Acetaminophen interacts with human hemoglobin: optical, physical and molecular modeling studies

Paromita Seal, Jyotirmoy Sikdar, Amartya Roy and Rajen Haldar*

Department of Physiology, University Colleges of Science and Technology, University of Calcutta, 92, A.P.C. Road, Kolkata 700 009, India

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Acetaminophen, a widely used analgesic and antipyretic drug has ample affinity to bind globular proteins. Here, we have illustrated a substantive study pertaining to the interaction of acetaminophen with human hemoglobin (HHb). Different spectroscopic (absorption, fluorescence, and circular dichroism (CD) spectroscopy), calorimetric, and molecular docking techniques have been employed in this study. Acetaminophen-induced graded alterations in absorbance and fluorescence of HHb confirm their interaction. Analysis of fluorescence quenching at different temperature and data obtained from isothermal titration calorimetry indicate that the interaction is static and the HHb has one binding site for the drug. The negative values of Gibbs energy change (ΔG°) and enthalpy changes (ΔH°) and positive value of entropy change (ΔS°) strongly suggest that it is entropy-driven spontaneous and exothermic reaction. The reaction involves hydrophobic pocket of the protein which is further stabilized by hydrogen bonding as evidenced from ANS and sucrose binding studies. These findings were also supported by molecular docking simulation study using AutoDock 4.2. The interaction influences structural integrity as well as functional properties of HHb as evidenced by CD spectroscopy, increased rate of co-oxidation and decreased esterase activity of HHb. So, from these findings, we may conclude that acetaminophen interacts with HHb through hydrophobic and hydrogen bonding, and the interaction perturbs the structural and functional properties of HHb.

Keywords: acetaminophen; human hemoglobin; spectroscopy; isothermal titration calorimetry; molecular modeling

1. Introduction

Drug–protein interactions play a pivotal role in the absorption, distribution, bioavailability, and free concentration of various drugs which are strongly influenced by the binding of different biological targets including hormone, peptide, and several protein molecules (Bao, Zhu, Li, & Chen, 2001). The effectiveness of drugs in the human body is measured by its pharmacokinetic and pharmacodynamics. Both of these parameters are affected by drug–protein interactions in the blood stream (Jasinska, Ferguson, Mohamed, & Szreder, 2009; Yasmeen Khan & Suresh Kumar, 2015; Basu & Suresh Kumar, 2015a). Therefore, the study of drug–protein interaction is one of the very useful tools in the field of medical sciences which can provide information about the nature of interaction and also the drug-induced probable structural as well as functional modifications of protein.

Acetaminophen (N-acetyl-p-aminophenol), commonly known as paracetamol is a most preferred analgesic–antipyretic drug and widely used as medicine for the treatment of pain and fever (Flower & Vane, 1972; Goyal & Singh, 2006). Acetaminophen consists of a benzene ring core with one hydroxyl group and an amide group in para position (Figure 1). The benzene ring is highly reactive towards electrophilic attack due to the presence of two activating groups (Bales, Nicholson, & Sadler, 1985). Furthermore, on the physiological aspect, it is considered safe for human use at a recommended dose of 4000 mg/day (FDA) (Dal Pan, 2013). It has been observed in some companion animals that acute acetaminophen overdose can cause fatal liver damage along with serious health problems, like tachycardia, methemoglobinemia, Heinz body formation, red cell destruction, and several other oxidative injuries (Harvey, French, & Senior, 1986; MacNaughton, 2003). Earlier studies have reported that acetaminophen upon binding with human serum albumin can incur conformational modifications of the latter (Daneshgara et al., 2009).

Human hemoglobin (HHb) is another important tetrameric globular protein component of red blood cells; and it is also considered as a model protein for gaining general fundamental insights into drug–protein interaction (Perutz, 1970). It comprises two identical dimmers and each dimer consists of two α chains (each of 141 amino acids) and two β chains (each of 146 amino acids)
endogenous and exogenous components, such as flavonoids (Yuan, Liu, Kang, Lu, & Zou, 2008), alkaloids (Das & Suresh Kumar, 2016; Hazra, Hossain, & Suresh Kumar, 2013; Hazra & Suresh Kumar, 2014), herbicides (Wang et al., 2007), and different food colorants (Basu & Suresh Kumar, 2014, 2015b, 2015c). Besides these, the hemoglobin is also found to interact with a variety of anticancer compounds, such as oxali-palladium (Abbasi-Tejarag, Divsalar, Saboury, Ghalandari, & Ghourchian, 2015), Pt(II) complex (Abazari et al., 2015), etc., anion exchange inhibitor, e.g. DIDS & antiseptic drug, e.g. DNP (Rashidipour, Naeeminejad, & Chamani, 2016), and even with falcipain-2, a member of major hemogloaminase of hemoglobin intoxication (Omotuyi & Hamada, 2015). However, in most cases, interaction of such molecules with hemoglobin renders the structural integrity of the later. Recently, we have shown that structural modification of HHb leads to increase the rate of its auto-oxidation i.e. formation of methemoglobin, which is again considered as an incidence of generating oxidative stress in its vicinity (Roy, Sikdar, Seal, & Haldar, 2015). On the other hand, the association between methemoglobinemia and acetaminophen overdose in animal model (as mentioned earlier) indicates the existence of direct or indirect interaction of hemoglobin with acetaminophen. However, the precise interaction of the protein and acetaminophen is not clearly known in such models, and notably nothing is known for HHb. Furthermore, under normal condition, the HHb is mostly encapsulated within the erythrocyte, and a small amount of the HHb (cell free) is readily available in the plasma; and the concentration of the cell-free HHb further increases under different pathological conditions, such as anaemia, malaria, thalassemia, etc. Therefore, this cell-free HHb is highly susceptible to interact with a number of compounds in blood plasma. Hence, the consumption of acetaminophen under different pathologic conditions may increase the risk of interaction of HHb with the former. Thus, we have undertaken a detailed study of the interaction of acetaminophen with HHb and its post-binding modifications by employing different optical, physical, and molecular modeling techniques.

