Interaction of glycyrrhizin with human haemoglobin

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This study investigates interaction of glycyrrhizin (an herbal therapeutic agent) with human haemoglobin. The interaction is confirmed by glycyrrhizin-induced quenching of absorbance and fluorescence data. Both hydrophobic and electrostatic interactions appear to be involved in glycyrrhizin–haemoglobin binding. The binding causes no change in the secondary structure of haemoglobin. The interaction decreases H_2O_2-induced iron release from haemoglobin and haemoglobin-mediated oxidative reactions. Glycyrrhizin inhibits ferryl-haemoglobin formation, peroxidase and esterase-like activities of the heme protein. Almost no oxygen is released from haemoglobin due to glycyrrhizin binding. The interaction thus reduces haemoglobin-mediated oxidative damage without affecting oxygen-binding capacity of the protein, and may be an advantage in therapeutic application of glycyrrhizin.

Keywords: Free iron, glycyrrhizin, haemoglobin, oxidative stress.

GLYCYRRHIZIN, a triterpenoid saponin, is the main water-soluble component of licorice (*Glycyrrhiza glabra*) root. It is composed of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid (Figure 1). Glycyrrhizin has long been studied for its medicinal activity in the treatment of liver diseases[1]. It is known to possess anti-oxidant and anti-inflammatory role in disease conditions like hepatitis B, C and hepatic steatosis[2]. Several studies, including our findings have shown that glycyrrhizin and glycyrrhetinic acid exhibit hypoglycemic and hypolipidemic effects in streptozotocin (STZ)-induced diabetes and fructose-induced metabolic syndrome in rat model[3–6]. There are reports on the interaction of glycyrrhizin with proteins, namely high-mobility group box 1 and serum complement C3 (refs 2 and 7). *In vitro* and *in vivo* studies from our laboratory[8–13] indicate that the structural and functional properties of haemoglobin (Hb) change in hyperglycemic condition and modified Hb by glucose, fructose and methyl glyoxal may be a source of free iron (Fe^{2+}) which, in turn, may cause free-radical generation through Haber–Weiss reaction[14]: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$, inducing oxidative stress. We have shown earlier that iron release from Hb in STZ-induced diabetes[5] and fructose-induced metabolic syndrome[6] is significantly reduced by glycyrrhizin, which may be due to its hypoglycemic effect. However, glycyrrhizin may also interact with Hb to reduce iron release from the heme protein. Since there has been no study on the interaction of glycyrrhizin with Hb, we have undertaken the present study to find if such an interaction is possible as well as its effect, if any, on iron-mediated free-radical reactions of the heme protein, which may be of significance for therapeutic evaluation of glycyrrhizin.

Materials and methods

Chemicals

Glycyrrhizin, arachidonic acid, *o*-dianisidine, para-nitro phenyl acetate, ferrozine and Sephadex G-100 were purchased from Sigma-Aldrich Company (St Louis, USA). Other chemicals used were of analytical grade and obtained from Sisco Research Laboratories Pvt Ltd (Mumbai, India).

Spectroscopic studies of Hb and glycyrrhizin interaction

Blood samples from healthy, non-smoking volunteers aged 25–28 years were used to prepare Hb by gel filtration[15]. The concentration of the Hb solution in 10 mM

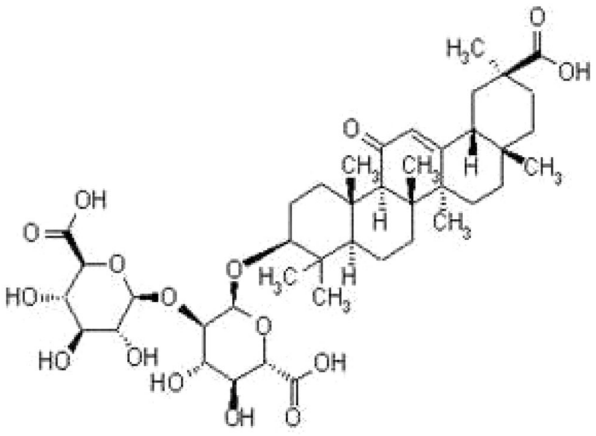

Figure 1. Chemical structure of glycyrrhizin.

