | 10             | 3.0                                                       | 1.07 | -6.09                    | -4.79                         | -1.30                         | -0.48 |
|           | 20             | 2.5                                                       | 0.98 | -5.99                    | -4.88                         | -1.11                         |       |
|           | 50             | 1.4                                                       | 1.24 | -5.64                    | -4.8                           | -0.84                         |       |
| SDZ–Mb    | 10             | 1.9                                                       | 1.17 | -5.83                    | -4.75                         | -1.08                         | -0.40 |
|           | 20             | 1.5                                                       | 1.30 | -5.69                    | -4.77                         | -0.92                         |       |
|           | 50             | 1.0                                                       | 1.31 | -5.45                    | -4.75                         | -0.70                         |       |

SDZ–Mb complexes, respectively. The higher values for \(\Delta G_{pe}^0\) than \(\Delta G_{npe}^0\) show that the non-polyelectrolytic interactions like hydrophobic, van der Waals, H-bonding, etc. dominate the binding process between the amino acids of the protein and the drugs and remain almost invariant at all salt concentrations.
where $k^2$ is the spatial orientation factor, $\eta$ is the quantum yield of the donor, $n$ is the refractive index of the medium, and $J$ is the overlap integral. $F(\lambda)$ is the emission intensity of the donor at wavelength $\lambda$, and $e(\lambda)$ is the absorption coefficient of the acceptor at $\lambda$. The values used in this case were $k^2 = 2/3$, $n = 1.33$, and $\eta = 0.14$ for Mb. The values of $E$, $J$, $R_0$, and $r$ for SMZ–Mb (Figure S5A) were calculated to be 0.0770, 3.4151 × 10$^{-15}$ cm$^3$ L/mol, 2.115 nm, 3.19 nm, whereas for SDZ–Mb (Figure S5B), the values obtained were 0.0425, 1.3982 × 10$^{-15}$ cm$^3$ L/mol, 1.822 nm, and 3.06 nm, respectively. The results obtained suggested that the distance $r$ between the antibiotic drug SMZ or SDZ and Mb was much less than 8 nm, signifying a high probability of energy transfer between the drug and the protein and also indicating drug residing nearby the protein sites. The relationship $0.5R_0 < r < 1.5R_0$ was obeyed in both the interaction cases, supporting well the energy transfer probability and their quenching efficiency with the drugs. The data thus obtained were in accordance with the FRET theory, confirming the transfer of energy between Mb and the drugs.

**Structural Changes from Synchronous Fluorescence.**

The change in the microenvironment of the protein structure due to the incorporation of the drug (SMZ/SDZ) was determined using synchronous fluorescence. This technique is an exceptionally helpful approach to determine the shift in the emission maximum of the protein upon adding different concentrations of the drug in relation to the change in polarity. Figure S2 shows the spectra for the scanning wavelength intervals set at 15 and 60 nm for SMZ–Mb and SDZ–Mb. The change in interval $\Delta \lambda = 15$ denotes the change in the tyrosine residue, whereas interval $\Delta \lambda = 60$ signifies the change in the tryptophan residue. For both the bindings, SMZ–Mb and SDZ–Mb, a slight red bathochromic and hypochromic shift was observed at both the wavelengths (i.e., $\Delta \lambda = 15$ and 60 nm). The gradual decrease in the quenching of spectra upon increasing concentrations of the drug with a bathochromic shift for all of the cases was due to the increase
in the polarity around the amino acid residues and the decrease in hydrophobicity. For SMZ–Mb binding, the spectra changed by red shifts of 3 nm for Δλ = 15 and 6 nm for Δλ = 60, whereas for SDZ–Mb binding, red shifts of 2 and 4 nm were observed, respectively. The greater shift in the SMZ–Mb binding may be due to better quenching affinity than that of SDZ–Mb binding as reported from the quenching experiment data.