2. Materials and methods

2.1. Materials and preparation of solution

Acetaminophen, Sephadex G-100, ANS (8-anilino-1-napthalenesulphonic acid) was purchased from Sigma–Aldrich and all other chemicals of analytical grade were purchased from local vendors.

The stock solution of acetaminophen (6.6 × 10⁻⁲ M) was prepared in 0.01 M phosphate buffer (PB), pH 7.4. All the measurements were repeated thrice.

2.2. Subject selection

Human healthy participants having age range between 20 and 30 years were selected for this study. All subjects were free from smoking habit, chronic diseases and use of any tranquillizers, drugs, and anesthetics. All the volunteers were well informed about the experimentation and their written consent was obtained. This study was approved by Institutional Ethical Committee (IHEC/PHY/CU/H-P39/14), University of Calcutta.

2.3. Hemoglobin purification

Venous blood was drawn by venipuncture and kept in a tube containing heparin as an anticoagulant. The freshly donated blood was washed three times in phosphate buffered saline to remove the buffy coat and plasma. The whole erythrocytes were then lysed using 0.01 M PB, pH 7.4. After hemolysis, the samples were centrifuged at 14,000 rpm for 25–30 min at 4 °C (Bhattacharyya, Bhattacharyya, Chakraborti, & Poddar, 1998). The supernatant was collected and purified using size-exclusion chromatography through Sephadex G-100 column (12 × 1.5 cm), pre-equilibrated with 0.01 M PB, pH 7.4. The concentration of purified HHb was determined from its Soret absorbance at 415 nm (ε₄₅₁₅ = 125 mM⁻¹ cm⁻¹) (Gebicka & Banasiak, 2009). The purity of HHb was confirmed using polyacrylamide gel electrophoresis.

2.4. UV–vis absorption spectroscopy

UV–vis spectrophotometric studies have been adopted in dual beam spectrophotometer (Model: UV-1800, Make: Shimadzu, Kyoto, Japan). For this experiment, 5 × 10⁻⁶ M purified HHb was incubated with varying concentrations of acetaminophen. The UV–vis spectrum of HHb in the presence of acetaminophen was recorded from 250 to 650 nm, with 1 nm bandwidth. Different concentrations of acetaminophen were prepared separately by appropriate dilution of stock solution in 0.01 M PB, pH 7.4.

2.5. Fluorescence spectroscopy

Fluorescence spectra of HHb in the presence of acetaminophen were recorded using Hitachi F 7000.
spectrofluorometer (Hitachi High Technologies America Inc., USA) equipped with a 1.0 cm optical path length and a constant Peltier thermostat bath for temperature regulation. The excitation wavelength was 295 nm, and the emission spectrum was recorded from 300 to 500 nm, where excitation and emission bandwidths were 5 nm and 10 nm, respectively. To quantify the binding parameters, HHb concentration was kept at $5 \times 10^{-6}$ M and a series of acetaminophen concentrations were prepared by diluting its stock solution ($6.6 \times 10^{-2}$ M) in 0.01 M PB, pH 7.4. All experiments were run at three different temperatures (298.15, 303.15, and 310.15 K). The binding constants, number of binding sites, and thermodynamic parameters were determined using the equations and their corresponding plots (described in results and discussion section).

2.6. Isothermal titration calorimetry study

Experiment on binding interaction of the acetaminophen with HHb was also done in MicroCal ITC 200 for isothermal titration calorimetry. The sample cell was loaded with $2 \times 10^{-5}$ M protein solution dissolved in 0.01 M PB. The titrations were carried out using a 40 μl autopipet, filled with the drug solution ($5 \times 10^{-3}$ M acetaminophen) keeping the stirring speed fixed at 300 rpm. Each experiment consisted of 3 μL consecutive injections of the drug solution at each 2-min interval. For all the experiments, the heat of the binding reaction between the drug and the protein was obtained after the corresponding buffer subtractions. The data were analyzed using the Origin 7.0 software provided by MicroCal ITC 200. The plot of change in enthalpy against temperature was used to calculate the value of change in heat capacity upon binding.