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phosphate buffer, pH 7.4, was determined from its soret absorbance at 415 nm ($\varepsilon_{415\ nm}$ = 125 mM^{-1} cm^{-1}). Differential spectrophotometric study of Hb and glycyrrhizin interaction was performed using a spectrophotometer (Hitachi U-2800). Absorbance of Hb (5 μM) in the soret region (410–420 nm) was titrated with gradual addition of glycyrrhizin (0–36 μM) dissolved in 10 mM phosphate buffer, pH 7.4. Titration of protein fluorescence was performed using a spectrofluorimeter (Hitachi F-3010) by adding glycyrrhizin (0–36 μM) to Hb (10 μM). The emission peak emerged at 330 nm with excitation at 285 nm. The spectra were recorded after baseline correction. Absorbance of the maximum concentration (36 μM) of glycyrrhizin at the excitation wavelength was less than 0.05. The binding affinity constant (K) and the number of binding sites (p) were determined from the spectrophotometric and spectrofluorimetric data. The binding affinity constants were also determined spectrophotometrically for the interaction of glycyrrhizin with Hb at different NaCl concentrations (0, 0.0375, 0.075, 0.15, 0.20, 0.25, 0.30 and 0.40 M). For thermodynamic parameters, the binding studies were done spectrofluorimetrically using Hb and glycyrrhizin at different temperatures (10°C, 15°C, 20°C, 25°C, 30°C and 35°C). Circular dichroism (CD) spectra (200–300 nm) of Hb (5 μM) were recorded in the absence and presence of glycyrrhizin (100 μM) using a spectropolarimeter (Jasco J-600).

Molecular docking for glycyrrhizin interacting with Hb

Molecular docking was carried out to elucidate the interaction of glycyrrhizin with Hb. Three-dimensional atomic coordinates of the protein were obtained from the Brookhaven Protein Data Bank (PDB id: 1GZX). An on-line server PatchDock (www.bioinfo3d.cs.tau.ac.il/PatchDock/) was used to identify the probable binding site of glycyrrhizin in Hb. Among the top ten solutions obtained by docking analysis, the best scored solution having minimum energy was considered. It was analysed using Discovery Studio 4.0 Visualizer software.

H_2O_2 induced iron release from Hb and Hb-catalysed iron-mediated oxidative reactions

Ferrozine-detected free iron released from Hb (50 μM) by varying the concentration of H_2O_2 (0–1.5 mM) was estimated in the absence and presence of glycyrrhizin following the method of Panter[16]. Glycyrrhizin (100 μM) was added to the reaction mixtures before the addition of H_2O_2. Plasmid DNA (pGEM plasmid, 3.7 kb) degradation was assayed by 1% agarose gel electrophoresis in the presence of Hb (25 μM), varying concentrations of glycyrrhizin (25–100 μM) and H_2O_2 (0.3%). The reaction mixtures were incubated at 37°C for 1 h, after which the reactions were stopped with 10% glycerol[17] and subjected to electrophoresis. For arachidonic acid breakdown, the reaction mixture containing Hb (10 μM), varying concentrations of glycyrrhizin (5–20 μM), arachidonic acid (160 μM) and H_2O_2 (1 mM) was incubated at 37°C for 1 h. Thiobarbituric acid (TBA) reactive substance formed in the reaction mixture was measured according to the method of Sadrzadeh *et al.*[18].

Assay of peroxidase and esterase-like activities of Hb and ferryl-Hb formation

Peroxidase activity of Hb was estimated following the method of Everse *et al.*[19]. The reaction mixture contained 50 mM citrate buffer (pH 5.4), 1.5 μM Hb and different concentrations of glycyrrhizin (0, 4, 8 and 12 μM). The reactions were initiated by adding a mixture of 0.002% *o*-dianisidine and 17.6 mM H_2O_2. The increase in absorbance at 450 nm was recorded. Ferryl-Hb formation by H_2O_2 was measured in the absence and presence of glycyrrhizin (12.5, 25 and 50 μM). H_2O_2 (0.5 mM) was added to the freshly prepared Hb (50 μM) and percentage of ferryl-Hb formation in 3 min was estimated[20] from the fall of oxyhaemoglobin content as measured by $\varepsilon_{540\ nm}$ = 12.3 mM^{-1} cm^{-1} and $\varepsilon_{577\ nm}$ = 12.6 mM^{-1} cm^{-1}. Esterase activity of Hb was estimated following the method of Elbaum and Nagel[21]. The reaction mixture contained 5 μM Hb in 50 mM phosphate buffer (pH 7.4) and various concentrations of glycyrrhizin (0, 4, 8 and 12 μM). The reactions were initiated by adding 1.5 mM para-nitro phenyl acetate dissolved in acetone. The increase in absorbance at 400 nm was recorded.

Measurement of oxygen release from Hb

The change in dissolved oxygen content due to oxygen release from Hb (2 ml, 200 μM) by different concentrations of glycyrrhizin was detected by Clark-type oxygen electrode using an oxygraph machine (Hansatech Oxygraph). The output signal was recorded in the oxygraph chart as a function of time. The phosphate buffer or the protein sample in the absence of glycyrrhizin showed no change in the output signal. Considering 250 nmol dissolved oxygen present in 1 ml buffer[22], the oxygraph chart was calibrated in terms of nmol of oxygen released from the change in output signal due to the total depletion of dissolved oxygen from 2 ml buffer when 0.1 g sodium dithionite was added.