**Circular Dichroism Spectroscopy.** Circular dichroism (CD) is a widely used method to determine the secondary structure of protein molecules. Protein structures are best determined in the far-UV region (190–250 nm) of spectra. To reveal the secondary structure of proteins, the required characteristic for the α-helix unit is the excitation-state coupling of π−π* transition, forming a perpendicular positive π−π* transition at 192 nm and also leading to negative transitions of π−π* and n−π* at 208 and 222 nm parallel to each other, respectively. However, the complication in determining the quantitative estimation of β-sheet is due to the massive design and spectral variations of β-sheet structures.

To understand the effect of antibiotics (SMZ/SDZ) on Mb, CD analysis of the far-UV region was carried out. The structural information associated with binding can be demonstrated by circular dichroism. Figure 6A,B shows the spectra of SMZ–Mb and SDZ–Mb complexes, respectively. A negative π−π* transition at 212 nm parallel to n−π* transition at 222 nm signifies the α-helical structure of Mb. Upon binding with SMZ and SDZ, the intensity decreased with increasing concentrations with no shift in the peak, signifying loss in α-helicity and suggesting changes in the secondary structure of the protein (Mb). However, the antibiotics are CD-inactive and do not exhibit any spectra. The α-helix contents of free and SMZ/SDZ-bound Mb were calculated in terms of the molar residual ellipticity (MRE) values at 222 nm using the following equation.

\[
\text{MRE} = \frac{\theta}{10 \times n \cdot l \cdot C_p}
\]  

(7)

where \(\theta\) is the observed CD expressed in millidegrees, \(n\) is the number of Mb amino acid residues (154), \(l\) is the path length of the cell (1 cm), and \(C_p\) is the protein concentration.

The α-helical structure content of the heme protein (Mb) from free to bound complex (SMZ–Mb and SDZ–Mb) at 222 nm was calculated quantitatively from the following equation.

\[
\% \alpha - \text{helix} = \frac{-\text{MRE}_{222 \text{ nm}} - 4000}{33000 - 4000} \times 100
\]  

(8)

Mb consists of approximately 70% α-helix and the rest of the chain forms turns between helices devoid of symmetry. However, three different physiological conformations of myoglobin, namely, deoxymyoglobin, oxymyoglobin, and metmyoglobin (ferrioxymyoglobin) can give different percentages of 55–80% under neutral and mildly acidic conditions.

The secondary structure (α-helix) content of Mb was found to be 66.66% at 222 nm. However, after the saturation point of the protein treated with 26 μM SMZ/SDZ, the loss of α-helicity for the bound SMZ–Mb complex reduced to 19.53% at 222 nm. However, for the bound SDZ–Mb complex, it decreased to 4.44% at 222 nm. Thus, the reduced percentage for both the complexes denotes unfolding and loss of the secondary structure of the protein molecule. The greater loss of the α-helix structure in the case of the SMZ–Mb complex as compared to that of the SDZ–Mb complex may be due to the better binding affinity for SMZ of Mb.

The change in the Soret CD band region of Mb was analyzed by taking the spectra ranging from 350 to 500 nm. This region was studied to measure the change in the heme group present in the protein and the aromatic side chains with the drugs. The spectra presented in Figure 6C,D showed a positive peak at around 400 nm, which arises due to the π−π* transition of the porphyrin ring and those of the aromatic side chains. Upon binding with the drugs at different concentrations, there was a gradual decrease in the peak signifying the change in the planarity of the porphyrin ring, which shifted toward a longer wavelength (red shift) for the SMZ–Mb complex and toward a shorter wavelength (blue shift) for the SDZ–Mb complex. This indicates changes in the microenvironment of the porphyrin region of the protein; the red shift may be due to the folding/aggregation of the peptide membranes of the protein upon binding with the SMZ drug, while the blue shift suggests changes in the steric pattern or denaturation of Mb with heme being more exposed to the solvent than in the native structure.

**IR Spectroscopy Study.** Infrared spectroscopy studies the measurement of vibrations of the atom. The vibrational transition in the molecule is caused by the absorption of infrared radiation. This technique is often used to analyze the binding of various secondary protein structures. The IR spectra of the unbound protein Mb and its complexes (SMZ–Mb/SDZ–Mb) with the drugs are shown in Figure S3.