2.7. Surface hydrophobicity

The surface hydrophobicity of HHb upon interaction with acetaminophen was studied using external fluorophore, ANS. The solution of $5 \times 10^{-6}$ M HHb and $1 \times 10^{-5}$ M acetaminophen in 0.01 M PB (pH 6.0) was incubated with five different concentrations of ANS for 60 min at 298.15 K and the fluorescence emission from 400 to 550 nm was recorded with excitation at 350 nm. The excitation and emission bandwidths were set at 5 and 10 nm, respectively. The different concentrations of ANS ($0–1 \times 10^{-4}$ M) were obtained from diluting its $3 \times 10^{-2}$ M stock.

2.8. Sucrose binding assay

The hydrogen bonding between acetaminophen and HHb was determined spectrofluorometrically. The concentrations of sucrose were $0–1 \times 10^{-3}$ M. The solution of $5 \times 10^{-6}$ M HHb in the absence and presence of $1 \times 10^{-7}$ M acetaminophen was titrated with gradual addition of sucrose, dissolved in 0.01 M PB, pH 7.4 at 298.15 K. The fluorescence emission spectra were recorded from 300 to 500 nm with excitation at 295 nm.

2.9. Molecular modeling study

To predict the binding site and the nature of interaction of acetaminophen with HHb, the complementary application of molecule docking using AutoDock 4.2 software, based on Lamarckian Genetic Algorithm, was employed (Forli & Olson, 2012; Solis & Wets, 1981). The structure of HHb was obtained from protein data bank (PDB) for molecular docking study (Shaanan, 1983). After selecting the macromolecule, all water molecules from HHb structure were removed and only polar hydrogens and Kollman partial charges were added to it. The small ligand, acetaminophen was also chosen from PDB [http://www.rcsb.org/pdb; entry code: 1TYL]. Then in the AutoDock tools package Gasteiger partial charges were added (Gasteiger & Marsili, 1980) and the torsion angle was determined through torsion root and finally rotatable bonds were assigned. For the potential binding site determination, the grid maps were selected as $104 \times 104 \times 104$ with a grid spacing of 0.375 Å (roughly a quarter of the length of a carbon–carbon single bond). After setting all parameters, 1000 independent runs were performed for docking. Distance calculations, images of structures were generated using PyMol [DeLano Scientific LLC, Palo Alto, CA, USA].

2.10. Synchronous fluorescence spectroscopy

Synchronous fluorescence measurements were carried out in order to get the information on the molecular microenvironment in the vicinity of the intrinsic fluorophore, tryptophan (Trp) and tyrosine (Tyr) in the protein molecule (Lloyd, 1971; Lu et al., 2007). Three ml of the reaction mixture contained $5 \times 10^{-6}$ M HHb and different concentrations of acetaminophen in 0.01 M PB, pH 7.4. Synchronous fluorescence spectra were recorded by simultaneous scanning the excitation and emission monochromator with wavelength difference (between excitation and emission wavelengths) of 15 nm ($\Delta$15) for monitoring tyrosine and 60 nm ($\Delta$60) for monitoring the tryptophan microenvironments of HHb (Ding, Liu, Liu, Li, & Sun, 2009).

2.11. Circular dichroism

CD spectra of HHb and their drug complexes were recorded in Jasco-815 CD spectrometer in a 0.1 cm pathlength quartz cell. Protein concentration was kept at $1 \times 10^{-7}$ M in 0.01 M sodium phosphate buffer, pH 7.4
at 298.15 K and the drug concentrations were 0–1.5 × 10^{-5} M. The CD spectra were recorded from 200 to 250 nm with a response time of 0.5 s. Each spectrum was the average of three scans and corrected by phosphate buffer background, 0.01 M, pH 7.4.

2.12. HHb co-oxidation study

HHb oxidizes spontaneously at a slower rate (auto-oxidation); but in the presence of oxidizing agent (co-oxidation), viz., nitrobluetetrazolium (NBT), the rate of oxidation increases (Wallace, Maxwell, & Caughey, 1974). Here we studied the co-oxidation process of HHb in the absence and presence of acetaminophen. Briefly, 4 × 10^{-5} M purified HHb in 0.01 M PB, pH 7.4 was titrated with two concentrations of acetaminophen (1 × 10^{-5} and 2 × 10^{-5} M). The reaction was initiated by adding 1 × 10^{-4} M NBT, and monitored spectrophotometrically at 630 nm. The formation of met hemoglobin was measured using molar extinction co-efficient, 3.7 mM^{-1} cm^{-1} at 630 nm (Elmer, Harris, Sun, & Palmer, 2009).