Results and discussion

Binding parameters for the interaction of glycyrrhizin with Hb were determined from the decrease of soret absorbance (at 415 nm) of Hb with gradual addition of

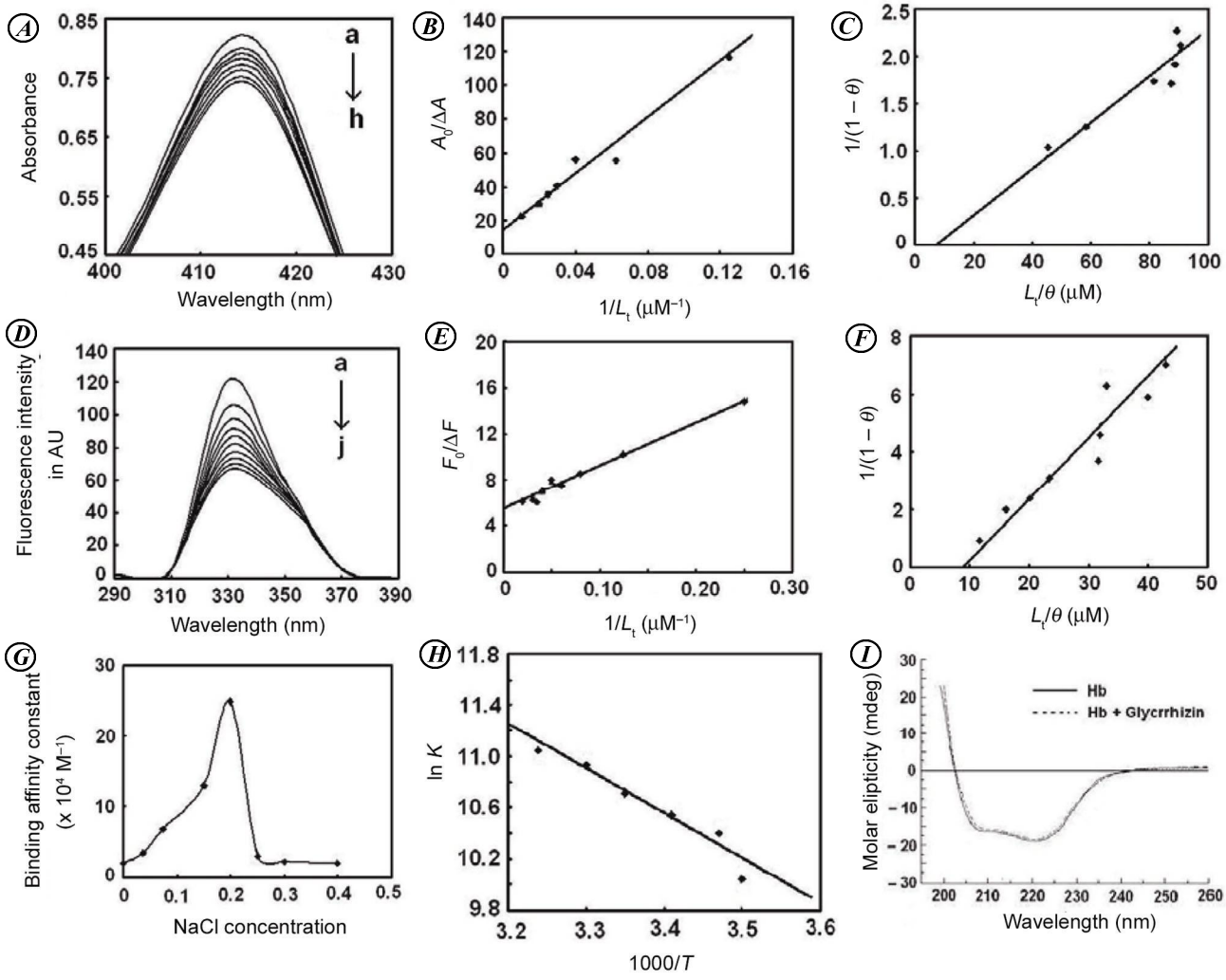

Figure 2. Spectroscopic studies for binding of glycyrrhizin with haemoglobin (Hb). ***A***, Representative spectra showing quenching of soret absorbance (at 415 nm) of Hb (5 μM) with gradual addition of glycyrrhizin: (a) no glycyrrhizin, (b) 4 μM glycyrrhizin, (c) 8 μM glycyrrhizin, (d) 12 μM glycyrrhizin, (e) 16 μM glycyrrhizin, (f) 20 μM glycyrrhizin, (g) 28 μM glycyrrhizin and (h) 36 μM glycyrrhizin. ***B***, Plot of $A_0/\Delta A$ versus $1/L_t$ for estimation of binding affinity constant *K*. ***C***, Plot of $1/(1-\theta)$ versus L_t/θ for estimation of the number of binding sites *p*. ***D***, Representative spectra showing quenching of Hb (10 μM) fluorescence at 330 nm (excitation 285 nm) with gradual addition of glycyrrhizin: (a) no glycyrrhizin, (b) 4 μM glycyrrhizin, (c) 8 μM glycyrrhizin, (d) 12 μM glycyrrhizin, (e) 16 μM glycyrrhizin, (f) 20 μM glycyrrhizin, (g) 24 μM glycyrrhizin, (h) 28 μM glycyrrhizin, (i) 32 μM glycyrrhizin and (j) 36 μM glycyrrhizin. ***E***, Plot of $F_0/\Delta F$ versus $1/L_t$ for estimation of binding affinity constant K. ***F***, Plot of $1/(1-\theta)$ versus L_t/θ for estimation of the number of binding sites *p*. ***G***, Change of binding affinity constant (*K*) for binding of glycyrrhizin with Hb in different NaCl molarities (0–0.4 M). ***H***, Plot of ln *K* versus 1000/*T* for binding of glycyrrhizin with Hb at different temperatures (10–35°C). ***I***, CD spectra of Hb (5 μM) without (solid line) and with (dashed line) 100 μM glycyrrhizin.

glycyrrhizin (Figure 2 *A*). Binding affinity constant (*K*) was estimated using the linear plot of $A_0/\Delta A$ versus $1/L_t$ (Figure 2 *B*) as follows[23]

$$A_0/\Delta A = A_0/\Delta A_{max} + A_0/\Delta A_{max}(1/K) \cdot (1/L_t),$$

where $\Delta A = A_0 - A$; A_0 and A represent the absorbance of Hb at 415 nm in the absence and presence of the added glycyrrhizin concentration (L_t). ΔA_{max} is the maximum change in absorbance. The intercept of the above plot on the $A_0/\Delta A$ axis corresponding to $1/L_t = 0$ gives an estimate of the $A_0/\Delta A_{max}$ and the slope measures the affinity constant, *K*. The possible number of binding sites (*p*) was estimated from the plot of $1/(1-\theta)$ versus L_t/θ (Figure 2 *C*) follows[23]