The protein molecule showed a sharp peak at 1638 cm\(^{-1}\), which represents the amide I region due to the C=O stretching coupled with C–N stretching and N–H bending. However, upon binding with the drugs, the amide I peak shifted to 1632 cm\(^{-1}\) for SDZ and 1629 cm\(^{-1}\) for SMZ, which may be due to the interaction between the drug and the amino acid residues. The decrease in the amide I (α-helix) region of the protein suggested a decrease in the α-helical structure, which is in accordance with the CD spectral results.

The protein molecule also showed a peak at 534 cm\(^{-1}\) (fingerprint region) and a broad peak at 3346 cm\(^{-1}\), which after forming a complex with SMZ/SDZ shifted to 548 and 3291 cm\(^{-1}\) for SDZ–Mb and 556 and 3270 cm\(^{-1}\) for SMZ–Mb, respectively. The broad peak at 3346 cm\(^{-1}\) is due to the NH stretching vibration of the amide group called amide A. The shift in the peak after drug binding may signify the possibility of hydrogen bonding between the amide group of the protein and the drugs. Amide vibrations are the backbone of the secondary structure of the protein, which is commonly used for structural analysis. These shifting of amide vibration peaks are an indication of structural perturbation of the protein due to binding and the interaction pattern between the protein and the drug molecules. This IR result is in accordance with the synchronous and CD data related to structural perturbation of the protein structure upon drug binding.

**Differential Scanning Calorimetric (DSC) Study.** DSC is a helpful and essential analytical technique for bio-macromolecules, such as proteins, to determine the thermodynamic parameters of heat-induced transitions. The phase transition energetics, structural changes, and quantification of temperature dependence are mainly determined by a DSC instrument. DSC records the changes in the heat capacity of bio-macromolecules, generally for the protein denaturation process after the protein–ligand complex formation. The effect of SMZ/SDZ on the thermal stability of Mb was
monitored using differential scanning calorimetric studies (Figure 7). DSC analysis was carried out to understand the structural stability of the protein upon binding with the drugs. Mb underwent melting under the experimental condition and was denatured with a single endothermic peak at 356.5 K. The thermal melting temperature (ΔT) of Mb decreased slightly upon binding with the antibiotic drugs. The decrease in the melting temperature was higher for the SMZ–Mb complex by 2.26 K than 1.04 K for the SDZ–Mb complex, which resulted in destabilization of the protein molecule upon binding. The destabilized state of the protein may be due to the unfolding or denaturation of the protein upon binding. The unfolding of the protein associated with the heat capacity variation is due to the changes in the hydration of side chains exposed to the solvent. The factors accountable for the stability and folding of the protein includes amino acid sequence, hydrogen bonding, hydrophobic interactions, and conformational entropy. Accordingly, the lower the Tm, the lower the thermodynamic stability of the complex and the more the susceptibility to unfolding and denaturation of the protein. The better effect (decrease) of SMZ drug with Mb than SDZ drug is in accordance with the results provided by the other characterization reports.

**Isothermal Titration Calorimetric (ITC) Study.** Isothermal titration calorimetry (ITC) is the most important tool to determine and characterize the interaction of small molecules to proteins. A single titration from this technique determines the overall thermodynamic properties, which provide the binding affinity of the interaction and the stoichiometry of the complex. Figure 8 represents the ITC thermogram that is characterized to be exothermic for both cases, showing negative enthalpy changes (ΔH = −24.17 kJ/mol for SMZ and −13.75 kJ/mol for SDZ) and positive entropy contributions (TΔS = 13.112 kJ/mol for SMZ and 1.54 kJ/mol for SDZ), indicating hydrogen-bonding, electrostatic, and hydrophobic interactions. The observed entropy contribution, particularly with SMZ, suggests significant conformational changes in the protein, which may reflect well from CD analysis. The Gibb's free energy changes were determined to be −37.28 kJ/mol (SMZ–Mb) and −15.29 kJ/mol (SDZ–Mb), signifying spontaneity of the reaction. The stoichiometry (N) values of binding for both cases were found to be 1.01 (SMZ–Mb) and 0.97 (SDZ–Mb), indicating a 1:1 binding ratio of the complex. The association constant or binding constant, also known as equilibrium constant, which is the measure of binding affinity between the protein and the drug molecules at equilibrium, was measured. It is associated with the binding and unbinding reactions of the protein (P) and drug (D) molecules, P + D ⇌ PD. The values obtained for binding affinity (K) between the protein (Mb) and the drugs (SMZ and SDZ) from the experiment at 298 K are 5.36 × 10^4 M^−1 for the SMZ–Mb complex and 3.23 × 10^5 M^−1 for the SDZ–Mb complex, slightly higher for SMZ than that for SDZ, in accordance with the spectroscopic data. This confirms the stronger structural perturbation of the protein structure with SMZ upon binding as compared to that with SDZ and their effect on the stability of the complexes as reported from the above studies.