2.13. Esterase activity study

Esterase activity of purified HHb in presence of different concentrations of acetaminophen was assayed through para-nitro phenyl acetate (p-NPA) hydrolysis. For this experiment, HHb concentration was kept at 5 × 10^{-6} M and acetaminophen concentrations used were 1 × 10^{-5} M and 2 × 10^{-5} M in 0.01 M PB, pH 7.4 at 298.15 K. In order to find out the kinetics of p-NPA hydrolysis by HHb, wide ranges of working solutions (1 × 10^{-4}, 2.5 × 10^{-4}, 5 × 10^{-4}, 7.5 × 10^{-4} and 1 × 10^{-3} M) of p-NPA were used. The assay was conducted in Shimadzu dual beam 1800 spectrophotometer. After addition of p-NPA immediately the solution was placed in the spectrophotometer to determine the rate of p-nitro phenol generation using molar extinction co-efficient 11.6 mM^{-1} cm^{-1} at 400 nm (Elbum & Nagel, 1981). The values of Michaelis Constant, K_m and maximum velocity, V_max were determined using Lineweaver–burk plot.

3. Results and discussion

3.1. Study of interaction of HHb with acetaminophen

3.1.1. Spectrophotometric analysis

UV–visible absorption spectroscopy was used to study the interaction between HHb and acetaminophen. Figure 2 represents the UV–visible absorption spectra of HHb in the absence and presence of acetaminophen. The absorption maximum of Soret band was found to decrease with gradual addition of acetaminophen, while the absorbances around 250–300 nm were increased. The latter is due to the summation of absorbances of HHb and acetaminophen, since, acetaminophen has absorbance at 240–300 nm (Figure 2, inset (A)). However, the decrease in absorbance at Soret region (Figure 2, inset (B)) upon addition of acetaminophen may clearly

![Figure 2](image-url)
indicate the interaction between acetaminophen and HHb, and upon interaction, it releases heme from HHb as a post-binding modification.

### 3.1.2. Spectrofluorometric assay of HHb–acacetaminophen interaction

HHb contains two $\alpha\beta$ dimers; and there are three tryptophan residues, i.e. $\alpha$-Trp$^{14}$, $\beta$-Trp$^{15}$, and $\beta$-Trp$^{37}$ in each $\alpha\beta$ subunit (Venkateshrao & Manoharan, 2004). The $\beta$-Trp$^{37}$ residue is located at the $\alpha_1\beta_2$ interface, which has been identified as the primary source of fluorescence emission of HHb (Cheng, Lin, Xue, Li, & Wang, 2001). This intrinsic fluorescence property could provide valuable information about the protein’s structural transition upon interaction with different ligands. As depicted in Figure 3, the tryptophan fluorescence at 333 nm was found to be quenched gradually in the presence of increasing concentrations of acetaminophen. We also observed that this quenching was accompanied by gradual increases in fluorescence intensity at 450 nm. These results strongly support the fact that acetaminophen interacts with HHb.

The fluorescence quenching is the decrease in the quantum yield of a fluorophore induced by a variety of molecular interaction with quencher through a dynamic or a static quenching process. The quenching process, whether dynamic or static can be distinguished by temperature-dependent quenching studies (Lakowicz, 2006). In dynamic quenching process, increasing the temperature results in faster rate of quencher diffusion, and hence larger amount of collision occurs, which resulted in rising of the quenching constant. In static one, increasing the temperature weakens the stability of the formed complex and hence reduces the quenching constant (Lakowicz, 2006). Therefore, to understand the binding process, fluorescence quenching of HHb by acetaminophen was studied at three different temperatures, viz., 298.15, 303.15, and 310.15 K; and the data were analyzed using Stern–Volmer plots (Figure 4(a)) following the Stern–Volmer equation (Equation 1):

$$\frac{F_0}{F} = 1 + K_{SV} \times [Q] \quad (1)$$

where, $F_0$ and $F$ are the fluorescence intensities before and after the addition of the acetaminophen, respectively. $K_{SV}$ is the Stern–Volmer quenching constant and $[Q]$ is the concentration of the quencher (acetaminophen). The Stern–Volmer quenching constants at different temperatures are presented in Table 1. Moreover, $K_{SV} = K_q \times \tau_0$, where $K_q$ is the quenching rate constant and $\tau_0$ is the average lifetime of the biomolecule without quencher. However, assuming $\tau_0 \times 10^{-8}$ s (Lakowicz & Weber, 1973), the $K_q$ values at all three temperatures were found to be much greater than the maximum diffusion rate constant of biomolecule i.e. greater than $2 \times 10^{10}$ M$^{-1}$ s$^{-1}$ (Table 1) (Zhang et al., 2013). Thus, it indicates that the fluorescence quenching of HHb was initiated by complex formation between HHb and acetaminophen (Lakowicz, 2006; Naik, Chimatadar, & Nandibewoor, 2009). Furthermore, the gradual decrease in $K_{SV}$ with increasing temperature (Table 1) also suggests us to conclude that the quenching mechanism of HHb by acetaminophen is mediated through static complex formation (Basu & Suresh Kumar, 2015b).

For the evaluation of number of binding sites, the fluorescence data were also analyzed using modified Stern–Volmer plot (Figure 4(b)) following the Equation 2:

$$\log \left( \frac{F_0 - F}{F} \right) = \log K_a + n \log [Q] \quad (2)$$

where $K_a$ is the association constant, and $n$ is the number of binding sites per molecule of HHb. All the calculated values of the number of binding sites at different temperatures were found to be about 1 (Table 1), from which only one binding site could be suggested. Therefore, we can conclude from this section that acetaminophen produces static complex with HHb at 1:1 ratio.