$$1/(1-\theta) = K \cdot L_t/\theta - K \cdot p \cdot A_t,$$

where θ is the fractional saturation of glycyrrhizin sites expressed as $\theta = \Delta A/\Delta A_{max}$ and A_t is the fixed concentration of protein. The values of *K* and *p* are shown in Table 1. Binding parameters for the interaction of glycyrrhizin with Hb were also determined from the quenching of protein fluorescence due to the addition of glycyrrhizin. Quenching of tryptophan fluorescence intensity of Hb in the presence of the added glycyrrhizin was measured

Table 1. Estimated values of binding parameters (K and p) for the interaction of glycyrrhizin with Hb

Glycyrrhizin concentration (μM)	Affinity constant (K) and binding sites (p) for glycyrrhizin binding to Hb			
	Spectrophotometric study		Spectrofluorimetric study	
	K ($\times 10^4$ M^{-1})	p	K ($\times 10^4$ M^{-1})	p
4–36	2.13 ± 0.41	1.53 ± 0.25	10.73 ± 2.11	1.80 ± 0.26

The results are mean ± SD of three independent observations.

from the change in the emission intensity at 330 nm (Figure 2 *D*). The quenching data were then analysed to obtain the binding affinity constant (K) and the possible number of binding sites (p). Figure 2 *E* shows a linear plot of $F_0/\Delta F$ versus $1/L_t$ following the equation given below[23]

$$F_0/\Delta F = F_0/\Delta F_{max} + F_0/\Delta F_{max}(1/K) \cdot (1/L_t),$$

where $\Delta F = F_0 - F$; F_0 and F represent the fluorescence intensity at 330 nm in the absence and presence of the added glycyrrhizin concentration (L_t). ΔF_{max} is the maximum change in fluorescence intensity. The intercept of the above plot on the $F_0/\Delta F$ axis corresponding to $1/L_t = 0$ gives an estimate of $F_0/\Delta F_{max}$ and the slope measures the affinity constant K. The possible number of binding sites (p) is estimated from the plot of $1/(1 - \theta)$ versus L_t/θ (Figure 2 *F*) as follows[23]

$$1/(1 - \theta) = K \cdot L_t/\theta - K \cdot p \cdot A_t,$$

where θ (the fractional saturation of glycyrrhizin sites) = $\Delta F/\Delta F_{max}$ and A_t is the fixed concentration of protein. The values of K and p are shown in Table 1. The binding parameters (K and p) obtained by spectrophotometric and spectrofluorimetric studies are comparable, indicating both ground state and excited state complex formation between Hb and glycyrrhizin.

