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A CRITICAL STUDY ON THE INTERACTIONS OF HESPERITIN WITH HUMAN HEMOGLOBIN: FLUORESCENCE SPECTROSCOPIC AND MOLECULAR MODELING APPROACH

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Abstract

Hesperitin, a ubiquitous bioactive flavonoid abundant in citrus fruits is known to possess antioxidant, anti-carcinogenic, hypolipidemic, vasoprotective and other important therapeutic properties. Here we have explored the interactions of hesperitin with normal human hemoglobin (HbA), using steady state and time resolved fluorescence spectroscopy, far UV circular dichroism (CD) spectroscopy, combined with molecular modeling computations. Specific interaction of the flavonoid with HbA is confirmed from flavonoid-induced static quenching which is evident from steady state fluorescence as well as lifetime data. Both temperature dependent fluorescence measurements and molecular docking studies reveal that apart from hydrogen bonding and van der Waals interactions, electrostatic interactions also play crucial role in hesperitin-HbA interactions. Furthermore, electrostatic surface potential calculations indicate that the hesperitin binding site in HbA is intensely positive due to the presence of several lysine and histidine residues.

Keywords

Pharmacologically important flavonoids; Hesperitin; Hemoglobin binding; Fluorescence lifetime; Molecular modeling

1. Introduction

In 1936, Szent-Györgyi [1] first drew attention to the therapeutically beneficial role of dietary flavonoids [2–5], which are polyphenolic compounds ubiquitous in plants of higher genera and present in common plant based food items and beverages, e.g., onions, apples, broccoli, tea and red wine. Recent years have witnessed an explosive growth of research on various bioactive flavonoids having important therapeutic activities [6–12]. Many recent

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Dedicated to Professor Michael Kasha on the occasion of his 90th birthday.

studies, both in vivo and in vitro, have established that flavonoids are powerful antioxidants effective against a wide range of free radical mediated and other diseases including various types of cancers, tumors, diabetes mellitus, atherosclerosis, ischemia, neuronal degeneration, cardiovascular ailments, and AIDS [13–14]. High potency and low systemic toxicity make these polyphenolic compounds viable alternatives to conventional therapeutics.

Hesperitin (structure shown in Scheme 1 inset), which is a bioactive plant flavonoid belonging to the chemical class ‘flavanone’ (abundantly present in citrus fruits), is rapidly emerging as an especially attractive therapeutic agent with a broad spectrum of activities. In particular, both in vitro and in vivo studies have been reported indicating pronounced chemopreventive potential of hesperitin against malignant melanoma [15]. In addition, hesperitin has been proved to be useful for the treatment of hemorrhoids, as a vasoprotective agent, and in the prevention of post-operative thromboembolism [16]. Hesperitin also exhibits strong lipid lowering activities by reducing cholesterol biosynthesis, as evident from low hepatic HMG-CoA reductase levels and acyl-CoA:cholesterol acyl transferase activities upon hesperitin intake [17].

Normal human hemoglobin (HbA), the major protein component of red blood cells, is a physiologically important globular protein which carries oxygen from the lungs to different tissues. Moreover, HbA reversibly binds many endogenous and exogenous molecules including various drugs [18] such as antioxidants and flavonoids. Thus, HbA can play an important role in the distribution and bioavailability of flavonoids [19]. In previous studies we have shown that various flavonoids can prevent glycosylation of HbA to a significant extent [11, 20–21], and thus can be potentially useful in the management of diabetic complications. The knowledge of the binding characteristics of therapeutically important flavonoids with HbA and other relevant functionally important cellular proteins is therefore crucial for understanding the mechanism of their drug actions. Moreover, since HbA is an important functional protein for reversible oxygen binding, it is of significant importance to ascertain the influence of potential therapeutically important flavonoids on the structural integrity of HbA.

In the present study we have explored the interactions of hesperitin with normal human hemoglobin (HbA), by exploiting the intrinsic fluorescence of tryptophan of HbA. Unlike flavonols which are intrinsically fluorescent [22], fluorescence studies presented here relied on the fluorescence of the protein (HbA) in order to study the binding characteristics of HbA with the flavanone hesperitin. Both steady state and time resolved fluorescence spectroscopy have been used in this investigation. We also present molecular modeling studies (encompassing molecular docking and electrostatic surface potential calculations) which provide critical insights regarding the interaction processes of hesperitin with HbA in atomic detail. Far UV circular dichroism (CD) study reveals some minor changes in the secondary structure of HbA in presence of hesperitin at physiologically relevant concentration (upto 10 μ M) [23], which strengthens the claim for the medicinal application of hesperitin. However at higher concentrations of hesperitin, the observed changes are much more noticeable than at lower concentrations.