### 3.1.3. Interacting forces between acetaminophen and HHb

There are essentially four main types of non-covalent interacting forces that could play a major role in drug–protein binding. These are hydrogen bonds, van der
Waals forces, electrostatic, and hydrophobic interactions (Leckband, 2000). The interacting forces involved in the acetaminophen and HHb complex were determined from the thermodynamic parameters. The changes in enthalpy ($\Delta H^0$) and entropy ($\Delta S^0$) were determined from the van’t Hoff’s plot (Figure 5) following the Equation 3; and the values are tabulated in Table 2.

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

where $R$ is the universal gas constant (8.314472 $\text{J mol}^{-1} \text{K}^{-1}$), $T$ is the temperature in kelvin (K), and $K_a$ is the effective quenching constant. The $K_a$ values, as shown in the Table 2, at different temperatures, were calculated from the Lehrer plot (Figure 6) following the Equation 4 (Lehrer, 1971):

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a}$$

where $F_0$ and $F$ are the maximum fluorescence in the absence and presence of the acetaminophen, $f_a$ is the fraction of accessible tryptophan, and [Q] is the concentration of acetaminophen. However, with the increase in temperature, the decrease in $K_a$ was found to be in accordance with the decrease in $K_{sv}$.

Table 1. Quenching parameters of the interaction of acetaminophen with HHb at three different temperatures.

| T (K)   | Stern-Volmer Constant ($K_{sv}$) | $K_q$ (M$^{-1}$ s$^{-1}$) | $R^2_a$ | Number of binding sites (n) | $R^2_b$ |
|---------|---------------------------------|--------------------------|---------|-----------------------------|---------|
| 298.15  | 0.90 × $10^3$ M$^{-1}$          | 0.90 × $10^{11}$        | 0.984   | 0.750                       | 0.988   |
| 303.15  | 0.74 × $10^3$ M$^{-1}$          | 0.74 × $10^{11}$        | 0.985   | 0.912                       | 0.986   |
| 310.15  | 0.43 × $10^3$ M$^{-1}$          | 0.43 × $10^{11}$        | 0.993   | 0.974                       | 0.986   |

$R_a$, Linear correlated coefficient of Stern-Volmer plots.

$R_b$, Linear correlated coefficient of modified Stern-Volmer plots.

Moreover, the Gibbs energy changes ($\Delta G^0$) as depicted in Table 2 were determined using the following Equation 5:

$$\Delta G = \Delta H - T\Delta S$$

The values of the thermodynamic parameters, viz., $\Delta G^0$, $\Delta H^0$, and $\Delta S^0$ are also shown in Table 2. The negative
values of $\Delta G^0$ and $\Delta H^0$ indicate that the interaction between acetaminophen and HHb is spontaneous and exothermic; and the positive $\Delta S^0$ indicates that the reaction is entropy-driven (Cheng, Liu, Bao, & Zou, 2011; Ross & Subramanian, 1981). Moreover, the positive $\Delta S^0$ also indicates that the randomness around the acetaminophen–HHb complex increases, which is again an evidence of hydrophobic interaction (Shahabadi, Maghsudi, Kiani, & Pourfoulad, 2011); and the negative $\Delta H^0$ may also support the existence of hydrogen bond in the binding reaction (Lehrer, 1971).

### 3.1.4. Isothermal titration calorimetric assay of the binding of acetaminophen with HHb

The Isothermal titration calorimetry provides an accurate and detailed thermodynamic binding profile of drug–protein interactions (Hazra & Suresh Kumar, 2014; Hazra, Hossain & Suresh Kumar, 2013). Figure 7(A), shows a representative isothermal titration calorimetric profile of the raw signals obtained from the titration of $5 \times 10^{-3}$ M acetaminophen with $2 \times 10^{-5}$ M HHb at 298.15 K. Each peak in the binding isotherm represents a single injection of the drug into the protein solution. Integration of the area of cell feedback by subtracting the dilution heats of both the acetaminophen and the HHb gives the differential curve shown in the bottom panel of Figure 7(B). The control data and only HHb-acetaminophen data without buffer subtracting were shown in the Figure S1 and S2 respectively (given as supplementary data). The data were analyzed by Origin 7.0 software as provided with the ITC device by the manufacturer. The data were found to be fitted satisfactorily to the theoretical model of “one set of binding sites.” The analysis used the nonlinear least square methodology, based on the Wiseman isotherm (Anbazhagan, Sankhala, Singh, & Swamy, 2011; Wiseman, Williston, Brandts, & Lin, 1989). Therefore, the number of binding sites, i.e. binding stoichiometry of HHb-acetaminophen complex is the same as calculated in fluoremetric studies as discussed in Section 3.1.2. The thermodynamic binding parameters, as obtained from

| T (K) | $K_a$ ($\times 10^2$ M$^{-1}$) | $\Delta G^0$ (kJ/mol) | $\Delta S^0$ (J/mol K) | $\Delta H^0$ (kJ/mol) |
|-------|------------------|------------------|------------------|------------------|
| 298.15 | 1.4 | -1.44 | 0.989 | |
| 303.15 | 1.1 | -1.45 | 0.984 | 2.12 |
| 310.15 | 0.6 | -1.47 | 0.996 | |
ITC for this interaction are presented in Table 3. The similarity of the nature of these thermodynamic parameters with those obtained from flurometric assays strongly evidence the spontaneous binding of acetaminophen with HHb. Moreover, the positive entropy and negative enthalpy values as obtained by ITC strengthen the involvement of hydrophobic interaction and hydrogen bonding in HHb–acetaminophen complex.