**ζ Potential Study.** The surface charge of proteins depends on their environmental condition. Partial ionization of various amino acids contributes to the surface charge of protein molecules. The study of charge distribution by the ζ potential method provides knowledge about the overall surface charge information of the protein particle and changes in the surface potential of the complex after adding the drug. The myoglobin molecule had a negative ζ potential charge of −5.0 mV; however, after addition of the antibiotic drug molecules, the net negative charge changed to −3.3 mV for the SMZ–Mb complex and −3.6 mV for the SDZ–Mb complex (Figure 9). The low charge obtained for the protein molecule may be because of the existence of the proteins in their aggregation form. However, after binding, there is coagulation of the drug and the protein, and the changes in charge signify the interaction between them. These results suggest structural modifications along with alteration of the ζ potential value after the complex formation, which are in agreement with the CD and IR spectroscopy results.

**Computational Methods. DFT Highest Occupied Molecular Orbital—Lowest Unoccupied Molecular Orbital (HOMO—LUMO) Study.** DFT is a computational quantum-mechanical modeling method useful for determining electronic structure, electronic transition, etc. The HOMO–LUMO energy gap signifies the compound stability index and its chemical activities and bioactive properties. The higher energy value of HOMO (SMZ = −6.179 eV, SDZ = −6.21018 eV) is associated with the electron-donating ability and is related to the ionization potential of the molecule, whereas the lower LUMO (SMZ = −1.193 eV, SDZ = −1.147 eV) energy is directly related to the electron affinity and its ability to accept electrons. Figure 10 shows the electronic excitation of the optimized ground and excited states of the antibiotic drugs, SMZ and SDZ. SMZ and SDZ show similar kinds (values) of HOMO–LUMO energy gaps, slightly lower for SMZ (ΔE = 4.98 eV) than that for SDZ (ΔE = 5.06 eV). The lower energy gap for SMZ denotes better stability of the molecule with better bioactive properties. Thus, we can conclude that SMZ is a better antibiotic drug than SDZ. However, the energy gap between HOMO and LUMO for both the drugs (SMZ and SDZ) indicates that they are capable of binding to biomacromolecules. These results support the above experimental data (spectroscopy, calorimetry, etc.), wherein successful protein–ligand interactions have been elucidated.

**Molecular Docking Study.** Molecular docking studies are usually performed to study the preferred orientation of the bound drug(s) and the protein. The model protein of the current report, myoglobin, is a single-polypeptide globular protein consisting of eight α-helices (subunits labeled as A–H). These subunits are connected through turns with an
oxygen-binding heme group at the centre and a hydrophobic core.98,99 Figures 11 and 12 (Figures S4–S9) show the possible docking results with drug–protein complex structures corresponding to SMZ–Mb and SDZ–Mb systems. Different types of docking software are used to obtain the optimal result. Moreover, various possibilities of docking sites are also discussed, and finally, the results are matched with the data obtained from different biophysical experiments.

Figure 8. ITC profiles for the binding of (A) SMZ–Mb and (B) SDZ–Mb complexes. The panel shows the rectified integrated heat result against the molar ratio of SMZ–Mb and SDZ–Mb complexes. The solid lines represent the best-fit data to the “one set of binding sites” model.