To determine the nature of glycyrrhizin–Hb interaction, binding studies were done spectrophotometrically at different molarities of NaCl solution (0–0.4 M). The K values for Hb–glycyrrhizin interaction increased steadily with the increase in NaCl molarity from 0 to 0.2 M, revealing hydrophobic mode of interaction as the major governing factor (Figure 2 *G*). However, electrostatic interactions could still have some contribution in the binding process as indicated by the sudden fall of K value as NaCl concentration exceeds 0.2 M. The decrease in K value might be due to the loss of electrostatic contribution at higher ionic strengths (0.25–0.4 M). No change in the K value in the higher range of NaCl molarity also indicated hydrophobic interaction.

Enthalpy and entropy changes accompanying the binding process were estimated to have further insight into the nature of interaction. The temperature dependence of the binding constant was measured spectrofluorimetrically from the emission spectra of Hb in the presence of different concentrations of glycyrrhizin in the temperature range 10–35°C. This temperature range did not have any effect on fluorescence emission spectra of Hb. Figure 2 *H* shows the van't Hoff isochore plot of ln K versus $1000/T$, where T is the absolute temperature at which glycyrrhizin binding to Hb is studied. The slope of this plot gives a measure of the standard enthalpy change ($\Delta H°$) as follows[24]

$$\ln K = -\Delta G°/RT = -\Delta H°/RT + \Delta S°/R,$$

where $\Delta G°$ is the standard free energy change upon binding of the ligand to the protein, $\Delta S°$ the corresponding standard entropy change and R is the universal gas constant. Assuming no significant temperature dependence of $\Delta H°$ in the temperature range used, the values of $\Delta G°$, $\Delta H°$ and $\Delta S°$ are estimated as shown in Table 2. Hb binding to glycyrrhizin appeared to be endothermic (with unfavourable positive $\Delta H°$ value). But $\Delta S°$ assumed such a high positive value that the entropic contribution ($T\Delta S°$) in the equation: $\Delta G° = \Delta H° - T\Delta S°$ ultimately enabled $\Delta G°$ to become negative, making the binding process a favourable one. Thus the binding of glycyrrhizin to Hb is largely an entropy-driven process and these features are expected for binding of ligands by primarily hydrophobic process[25]. Such hydrophobic interaction possibly involves an intermediate collisional process which is enhanced with the rise in temperature resulting in gradually increased binding constant.

To find the possible effect of glycyrrhizin binding on the secondary structure of Hb, far UV–CD spectroscopy was performed (Figure 2 *I*). The CD spectrum (shown by solid line) of Hb sample showed two characteristic peaks of negative elipticity at 208 and 222 nm, indicating its predominantly α-helical secondary structure. The α-helix content appeared to be 74.52%, as estimated by the method of Chen *et al.*[26]. The CD spectrum of Hb obtained in the presence of glycyrrhizin (100 μM; shown by dashed line) indicated almost identical α-helix content (73.38%). The interaction thus occurs without much distortion of the secondary structure of the protein, which may be of significance for therapeutic application of glycyrrhizin.

Table 2. Estimated values of thermodynamic parameters for Hb–glycyrrhizin interaction

Glycyrrhizin concentration (μM)	Thermodynamic parameters for binding with Hb		
	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/deg/mol)	$\Delta G°$ (kcal/mol)
4–36	+5.25 ± 0.13	+25.24 ± 4.51	–6.59 ± 0.79

The results are mean ± SD of three independent observations.

Table 3. Amino acid residues of Hb interacting with glycyrrhizin and nature of interaction

Amino acid residue	Distance from the ligand (Å)	Nature of interaction
Pro 95, α_1 chain	3.8	Hydrophobic (alkyl–alkyl)
Ala 130, α_2 chain	4.3	Hydrophobic (alkyl–alkyl)
Leu 105, β_2 chain	4.3	Hydrophobic (alkyl–alkyl)
Tyr 35, β_2 chain	3.2	Hydrophobic (pi–alkyl)
Trp 180, β_2 chain	3.4	Hydrophobic (pi–alkyl)
Thr 134, α_2 chain	3.4	Hydrogen bonding
Asn 108, β_2 chain	3.0	Hydrogen bonding
Val 1, α_2 chain	3.7	Electrostatic