2. Materials and methods

2.1 Materials

Lyophilized powder of human hemoglobin (molecular weight 64,500 D), hesperitin, and phosphate buffer were purchased from Sigma Chemicals, USA. Solvents used were of spectroscopic grade and obtained from Sigma–Aldrich.

2.2 Interaction of flavonoid with HbA

Aliquots of concentrated methanolic solution of hesperitin were added to 1 ml solutions of various concentrations of HbA. The final concentrations of methanol were kept <1% (by volume) in all samples. After adding the hesperitin, the solutions were allowed to equilibrate for one hour at 25 °C before carrying out spectroscopic measurements. All the concentrations were calculated using the standard molecular weight as well as using the molar extinction coefficient of HbA at 276 nm ($120808 \text{ M}^{-1} \text{ cm}^{-1}$ [W. B. Gratzer, Med. Res. Council Labs, Holly Hill, London]).

2.3 Spectroscopic Measurements

Steady state absorption spectra were recorded with Cecil model 7500 and Shimadzu UV2550 spectrophotometers. Steady state fluorescence measurements were carried out with Varian Cary Eclipse and Shimadzu RF5301 (equipped with a Fisher temperature controlled accessory) spectrofluorometers. The fluorescence readings were taken by exciting the samples and measuring the emissions at relevant wavelengths and appropriate blanks were subtracted for respective measurements. Quartz cuvettes of 1 cm path length were used for all experiments.

Time resolved fluorescence decay measurements were carried out with an Edinburgh Instruments nano-second time correlated single photon counting (TCSPC) spectrometer, using 295 nm diode excitation source (IBH, UK, nano-LED, pulse FWHM ~ 1.2 ns). An emission monochromator was used to block scattered light and isolate the emissions. Fluorescence intensity decay curves were deconvoluted with the instrument response function and fitted to a multiexponential decay function, $F(t) = \sum_i A_i \exp(-t/\tau_i)$, where A_i and τ_i represent the amplitudes and times of the decay components such that $\sum_i A_i = 1$. The goodness of fit was estimated by using χ^2 values.

Average lifetimes $\langle \tau \rangle$ for biexponential decays were calculated from the decay times and preexponential factors using the expression [24, 25]:

$$\langle \tau \rangle = \frac{A_1 \tau_1^2 + A_2 \tau_2^2}{A_1 \tau_1 + A_2 \tau_2}$$

Circular dichroism (CD) spectra were recorded on a Jasco J-600 spectropolarimeter. All spectral measurements were carried out at room temperature (298 K) and three independent measurements were done in each case and the results were averaged. 1 mm pathlength quartz cells were used for the CD studies in the far UV region.

2.4 Molecular modeling

Molecular modeling studies were carried out to elucidate the interaction of hesperitin with normal human hemoglobin (HbA), according to the procedure described in our recently published article on HbA-flavonoid interactions [26]. Briefly, an online server Q-siteFinder [27] was used to identify the probable binding pockets of hemoglobin. 3-D atomic coordinates of the protein were obtained from the Brookhaven Protein Data Bank (PDB id 2D60). Adaptive Poisson-Boltzmann Solver (APBS) software [28] was used to calculate electrostatic surface potential on the hemoglobin. Default values for charge (full charge), salt concentration (0.0), interior dielectric (2.0), and exterior dielectric (80.0) were used.

AutoDock4 [29] was employed to dock hesperitin with the receptor (HbA). All the hetero atoms were deleted and non-polar hydrogens were merged Kollman united-atom charges, atomic solvation parameters and fragmental volumes were assigned to the receptor. A grid

map of $100 \times 100 \times 100$ grid points in size with a grid-point spacing of $0.375 \text{ \AA}$ was created for the protein. The molecular structure of the ligand (hesperitin) was built using HYPERCHEM [30] molecular builder module and optimized using AM1 semi-empirical method to an rms convergence of $0.001 \text{ kcal/ \AA mol}$ with Polak-Ribiere conjugate gradient algorithm implemented in the HYPERCHEM 7.5 package. Rotatable bonds were assigned and partial atomic charges were calculated using Gasteiger-Marsili method. 100 docking runs were carried out for HbA-flavonoid system and for each run, a maximum of 2,500,000 GA operations were performed on a single population of 150 individuals. The weights for crossover, mutation and elitism were default parameters (0.8, 0.02 and 1 respectively).