Figure 9. ζ potential curves for (A) myoglobin, (B) SMZ–Mb complex, and (C) SDZ–Mb complex.
Results from “Swissdock” on SMZ-Hb and SDZ-Mb Complexes. The docking exercises performed at the Swissdock web server for SMZ-Mb binding resulted in 32 ligand binding sites, out of which only five sites were found with higher densities of ligand occupancy or clustering in comparison to the remaining surface area (Figure 11). Among these sites, top three scoring ligands (according to free energy scores obtained from Swissdock) were prepared for the AutoDock (version 1.5.6) ligand docking platform along with the protocol of the flexible ligand and rigid receptor (here protein) docking approach.

Likewise, for SDZ, 32 top docking clusters were predicted by Swissdock software; however, only four sites on the surface showed high-density ligand clustering (Figure 12). Among these sites, top three scoring ligands from three different sites were prepared for AutoDock analyses based on the flexible ligand and rigid receptor docking protocol and are presented below.

Autodock Results on SMZ-Mb and SDZ-Mb Complexes. First among Three Top Surface Sites/Spots. The top scoring binding sites showed that the antibiotic drug binds to the pocket near the porphyrin ring where the main function of myoglobin takes place, forming complexes with the protein. This docking position holds the potential of interaction directly with the ring and additionally with the three helices of myoglobin, namely, H, E, and F, which may influence the stability of the myoglobin active site. Figures S4 and S5 show the SMZ-Mb/SDZ-Mb complexes, where both SMZ and SDZ form hydrogen bonds with Ala71(A) and hydrophobic interactions with Pro88(A), Leu89(A), Hem154(B), Leu86(A), Ile75(A), and Glu85(A), as reported from the LIGPLOT analyses. The estimated free energy of binding was found to be $-7.44$ kcal/mol for the SMZ-Mb complex and $-6.99$ kcal/mol for the SDZ-Mb complex, slightly higher for SMZ (more details on this solution are in Table S1).

Second among Three Top Surface Sites/Spots. Figure S6 shows the docking solution of SMZ with Mb, binding to the C-terminal end of the Mb protein chain with interaction status on helices G, H, and F, respectively. SMZ also sits on the spot wherein the joining loop of helices F and G directly interacts, forming hydrogen bonding with Ala143(A) and Tyr151(A) residues. Therefore, as an effect of this binding, disruption of the joining region between G, H, and F helices can be seen and a partial disorder of the conventional myoglobin interaction occurs. The estimated free energy of binding was found to be $-7.48$ kcal/mol for the SMZ-Mb complex (more details on this solution are in Table S1).

However, for SDZ, Figure S7 shows the binding to the N-terminal end of the Mb protein chain with interacting distances on specific helices A and H; SDZ also sits on the spot wherein the joining loop of helices F and G directly interacts and is directly interacting with it, forming hydrogen bonding with Trp7(A), Leu2(A), and Glu83(A) residues. Therefore, as an effect of this binding, a disruption of the joining region between A, H, E, and F helices is seen and a partial disorder of the conventional myoglobin interaction occurs. The estimated free energy of binding was found to be little less as compared to that for SMZ-Mb, $-7.27$ kcal/mol for the SDZ-Mb complex (more details on this solution are in Table S1).

Third among Three Top Surface Sites/Spots. In this solution, it is found that SMZ/SDZ binds to the pocket made of three helices: G, B, and D (Figures S8 and S9). Though D is a bit distant for influencing the active site, G and B helices take a direct part in active site construction and interact with the ring. Therefore, binding to this pocket can directly affect ring binding and the three-dimensional conformation of the active site, which may lead to the impaired activity of myoglobin. The
estimated free energy of binding is $-7.42 \text{ kcal/mol}$ for SMZ–Mb forming hydrogen bonding with Ser35(A), Glu52(A), Arg31(A), and Lys56(A) and $-6.77 \text{ kcal/mol}$ for the SDZ–Mb complex forming hydrogen bonding only with Arg31(A) residues (more details on this solution are in Table S1).