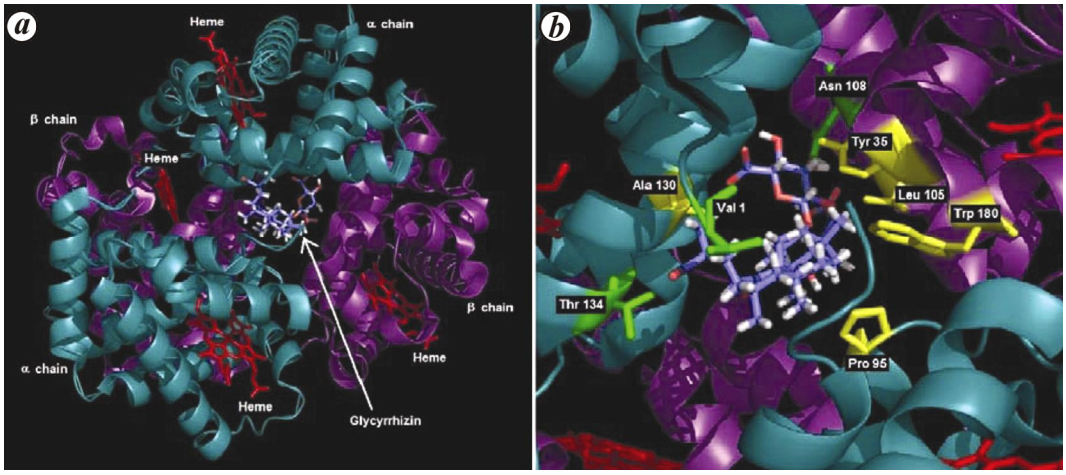

Figure 3. Molecular docking of Hb and glycyrrhizin interaction. ***a***, Binding site of glycyrrhizin within Hb molecule. ***b***, Amino acid residues of Hb involved in interaction with glycyrrhizin. The amino acid residues were within 6 Å from the bound triterpene. The amino acids with green colour established hydrophilic (electrostatic and hydrogen bonding) interaction and those acids with yellow colour established hydrophobic interaction with glycyrrhizin.

Molecular docking of glycyrrhizin–Hb interaction is shown in Figure 3 and Table 3. The binding of glycyrrhizin with Hb might involve both hydrophobic and electrostatic interactions. Possible hydrophobic interactions of the triterpene were found to be with the hydrophobic side chains of Pro 95 (α_1 chain), Ala 130 (α_2 chain), Leu 105 (β_2 chain), Tyr 35 (β_2 chain) and Trp 180 (β_2 chain) residues of Hb. Glycyrrhizin binding might also involve hydrogen bonding with Thr 134 (α_2 chain) and Asn 108 (β_2 chain) residues of the heme protein. Molecular docking indicated a possibility for electrostatic interaction between the free amino group of N-terminal residue (Val 1) of the α_2 chain of Hb and carboxyl group of glycyrrhizin. Similar binding nature involving both hydrophobic and electrostatic interaction of glycyrrhizin has been reported with high-mobility group box 1 protein[2]. The decrease in K value at higher ionic strength, as shown in Figure 2 *G*, may be due to the loss of hydrogen bonding and electrostatic interaction between the protein and glycyrrhizin. Increased Na^+ and Cl^- ions may mask the oppositely charged groups and thereby hydrogen bonding and electrostatic interactions become less feasible.

H_2O_2 is known to release iron from heme proteins like Hb and myoglobin[8,9,27]. H_2O_2-induced iron release from Hb was measured by ferrozine reaction in the absence and presence of glycyrrhizin. Iron release from Hb significantly increased with gradual increase of H_2O_2 concentration (0, 0.25, 1.0, 1.25, 1.5 mM) as shown in Figure 4 *a*. Addition of glycyrrhizin (100 μM) resulted in significant diminution of iron release at each H_2O_2 concentration.