3. Results

In the present work we have explored the binding of the plant flavonoid hesperitin to HbA under physiological conditions via steady-state and time-resolved fluorescence methods, in combination with far UV-CD, and docking studies. The results show that hesperitin binds to the protein with high affinity and strongly quenches its intrinsic (tryptophan) fluorescence.

3.1 Steady state and time resolved fluorescence results

Scheme 1 shows the absorbance of HbA (—, $\sim 4 \text{ }\mu\text{M}$), hesperitin (- - -, $10 \text{ }\mu\text{M}$) and HbA with hesperitin (-.-) in 10 mM phosphate buffer at pH 7. There is a significant difference in the absorption profile of hesperitin bound to HbA when compared to unbound HbA. Quenching studies of protein tryptophan fluorescence by ligands is a convenient means for exploring ligand-protein interactions [31–32]. Figure 1 presents spectra which show quenching of HbA fluorescence in the presence of increasing concentrations of the flavonoid hesperitin. The excitation wavelength is 280 nm where hesperitin has comparatively low absorbance. Figure 2a presents corresponding Stern-Volmer plot based on the equation,

$$\frac{F_0}{F} = 1 + K_{sv}[\text{Hesperitin}]$$

where K_{SV} is the Stern-Volmer quenching constant for the quenching of HbA tryptophan fluorescence by flavonoid [24]. The Stern-Volmer plot is essentially linear for flavonoid concentrations upto $25 \text{ }\mu\text{M}$ (Figure 2a) which indicates that only one type of quenching occurs [24]. However, at higher flavonoid concentrations ($> 25 \text{ }\mu\text{M}$), the Stern-Volmer plots become non-linear (data not shown), which may be attributed to two complicating factors: (i) existence of more than one type of quenching mechanisms at higher flavonoid concentrations, (ii) attenuation of tryptophan fluorescence due to inner filter effect arising from significant absorption of flavonoids in the tryptophan emission region with these ($> 25 \text{ }\mu\text{M}$) quencher (flavonoid) concentrations. Hence, our analysis has been restricted to flavonoid concentrations $< 25 \text{ }\mu\text{M}$.

The K_{SV} value obtained is $\text{ca. } 2.17 \pm 0.76 \times 10^4 \text{ M}^{-1}$ (Table 1) for hesperitin which indicates that HbA tryptophan fluorescence is efficiently quenched by the flavonoid. We further performed lifetime measurements of HbA tryptophan fluorescence both in presence and absence of the flavonoid. Table 2 shows that the protein tryptophan fluorescence decay is bi-exponential with decay times of 0.725 ns and 3.087 ns . It is noteworthy that in presence of hesperitin, no significant changes are observed in the tryptophan fluorescence decay parameters. (Table 2). Thus the average lifetimes (τ) computed from the decay parameters remain essentially unchanged. This indicates that a static mechanism is principally responsible for the observed steady-state fluorescence quenching when hesperitin binds to hemoglobin.

The apparent binding constant ' K ' and the number of binding site(s) ' n ' were estimated from fluorescence titration studies, using the plot of $\text{Log}(F_0-F)/F$ vs. $\text{Log}(1/([D_t]-(F_0-F))[P_t]/F_0)$ [31] (Figure 2b) which is based on the equation:

$$\text{Log} \frac{F_0 - F}{F} = n \text{Log} K - n \text{Log} \left(\frac{1}{[D_t] - (F_0 - F)[P_t]/F_0} \right)$$

where F_0 and F are the fluorescence intensity of HbA in absence and presence of flavonoid (D) respectively, $[D_t]$ is the total flavonoid concentration and $[P_t]$ is the total protein concentration, n is the number of binding sites and K is the binding constant. Table 1 shows the binding constants ' K ' and number of binding sites ' n ' for the binding of hesperitin with HbA within the studied concentration range of the flavonoid.