3.1.5. Hydrophobicity of HHb upon interaction with acetaminophen

In order to check the involvement of hydrophobic surface of HHb in acetaminophen–HHb complex, a competitive binding assay was carried out using an external hydrophobic probe, ANS. The ANS, when remains in unbound or free form, exhibits a little fluorescence in aqueous solution; but it exhibits remarkable fluorescence ($\lambda_{ex}$ 350 nm) when it is bound to the hydrophobic surface of protein (Freedman & Radda, 1969; Stryer, 1965). As shown in Figure 8, the fluorescence intensities of different concentrations of ANS were found to be lower in HHb–acetaminophen complex than the control HHb. This may be due to the fact that acetaminophen occupies part of the hydrophobic surface of HHb and as a result, the binding of ANS to the HHb reduces. Therefore, the possibility of hydrophobic interaction may also exist. Moreover, surface hydrophobicity of a protein is related to its structural integrity, and thus this interaction may also influence the structural integrity of HHb.

3.1.6. Existence of hydrogen bond in HHb–acetaminophen interaction

In order to elucidate the involvement of hydrogen bond between HHb and acetaminophen complex, we carried out competitive experiments in the presence of sucrose. The sucrose is known to quench fluorescence of a protein upon its interaction with the latter through hydrogen bonding (Lager, Looger, Hilpert, Lalonde, & Frommer, 2006). As depicted in Figure 9, the rate of quenching of Trp intrinsic fluorescence (of HHb) by sucrose decreased in the presence of acetaminophen, indicating the presence of hydrogen bond in acetaminophen–HHb complex.

3.1.7. Molecular modeling study

In order to strengthen our experimental findings, the complementary application of computational modeling has also been employed. Cluster analysis was performed on the docked results using a root mean square tolerance of 0.2 Å with lowest energy and highest clustering. About 7 unique binding sites were found from the clustering; but, rank 1 consists of 822 conformations (out of 1000) with highest clustering and lowest binding energy. Therefore, it was selected as the most possible binding site of acetaminophen. This site is in close proximity to $\beta$-Trp$^{37}$. The conformations were ranked using the scoring function from which the free binding energy

| T (K) | K ($\times 10^3$ M$^{-1}$) | $\Delta H^0$ (kcal/mol) | $\Delta S^0$ (cal/mol/deg) | $\Delta G^0$ (kcal/mol) | n  |
|------|-----------------|-----------------|-----------------|-----------------|---|
| 298.15 | 6.40 ± 1.43 | $-1.163 \pm 0.00182$ | 13.5 | $-5.186$ | 1.20 |
was estimated. Figure 10 illustrates an overview of the binding of acetaminophen with HHb. The distance between acetaminophen binding site and two β-Trp$_{37}$ molecules were found to be 14.0 Å (Trp$^{580}$) and 11.5 Å (Trp$^{180}$). As shown in Figure 11(A), the benzene ring of the drug makes hydrophobic interactions with Trp$^{180}$, Pro$^{494}$, Ser$^{533}$, Tyr$^{539}$, and Phe$^{498}$ residues. The nature of interaction between acetaminophen and different amino acids in close proximity are tabulated in Table 4.

On the other hand, Figure 11(B) shows that the drug is surrounded by Lys$^{499}$ and Thr$^{337}$ which make hydrogen bonds with acetaminophen to stabilize the hydrophobic interaction. Therefore, these data support our experimental results of ANS binding (for hydrophobic interaction) and sucrose binding (for hydrogen bonding) assays. The hydrogen bonding or electrostatic interaction acts as an anchor and this helps to attain the 3D position of acetaminophen in its binding pocket (Zhang et al., 2008).

Figure 10. Representative diagram of best docked conformations of HHb–acetaminophen complex showing the distance between acetaminophen and β-Trp$_{37}$.
Note: The heme residues are shown as red spheres and the acetaminophen as purple one.