Hence, molecular docking can complement the experimental results via estimation of possible binding location(s) of the ligand inside the protein moiety. Thus, the present research portrays the binding characteristics of various sulfa antibiotic drugs with the heme protein, myoglobin, correlating the structural aspects and energetics. The role of substitutions in the aromatic organic molecules with potential capacity (toward bio-macromolecules, here proteins) has been discussed quantitatively using biophysical and computational methods.

## CONCLUSIONS

The interaction study of sulfonamide drug derivatives SMZ and SDZ with heme protein Mb was carried out using various biophysical and computational techniques. This work presented a comparison between two comparable structured sulfonamide drugs that differ in the structure of the methyl group and delivered the idea of how the presence of two extra methyl groups in the structure changes the whole structural–functional relationship between the protein and the drug. The difference in the structure led to fascinating outcomes upon several spectrophotometric and computational analyses. The study concluded that SMZ has a slightly higher binding affinity than SDZ with Mb. The binding of both drugs induced strong conformational changes in the protein structure, resulting in destabilization of the protein structure. Thermodynamic parameters revealed negative enthalpy and positive entropy changes, contributing to spontaneous binding Gibb’s free energy for both complexes. The data obtained from several experimental analyses were well supported and confirmed by the computational (DFT, molecular docking) method. The research work provides a quantitative method to determine the protein damage and a satisfactory understanding of the interactions between the compared sulfonamide drugs and Mb, which may be helpful for future investigations of antibiotic drugs in the pharmacological field.

## MATERIALS AND METHODS

### Materials

High-purity standard myoglobin (95–100%), sulfamethazine (SMZ), and sulfadiazine (SDZ) were procured from Sigma-Aldrich Corporation. All of the chemicals and reagents used in this study were analytical grade and purchased from Sigma-Aldrich. Double distilled and deionized (Milli-pore) water was used for the preparation of buffer for the experiment.

### Methods

UV–vis absorbance spectra were recorded on an Agilent, Cary 100 series UV–vis spectrophotometer. Fluorescence quenching studies in the steady state, salt and pH dependence, synchronous fluorescence spectroscopy, and fluorescence resonance energy transfer analysis were performed on an Agilent Cary eclipse spectrophluorophotometer at 25 ± 0.5 °C. An Agilent Cary 630 Fourier transform infrared spectrometer was used to measure the vibration of Fourier transform infrared spectra of Mb upon binding with SMZ and SDZ. The changes in the conformation of the protein molecule upon interaction with SMZ and SDZ drugs were analyzed using a Jasco J815 spectropolarimeter (Jasco International Co., Ltd.) equipped with a temperature controller PFD 425 L/15 and a Peltier cell holder. The stabilization or destabilization of the protein Mb upon interaction with SMZ and SDZ drugs was monitored using a MicroCal VP-differential scanning calorimeter (DSC) (MicroCal, Inc., Northampton, MA). ITC experiment results were obtained using a MicroCal VP-ITC unit, and the solutions were degassed on MicroCal’s Thermovac unit. The charge distribution of the protein (Mb) with and without the drugs, SMZ/SDZ, was analyzed using a Horiba Particle Zetasizer SZ-100 instrument. The Gaussian 09W program package was used for the DFT calculations utilizing the hybrid method B3LYP function and keeping the basis sets as 6-31G(d,p). Docking analyses for the interaction of myoglobin with antibiotic drugs SMZ and SDZ were performed using Autodock 1.5.6 software and viewed in PyMOL, LIGPLOT, and chimera for better visualization. The structure of myoglobin was taken from the RCSB Protein Data Bank under PDB ID: 1A6N.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05554.

Materials and methods and final genetic algorithm docked state for the three top surface sites/spots (PDF)

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https://doi.org/10.1021/acsomega.1c05554

ACS Omega 2022, 7, 4932–4944
Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.1c05554

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

J.B. is thankful to the Twinning Project of the DBT, Govt. of India (BT/PR25026/NER/95/963/2017). Financial assistance from UGC-DAE, Mumbai Center, Bhabha Atomic Research Center (BARC), Mumbai (Research scheme, CSR-t) and Antimicrobial Evaluation of a New Series of N-Sulfonamide 2-Pyridones as Dual Inhibitors of DHPS and DHFR Enzymes.

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