The binding forces contributing to interactions of flavonoids with proteins often include van der Waals interaction, hydrophobic force, electrostatic interactions, hydrogen bond, etc. The thermodynamic parameters, namely enthalpy change (ΔH^0), entropy change (ΔS^0) and free energy change (ΔG^0) of reaction provide the main lines of evidence for confirming the binding force. The thermodynamic parameters are evaluated using the following equations:

$$\ln K_T = -\Delta H^0 / RT + \Delta S^0 / R,$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0,$$

where K and R are the binding constant and gas constant, respectively. The temperature-dependence of the binding constant was studied at three different temperatures (288, 293, 298 K). The thermodynamic parameters were determined using van't Hoff plot (Figure 2b, inset) and are presented in Table 1. As shown in Table 1, the values of standard enthalpy changes (ΔH^0) is negative while standard entropy changes (ΔS^0) of the binding reaction between hesperitin and HbA is found to be low and positive.

Ross and Subramanian [33] have characterized the sign and magnitude of the thermodynamic parameters associated with various kinds of interactions that play an important role in the protein-ligand interaction. A positive ΔH^0 and ΔS^0 values are associated with hydrophobic interaction. The negative ΔH^0 and ΔS^0 values are associated with hydrogen bonding and van der Waals interaction in low dielectric medium. Finally very low positive or negative ΔH^0 and ΔS^0 values are characterized by ionic interactions. Therefore, it is difficult to characterize the HbA-hesperitin interaction with a single intermolecular force ; rather, it is complex in nature involving various intermolecular forces.

The binding process is spontaneous as evident by the negative sign of ΔG^0 and the relative contributions of ΔH^0 and ΔS^0 suggest that the binding of hesperitin with HbA is enthalpy driven. Negative enthalpy changes ΔH^0 ($-3.16 \text{ kcal. mol}^{-1}$) obtained in our study show that van der Waals and hydrogen bonding interactions play a major role in the binding of hesperitin to HbA. Furthermore, the small values of both ΔH^0 ($-3.16 \text{ kcal. mol}^{-1}$) and ΔS^0 ($0.009 \text{ kcal. mol}^{-1}$) suggests that electrostatic interactions are also involved in the HbA flavonoid binding. Recently, Mandal *et. al.* [34] have also observed similar changes in thermodynamic parameters using van't Hoff equation for Rhodamine 6G binding with hemoglobin and the binding pattern has been interpreted as combined effect of hydrophobic association and electrostatic interaction.

3.2 Far ultraviolet Circular Dichroism (CD) spectroscopic studies

To investigate the possible effect of the flavonoid binding on the secondary structure of HbA, we used far-UV CD spectroscopy. The CD spectrum of HbA in aqueous buffer (in the

absence of flavonoids) has two characteristic peaks of negative ellipticity at 208 nm and 222 nm indicating its predominantly α -helical secondary structure (Figure 3). As shown in the figure, the CD spectrum of the HbA shows slight changes upon addition of different concentrations of hesperitin (at fixed hemoglobin concentration) upto 1×10^{-5} M. The CD profiles of HbA obtained in presence of 5×10^{-6} M and 1×10^{-5} M of hesperitin (shown by dashed and dotted lines respectively in Figure 3) indicate similar minor effects of hesperitin addition on the secondary structure of the protein. However at hesperitin concentration of 2.5×10^{-5} M, the 208 nm peak undergoes a shift of 3 nm and an increase in the negative ellipticity.

3.3 Molecular modeling studies

Molecular modeling of the hesperitin binding site in HbA—Probable binding sites of hemoglobin were explored by Q-siteFinder which predicts a total of 10 probable binding pockets in the protein [26]. We chose a sufficiently large grid volume that covers all the probable binding sites of the protein and thus accounting for all possible binding of flavonoid molecule to the protein's binding cavity. It is noteworthy that, in our previous study on the interactions of HbA with two other flavonoids of related interest (3-hydroxyflavone and fisetin), it was found that the flavonoids bind to the β chain of HbA enclosed between helix 4 and 5 while the C-terminal segment of helix 6 and N-terminal segment of helix 7 form the base of the catalytic cavity [26]. In contrast to our previous result, hesperitin is predicted to bind to a different site, in the α chain of HbA. Compared to the usual flavonoid binding site located in the β chain, this site (in the α chain) is significantly wider, with a molecular volume of $653 \text{ \AA}^3$. To get an insight into the molecular basis for the different binding site of hesperitin, surface area calculations were carried out on the flavonoids. Interestingly, it turns out that hesperitin is comparatively larger than other flavonoids of related interest (surface area of hesperitin is $414.97 \text{ \AA}^2$ while those for 3-hydroxyflavone and fisetin considered in our previous study are $326.38 \text{ \AA}^2$ and $363.65 \text{ \AA}^2$ respectively). The larger size of hesperitin dictates its binding to the relatively larger binding pocket of α chain that allows maximum surface complementarities between the receptor and the ligand.