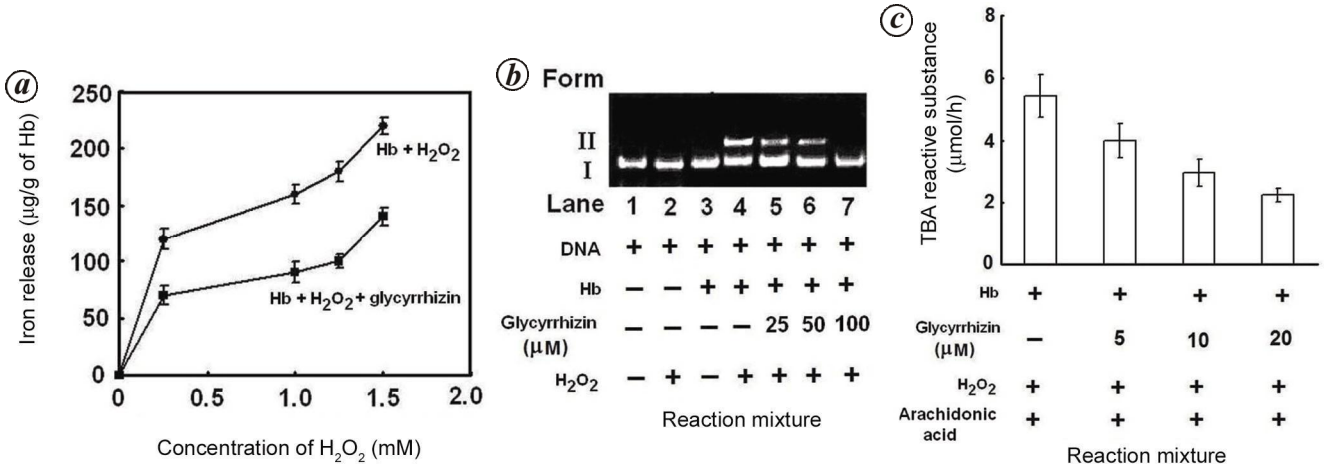

Figure 4. Effect of Hb–glycyrrhizin interaction on iron release from Hb and Hb-mediated free radical reactions. ***a***, H_2O_2-induced iron release from Hb in the absence and presence of glycyrrhizin. The results are mean ± SD of five independent observations. ***b***, H_2O_2-induced plasmid DNA breakdown by Hb in the absence and presence of glycyrrhizin. This is a representative picture from three different experiments. ***c***, H_2O_2-induced arachidonic acid degradation by Hb. The results are mean ± SD of three independent observations.

Hb–glycyrrhizin interaction may be responsible for preventing H_2O_2-induced iron release from the heme protein. Free iron, in the presence of H_2O_2, produces hydroxyl radical ($^{\cdot}OH$) which may cause oxidative reactions[14]. H_2O_2-induced Hb iron-mediated oxidative reactions (DNA and arachidonic acid degradation) were assayed in the absence and presence of glycyrrhizin. Figure 4 *b* shows plasmid DNA degradation in the presence of Hb, H_2O_2 and varying concentrations of glycyrrhizin. Plasmid DNA (lane 1) was not degraded in the presence of only H_2O_2 (lane 2) or Hb (lane 3), as shown by the presence of form I only. DNA degradation occurred considerably in the presence of both Hb and H_2O_2 (lane 4), as indicated by the appearance of form II. Glycyrrhizin, when added prior to H_2O_2, resulted in significant inhibition of DNA breakdown (lanes 5–7). Arachidonic acid degradation by Hb in the presence of H_2O_2 decreased with increasing concentration of glycyrrhizin (Figure 4 *c*). Interaction of glycyrrhizin with Hb might be associated with the inhibition of plasmid DNA and arachidonic acid degradation. Glycyrrhizin did not exhibit ferrous (Fe^{2+}) ion chelation property, as indicated by almost no change in absorbance at 560 nm (ferrozine reaction) in different reaction mixtures containing 0.1 mg ferrous iron and different concentrations (0, 50, 100 and 200 µM) of glycyrrhizin (result not shown). Therefore, Hb–glycyrrhizin binding reduces H_2O_2-induced Fe^{2+} release from the heme protein, which in turn decreases Fe^{2+}-mediated hydroxyl radical ($OH^{\bullet}$) generation causing reduced DNA damage and arachidonic acid peroxidation. We have shown earlier that both in diabetic patients[8,9] and animal (rat) models for diabetes[5,12] and metabolic syndrome[6], glycated Hb is a source of free iron and oxidative stress. Kell[28] has also discussed the role of poorly liganded iron in generating oxidative stress in different pathological conditions. Glycyrrhizin is known to ameliorate the harmful effects of glycated Hb by lowering blood glucose, Hb glycation and associated free-radical reactions[5,6]. Our present findings, however, indicate that besides hypoglycemic activity, glycyrrhizin may also be effective to reduce oxidative stress by its interaction with Hb.

Peroxidase activity of Hb was estimated and found to be reduced in the presence of glycyrrhizin, as demonstrated by a decrease in absorbance at 450 nm (Figure 5 *a*). H_2O_2 interacts with Hb to yield a potent oxidant ferryl-Hb capable of oxidizing a wide variety of electron donors resembling peroxidase-like activity[20]. Addition of H_2O_2 to oxy-Hb in a molar ratio of 10 : 1 causes a decrease in the absorbance at 540 and 577 nm (Q-bands). This is due to oxidation of oxy-Hb to ferryl-Hb. Percentage of ferryl-Hb formation was reduced with increasing concentration of glycyrrhizin (Figure 5 *b*). Glycyrrhizin itself had no effect on the degradation of H_2O_2 (result not shown). Reduction of peroxidase-like activity and ferryl-Hb formation with increasing concentration of glycyrrhizin may thus be related to its interaction with Hb. Ferryl-Hb is able to oxidize proteins, nucleic acids and lipids[19,20,29], and can also generate oxidative stress in different pathological conditions with increased H_2O_2 production. Inhibition of ferryl-Hb formation by glycyrrhizin may thus help in reducing oxidative stress in disease conditions, particularly in diabetes, in which glycated heme proteins exhibit enhanced peroxidase activities through ferryl forms[9,27].

Hb possesses esterase activity[21] and the activity was found to be decreased with increasing concentration of glycyrrhizin, as shown by a decrease in absorbance at 400 nm (Figure 5 *c*). Earlier report from our laboratory indicates that esterase activity of Hb enhances during diabetic condition and may aggravate the diabetic complications[30]. Reduction of esterase activity by glycyrrhizin may, therefore, be advantageous in such a condition.