Figure 11. The binding mode of HHb–acetaminophen interactions.
Note: [A] Representation of the close amino acid residues, establishing hydrophobic interaction (cyan colored), and electrostatic interaction (green colored) with acetaminophen (purple colored). [B] Schematic representation the calculated distances of hydrogen bondings between acetaminophen and Lys$^{499}$ and Thr$^{337}$ residues of HHb.
Table 4. Amino acid residues involved in HHb–acetaminophen interaction.

| Amino acid residue | Distance from ligand (Å) | Nature of interaction |
|--------------------|--------------------------|-----------------------|
| Pro<sup>494</sup>   | 3.8                      | Hydrophobic           |
| Trp<sup>380</sup>   | 16.5                     | Hydrophobic           |
| Tyr<sup>359</sup>   | 7.1                      | Hydrophobic           |
| Ser<sup>333</sup>   | 5.2                      | Hydrophobic           |
| Phe<sup>398</sup>   | 3.7                      | Hydrophobic           |
| Val<sup>400</sup>   | 3.9                      | Electrostatic         |
| Lys<sup>399</sup>   | 2.8                      | Hydrogen bonding      |
| Thr<sup>337</sup>   | 3.2                      | Hydrogen bonding      |

From the theoretical calculation, the values of Gibbs energy, electrostatic energy, and intermolecular energy of the interaction between acetaminophen and HHb complex are given in Table 5. Therefore, the molecular docking strongly supports the experimental (fluorometric and calorimetric) evidences for the complex formation of HHb and acetaminophen.

Table 5. Cluster analysis of HHb–acetaminophen complex.

| Cluster Rank | Number of conformation | Lowest binding free energy (kcal/mol) | Electrostatic energy (kcal/mol) | Intermolecular energy (kcal/mol) |
|--------------|------------------------|--------------------------------------|-------------------------------|----------------------------------|
| 1            | 822                    | −4.49                                | −0.28                         | −4.79                             |
| 2            | 148                    | −3.89                                | −0.25                         | −4.19                             |
| 3            | 15                     | −3.81                                | −0.04                         | −4.11                             |
| 4            | 4                      | −3.69                                | −0.10                         | −3.99                             |
| 5            | 8                      | −3.68                                | −0.21                         | −3.98                             |
| 6            | 1                      | −3.60                                | −0.08                         | −3.90                             |
| 7            | 2                      | −3.55                                | −0.25                         | −3.84                             |

3.2. Effect of acetaminophen on conformation of protein

3.2.1. Synchronous fluorescence measurement

The synchronous fluorescence spectroscopy is another conventional technique of simultaneous scanning of excitation and emission wavelengths to distinguish the overlapped excitation peaks of aromatic residues in fluorescence spectra. It is used to obtain the information about the microenvironments in the vicinity of aromatic fluorophores. When the differences between excitation and emission wavelengths are set to 60 and 15 nm, it gives characteristic information, respectively, about tryptophan and tyrosine microenvironments of protein (Shahabadi, Maghsudi, Kiani & Pourfoulad, 2011). The influence of acetaminophen on the tryptophan and tyrosine microenvironments of HHb is shown in Figure 12. It is apparent from Figure 12 (A) and (B) that the intensities of emission maxima of Δ60 and Δ15 decrease with increasing concentrations of acetaminophen with signifi-

Figure 12. The synchronous fluorescence spectra of HHb–acetaminophen complex at Δλ = 60 nm (A) and Δλ = 15 nm (B). Note: HHb concentration was 5×10<sup>−6</sup> M in 0.01 M PB, pH 7.4. Acetaminophen concentrations were 0, 1×10<sup>−7</sup>, 2×10<sup>−7</sup>, 4×10<sup>−8</sup>, 6×10<sup>−8</sup>, 8×10<sup>−6</sup>, 1×10<sup>−5</sup> and 1.5×10<sup>−5</sup> M respectively from a to h. The down arrow (↓) indicates the decrease of fluorescence intensities with increasing concentrations of acetaminophen.
cant right shift (bathochromic shift), respectively, from 339 to 344 nm (for Trp) and from 301 to 310 nm (for Tyr). The bathochromic shift in the emission maxima suggests a more polar (or less hydrophobic) environment of the Trp and Tyr molecules (Basu & Suresh Kumar, 2015b). Therefore, it indicates that the binding of acetaminophen to HHb influences the structural conformation at least in the vicinity of β-Trp\textsuperscript{37} and α-Tyr\textsuperscript{42, 140} residues at α,β\textsubscript{2} interface of HHb.

### 3.2.2. CD spectroscopy

To understand the influence of acetaminophen on the secondary structure of HHb, CD spectroscopy was carried out. One of the most successful applications of CD is to study the structural characterization of proteins at far-UV (for secondary structure) and near-UV (for tertiary structure) region. The CD spectra exhibit two distinct negative ellipticity minima in the far-UV region at 208 and 222 nm, which are due to the helix structure of protein. More precisely, the 208 nm band corresponds to \(\pi-\pi^*\) transition of the α-helix and the 222 nm band is responsible for \(n-\pi^*\) transition for both the α-helix and random coil; which are related to the transition of the peptide bond of the α-helix (Basu & Suresh Kumar, 2015b; Gaudreaua, Neaulta, Ramaswamy, Sarma, & Tajmir-Riahia, 2002). Upon gradual addition of acetaminophen to the protein solution, the ellipticity at 208 and 222 nm was found to be decreased as shown in Figure 13. This suggests that acetaminophen can modify the secondary structure of HHb. The α-helix contents of HHb in the absence and presence of acetaminophen were calculated from mean residue ellipticity (MRE) values at 222 nm using the following Equation 6:

\[
\text{\alpha\, helix\%} = \frac{\text{MRE}_{222} - 2340}{30,300}
\] (6)

The MRE (deg cm\(^2\) dmol\(^{-1}\)) was obtained using the following Equation 7.

\[
\text{MRE} = \frac{\text{Observed CD (mdeg)}}{C_p \times n \times l \times 10}
\] (7)

where \(C_p\) is the molar concentration of HHb; \(n\) is the number of amino acid residues of HHb; and \(l\) is the path length.