The list of residues that lines the ligand binding site in the α chain of HbA are listed in Table 3. In hemoglobin both the α and β chains possess similar structural fold consisting of seven consecutive helices and both the chains contain an interstitial binding pocket enclosed by helix 4 and 5 while the base of the cavity is formed by the C-terminal segment of helix 6 and N-terminal segment of helix 7. Four residues namely, Thr 39, Lys 40, Tyr 41, Phe 43 of helix 3 line the opening of the cavity of the hesperitin binding site in α chain. It is noteworthy that the intrinsic fluorescence of Trp 37 is normally used as an optical probe to monitor ligand binding to the binding sites of HbA. Our docking studies reveal that the hesperitin binding site is $13.5 \text{ \AA}$ away from Trp37 (β chain). Thus the fluorescence of Trp 37 can be used as a good optical probe to monitor the flavonoid binding to HbA. Also, this new binding pocket of HbA in the α chain is intensely positive. Detailed examination of the binding pocket reveals that there are mainly four positively charged residues, namely Lys 40, His 58, Lys 61, His 87, which contribute to the highly intense positive charge on the surface of the flavonoid binding site of HbA (Figure 4).

Hesperitin interaction with HbA—It is evident from figure 5 that hesperitin is docked with the HbA in such a way that its B-ring is coplanar with the chromone moiety formed by (A+C) ring of polyphenols. The phenyl ring interacts with helix 4 and 5 while the chromone ring is dipped inside the cavity and interacts with helix 6 and 7. Docking interaction energy reveals that the main binding forces for complexation are the van der Waals as well as hydrogen bonding interactions. The lowest energy docked model suggests that one hydrogen

bonding possibility in case of hesperitin and HbA, namely between the 7-OH group of hesperitin and Ser102. All the residues interacting with hesperitin by van der Waals interactions are listed in Table 4. Flavonoid binding site in the α -chain of HbA is intensely positive which is contributed by mainly four positively charged residues, namely Lys 40, His 58, Lys 61, His 87 as mentioned in the earlier section. Our docking study reveals there are electrostatic interactions between hesperitin and HbA with the calculated electrostatic interaction energy being ~ -0.12 kcal/mol. This study clearly reveals the contrasting binding mode of hesperitin with the normal human hemoglobin when compared with other flavonoids of related interest like fisetin and its model compound 3-hydroxyflavone [26]. The flavanone hesperitin prefers to bind at the α -subunit of HbA compared to the β -subunit binder flavonols like fisetin and 3-hydroxyflavone.

4. Discussion

The typical absorption spectra of the protein hemoglobin and ligand hesperitin is different when hesperitin is bound to it as displayed in Scheme 1. The main absorbance peak for hesperitin is ~ 322 nm with a shoulder at ~ 288 nm. In presence of HbA the band of hesperitin at 322 nm is shifted to 327 nm. The occurrence of this band with λ_{max} at ~ 327 nm in the absorption profile of the conjugate is clearly an indication of the ground state complexation between the drug hesperitin and the protein HbA. Furthermore this binding gives rise to the tryptophan fluorescence quenching of HbA with increasing concentrations of the flavonoid which is shown in Figure 1. A linear Stern-Volmer plot (Figure 2A) can be expected to arise from either collisional or static quenching [24, 25]. However, the K_q values obtained here are too large to be due to collisional quenching (Table 1), especially for an unquenched lifetime (τ_0) of tryptophan ca. 2.7 ns [24, 25]. For this value of τ_0 , the bimolecular quenching constant, K_q value is found to be $(8.04 \pm 0.75) \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$ for hesperitin (Table 1) which is nearly 100-fold larger than the maximum value possible for diffusion-limited quenching in water ($2.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) [24, 25]. Therefore, the quenching observed for HbA in presence of hesperitin must be due to some specific interaction that increases the local concentrations of the flavonoids around the tryptophan residue(s) in hemoglobin. Thus, the large K_q value indicates that the observed quenching is static in nature, in the concentration ranges of the flavonoid we used. Fluorescence lifetime measurements were used to obtain further confirmation regarding the static nature of the quenching. The fact that no significant changes occur in the HbA tryptophan fluorescence decay parameters in presence of the flavonoids corroborate the conclusion drawn from the steady state fluorescence data that a static mechanism is principally responsible for the observed fluorescence quenching, implying that flavonoids do indeed bind to hemoglobin. However, we observed a red shift in the $\lambda_{\text{max}}^{\text{em}}$ of HbA with increase in hesperitin concentrations. At $2.5 \times 10^{-5} \text{ M}$ hesperitin, a red shift of 10 nm is observed which suggests that at such high concentration of ligand, the microenvironment of β -Trp 37 residue in HbA undergoes a ligand induced change toward more polar side.