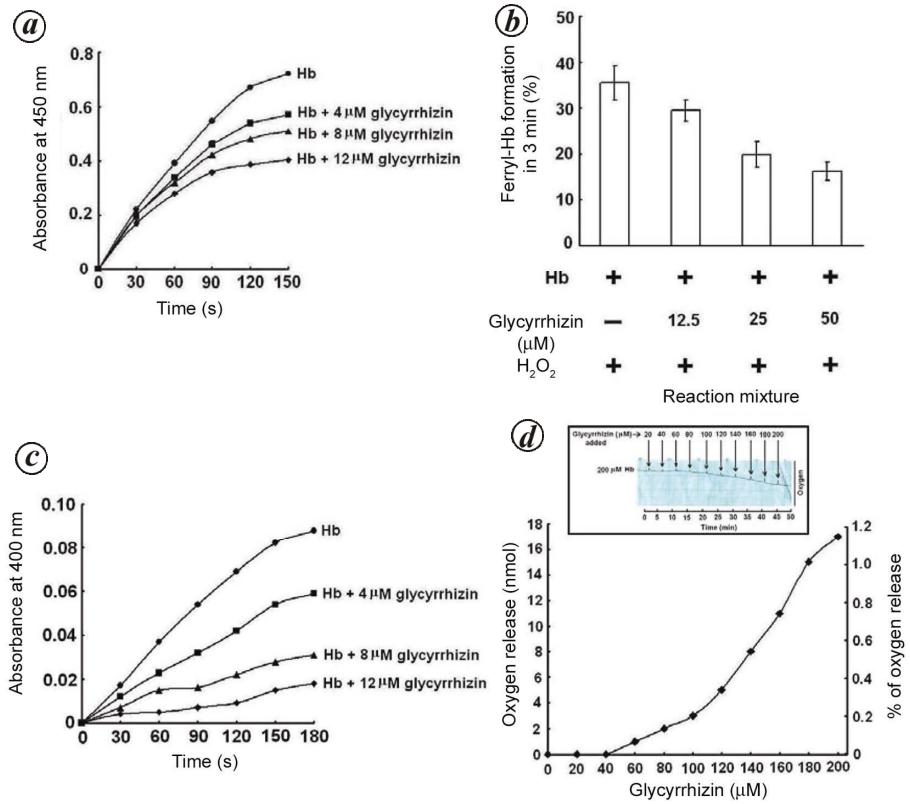

Figure 5. Effect of glycyrrhizin on enzymatic activities and oxygen-binding capacity of Hb. ***a***, Peroxidase-like activity of Hb in the absence and presence of glycyrrhizin. ***b***, H_2O_2-induced ferryl-Hb formation in the absence and presence of glycyrrhizin. The results are mean ± SD of three independent observations. ***c***, Esterase activity of Hb in the absence and presence of glycyrrhizin. ***d***, Extent of oxygen release from Hb (200 μM, 2 ml) due to interaction with different concentrations of glycyrrhizin. (Inset) Representative oxygraph chart of oxygen release from Hb (200 μM, 2 ml) using different concentrations of glycyrrhizin.

Glycyrrhizin-induced oxygen release from Hb was estimated using an oxygraph instrument. Up to 40 μM glycyrrhizin, no oxygen was released from 200 μM Hb then there was slow release (Figure 5 *d*). However, even with 200 μM glycyrrhizin the percentage of oxygen release (approximately 1.2%) was not significant, indicating no adverse effect on the heme protein. This is in contrast with significant oxygen release from Hb due to interaction with different drugs like phenothiazines[31], porphyrins[32], etc.

The mechanisms of decreased iron release, ferryl formation and reduction of peroxidase and esterase-like activities of Hb in the presence of glycyrrhizin are not clearly understood. Molecular modelling indicates that glycyrrhizin-binding site in Hb is far apart (more than 10 Å) from the heme groups. Therefore, glycyrrhizin binding may not directly affect the heme group of the protein, which is also supported from the negligible amount of oxygen released from Hb due to the addition of glycyrrhizin (Figure 5 *d*). However, presence of glycyrrhizin may cause steric hindrance or local perturbation that inhibits the binding of substrates like H_2O_2, *o*-dianisidine or para-nitro phenyl acetate at the active sites of the protein. Further studies are therefore necessary to elucidate glycyrrhizin-induced changes in enzymatic activities of Hb.

Conclusion

Glycyrrhizin interacts with Hb. The nature of interaction is predominantly hydrophobic with electrostatic contribution to some extent. The interaction does not change the protein with respect to its secondary structure and oxygen content. However, the interaction exhibits some beneficial effects like reduced H_2O_2-induced Hb-mediated Fe^{2+} release, free radical generation and oxidative stress, which may contribute to the therapeutic activity of glycyrrhizin.

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