The calculated α helix contents in HHb without and with acetaminophen were found to be, respectively, 76.2%, which is almost similar to the earlier reports (Basu & Suresh Kumar, 2015b; Roy, Sikdar, Seal & Haldar, 2015; Shen, Liou, Ye, Liang, & Wang, 2007) and 73.7%. Therefore, this decrease in α-helix content upon interaction with acetaminophen may reflect the internal folding pattern of the HHb.

### 3.3. Functional modifications of HHb

From the above experiments, it is quite clear that HHb shows its structural modifications upon interaction with acetaminophen. However, functional aspects of a protein depend on its structural integrity. Therefore, we have also investigated some of the functional properties as follows:

#### 3.3.1. Co-oxidation assay of HHb

Hemoglobin oxidizes to methemoglobin along with concomitant production of superoxide radical (Abugo & Rifkind, 1994). This mechanism is associated within the distal side of heme, which facilitates the nucleophilic displacement of bound oxygen by the histidine (Levy, Kuppusamy, & Rifkind, 1990). The rate of NBT-induced co-oxidation of HHb was found to be significantly higher in the presence of acetaminophen as depicted in Figure 14. This may be due to the fact that (i) acetaminophen carries one hydroxyl group in its structure, which may be involved in the oxidation process through proton donation and thus enhancing the rate of oxidation, or, (ii) the acetaminophen modifies the structure of HHb in such a way that it leads to augmentation of the oxidation rate, or both. However, this increased rate of oxidation indicates that HHb may further induce oxidative damage of other biomolecules in its vicinity due to enhanced production of superoxide radical.

#### 3.3.2. Assay of esterase like activity of hemoglobin

The esterase activity of purified HHb in presence of acetaminophen was measured from hydrolysis of \(p\)-NPA (Figure 15) (Fife, 1965). The catalytic activity of HHb...
was found to be reduced in the presence of increasing concentration of acetaminophen. The esterase activity of HHb is mainly due to the presence of Acid–Base–Nucleophile triad or catalytic triad composed of basic three amino acids, His, Ser, and Asp (Dodson & Wlodawer, 1998). These residues form a charge-relay network to activate the nucleophile (Ser), which attacks the substrate, and thus forming a covalent intermediate, which is then hydrolyzed. The imidazole group of histidine takes part in this catalytic reaction by removal of a proton from the attacking reagent in the transition state (Buller & Townsend, 2013). The kinetics of p-NPA hydrolysis by HHb in absence and presence of acetaminophen were determined from Lineweaver–Burk plots, following the Equation 8.

\[
\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \tag{8}
\]

where \(v\) is the velocity of the reaction; \(V_{max}\) is the maximum velocity; \(K_m\) is the Michaelis constant; and \([S]\) is the molar concentration of p-NPA. This study demonstrates that acetaminophen plays an inhibitory role as \(K_m\) increases and \(V_{max}\) decreases with increasing concentration of acetaminophen (Table 6). This phenomenon can be attributed to the fact that binding of acetaminophen to a distinct inhibitory site of HHb causes a conformational change in the protein molecule, which distorts or masks the substrate binding sites, and leads to the increase of \(K_m\), and also hampers the catalytic activity of HHb, as evidenced by the reduction in \(V_{max}\). Therefore, from this experiment it may be interpreted that acetaminophen may induce a post-binding modification, which directly interferes with the catalytic activity or substrate binding site of HHb.

### 4. Conclusion

From the findings of the present study, we conclude that HHb possesses one binding site for acetaminophen. The binding of acetaminophen to HHb is mediated through hydrophobic interaction, which is further stabilized by hydrogen bonds. Upon interaction, the acetaminophen influences the structural integrity as well as functional properties of HHb. Therefore, this study will definitely help to interpret the structural basis for a more rational approach to the design of phenolic compounds in clinical and pharmacological researches. Moreover, these findings also provide a new insight into the possible mechanism of acetaminophen with physiologically essential biomolecule, HHb.

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| \(K_m\) (mM) | \(V_{max}\) (mM/min) |
|---|---|
| Control | 30.03 | 0.65 |
| HHb+10 μM acetaminophen | 49.92 | 0.62 |
| HHb+20 μM acetaminophen | 72.3 | 0.55 |
cooperating in accessing the ITC. The authors are also grateful to Prof. Ansuman Lahiri, Professor of the department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, and his research fellow, Mr. Aditya Kumar Sarkar for helping in AutoDock analysis.

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