Since the intrinsic fluorescence of human hemoglobin originates primarily from β -37 tryptophan, it seems reasonable to infer that the β -37 tryptophan residue is presumably at or near the binding site of the flavonoid. This conclusion is corroborated by the theoretical (docking) studies which show that the β -37 Trp residue occurs in close proximity to the flavonoid binding site (see Figure 4) suggesting that the fluorescence of Trp 37 should be capable of 'sensing' ligand binding to that pocket. Far-UV circular dichroism (CD) spectra indicate that in the physiologically relevant concentration range (upto 10 μM), hesperitin induces only slight changes in the secondary structure of HbA, which is a positive aspect in the context of the medicinal application of hesperitin. However, at higher concentrations, there is a more significant change in the secondary structure of HbA, which is induced by the binding of the relatively bulky hesperitin (when compared to other flavonoids of related

interest like fisetin and its model compound 3-hydroxyflavone [26]) to the α -subunit of HbA. The fluorescence spectroscopic study reveals that there is only one binding site ($n \approx 1$, Table 1) for hesperitin in HbA at the studied concentration.

Molecular modeling calculations predict that hesperitin binds in the α chain of HbA. The larger size of hesperitin compared to other flavonoids dictates the binding of hesperitin to the larger binding site in the α chain of HbA. The binding process is spontaneous as evident by the negative sign of ΔG^0 . Furthermore, negative value for ΔH^0 ($-3.16 \text{ kcal. mol}^{-1}$) obtained in our study suggests that van der Waals and hydrogen bonding interactions play a major role in the binding of hesperitin to HbA. These results are in accordance with our docking study which also predicts that the main binding forces for complexation are the van der Waals and hydrogen bonding interactions. The lowest energy docked model suggests one hydrogen bonding possibility in case of hesperitin and HbA, namely between the 7-OH group of hesperitin and Ser102. Furthermore, the small values of ΔH^0 and ΔS^0 suggest that electrostatic interactions are also involved in the HbA-flavonoid binding. A critical look reveals that the hesperitin binding site contains several positively charged residues, namely Lys 40, His 58, Lys 61, His 87 which contribute to the highly intense positive potential on the surface of the hesperitin binding site of HbA.

5. Concluding remarks

We present, for the first time, a comprehensive study on the interactions of the therapeutically important citrus flavonoid, hesperitin with normal human hemoglobin (HbA), a carrier protein which plays a crucial role in the distribution and bioavailability of flavonoids and other therapeutic drugs. The present study demonstrates the usefulness of hemoglobin (tryptophan) fluorescence in combination with molecular modeling techniques, for characterizing the binding of hesperitin with HbA in critical detail. Finally, it is worth noting that the experimental results (involving fluorescence spectroscopic measurements) are in excellent agreement with predictions from theoretical (molecular modeling) studies. Most particularly, the thermodynamic parameters obtained from temperature dependent fluorescence measurements, as well as the molecular docking studies reveal that hydrogen bonding and van der Waals along with electrostatic interactions are the principal driving forces for the hesperitin-HbA interaction.

We can envision promising extension of this approach to other flavonoid derivatives, which should open the door to new avenues for the 'screening and design' of the most suitable flavonoid derivatives from among numerous structural variants of this new generation of rapidly emerging therapeutic drugs of immense importance in modern medicine.

Research Highlights

- ▶ Absorption spectra of hesperitin bound HbA indicates ground state complex formation.
- ▶ Binding induces static quenching of intrinsic fluorescence of the tryptophan of HbA.
- ▶ Molecular docking and electrostatic surface potential calculations were performed.
- ▶ Contrasting binding modes of hesperitin compared to other flavonoids were observed.

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Figure 1. Quenching effect of hesperitin on HbA ($\sim 3.0 \mu\text{M}$) fluorescence emission spectra ($\lambda_{ex} = 280 \text{ nm}$).

Figure 2.

(a) Stern-Volmer plot of the HbA tryptophan fluorescence quenching. (b) The plot of $\log(1/([D]-(F_0-F))[P]/F)$ vs. $\log(F_0-F)/F$ of the fluorescence quenching data. The concentration of HbA, $[P] = 3.0 \times 10^{-6}$ M. 2b Inset: van't Hoff plot of the binding of hesperetin with 3.0×10^{-6} M HbA. Each data point indicates the average of three determinations where error bars indicate the standard deviation.

Figure 3.

Circular dichroism spectra of hesperitin-treated hemoglobin (HbA) at different [Flavonoid] / [HbA] ratios: the concentration of HbA = 3.0×10^{-6} M in 0.01 M phosphate buffer, pH 7.4, was kept fixed, while the molar concentrations of flavonoids were varied accordingly. Solid for free HbA, Dash for 5×10^{-6} M, Dot for 10×10^{-6} M and Dash dot for 25×10^{-6} M hesperitin respectively.

Figure 4.

(A) Predicted binding pockets of hesperitin in HbA (PDB id 2D60). The red colored section represents the α chain while blue ribbons represent β chain. Bound hesperitin is shown as green stick. Magenta sticks represent the chromophoric TRP 37. (B) Electrostatic surface potential of HbA. Blue and red colors are used to indicate the most positive and negative electrostatic potentials, respectively.

Figure 5. Lowest energy docked conformation representing the interaction profile of hesperitin with the binding pocket of HbA.

Scheme 1.

Absorption spectra of hesperitin (10 μM, dashed, right axis), hesperitin with HbA (dash with dots) and HbA (solid) in phosphate buffer. Inset: Structure of hesperitin.

Table 1

Binding and thermodynamic parameters for Hesperitin-HbA interactions

$^{\dagger}K_{SV}, M^{-1}$	$^{\dagger}K_q, M^{-1}s^{-1}$	$^{\dagger}K, M^{-1}$	$^{\dagger}n$	$^{\dagger}\Delta H^{\theta}$ (kcal/mol)	$^{\dagger}\Delta S^{\theta}$ (kcal/mol/K)	$^{\dagger}\Delta G^{\theta}$ (kcal/mol)
2.17×10^4	8.04×10^{12}	2.24×10^4	1.01	-3.16	0.009	-5.91

$^{\dagger}K_{SV}$; the Stern-volmer quenching constant; K_q , the bimolecular quenching constant; K , the binding constant and n , the number of binding sites. ΔH^{θ} and ΔS^{θ} are the enthalpy and entropy changes respectively and ΔG^{θ} is free energy change

Table 2

Fluorescence lifetime of HbA tryptophan at different flavonoid concentrations.

[Hesperitin], μM	A_1	τ_1 (ns) ^a	A_2	τ_2 (ns) ^a	χ^2
0 (HbA in Buffer)	0.775	725 ± 0.016	0.225	3.087 ± 0.024	1.01
12	0.793	690 ± 0.008	0.207	3.032 ± 0.017	1.121
24	0.792	693 ± 0.011	0.208	3.202 ± 0.031	1.09

^a λ_{ex} = 295 nm, λ_{em} = 335 nm, HbA concentration was 3 μM .

Table 3

List of residues forming the hesperitin binding pocket of HbA in α chain.

Helix No.	Helix 3	Helix 4	Helix 5	Helix 6	Helix 7
Residues present in the binding pocket of Hemoglobin	Thr 39, Lys 40, Tyr41, Phe43	His 58, Gly 59, Lys 61, Val 62, Ala 63, Leu 66, Ala 69, Val 70	Leu 80, Ala 82, Leu 83, Ser 84, Leu 86, His 87, Leu 91	Asn 97, Phe 98, Leu 101, Ser 102, Leu 105	Leu 129, Val 132, Ser 133, Leu 136

Table 4

Hesperitin-HbA specific interactions predicted by molecular docking.

HbA amino acid residues falling within 6 Å from bound flavonoids	Hydrogen bonding interactions (Number and details)
Leu 66, Val 62, His 58, Leu 105, Leu 29, Met 32, Ser 102, Leu 101, His 103, Leu 100, Leu 129, Val 132, Leu 136, His 87, Ser 133, Ala 130, Phe 98, Val 93, Lys 99, Asn 97, Asp 94, Val 96	One (between Ser 102 of HbA and 7-OH of hesperitin).