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Characterization of diadzein–hemoglobin binding using optical spectroscopy and molecular dynamics simulations

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ABSTRACT

The present study establishes the effectiveness of natural drug delivery mechanisms and investigates the interactions between drug and its natural carrier. The binding between the isoflavone diadzein (DZN) and the natural carrier hemoglobin (HbA) was studied using optical spectroscopy and molecular dynamics simulations. The inherent fluorescence emission characteristics of DZN along with that of tryptophan (Trp) residues of the protein HbA were exploited to elucidate the binding location and other relevant parameters of the drug inside its delivery vehicle HbA. Stern–Volmer studies at different temperatures indicate that static along with collisional quenching mechanisms are responsible for the quenching of protein fluorescence by the drug. Molecular dynamics and docking studies supported the hydrophobic interactions between ligand and protein, as was observed from spectroscopy. DZN binds between the subunits of HbA, ~15 Å away from the closest heme group of chain α1, emphasizing the fact that the drug does not interfere with oxygen binding site of HbA.

1. Introduction

Understanding and designing drug delivery systems is an increasingly promising discipline in pharmaceutical development, allowing sensible and reasonable handling of the pharmacological profiles of drugs and the beneficial roles associated with them. Recently, erythrocytes (red blood cells) have come into prominence as drug delivery systems, due to their biocompatibility, biodegradability, usability and easy loading capability [1–4]. But the factor that critically limits their use as drug carrier is their degradation *in vivo* by the reticuloendothelial system (RES) [5] and in some cases the toxic effects caused by erythrocytes under certain conditions [6,7]. In order to design and improve an erythrocyte-based drug delivery system, the binding interactions of drugs with the main component of erythrocytes, hemoglobin (HbA), have to be fully understood. Hemoglobin is the most abundant blood protein and consists of two α and two β subunits which are noncovalently associated within erythrocytes as a 64.5 kDa tetramer [8–10]. Delivery of oxygen from the lungs to respiring tissues is the main function of the heme group of hemoglobin. Recently, using radiolabeled HbA, the liver was confirmed as the main site for HbA uptake [7]. By using HbA as a drug carrier, the natural mechanisms of HbA uptake and decomposition, the latter of which leads to unloading of any bound compounds, can be employed to selectively transport therapeutic drugs to physiological sites of interest for the treatment of diseases.

Isoflavones are naturally occurring polyphenolic compounds with antioxidant properties, and exert many health benefits such as lowering the risk of breast and prostate cancers, osteoporosis and cardiovascular diseases [11,12]. Genistein and daidzein (DZN) are isoflavonoids commonly found in legumes. Both of these isoflavones have a wide spectrum of physiological and pharmacological functions including antiestrogenic [13,14], anticancer [15,16], anti-inflammatory [17], cardioprotective [18] and enzyme-inhibitory effects [13,14]. The Dietary Reference Intakes, established by the National Academy of Sciences [19], highlight that plant based polyphenols are important dietary constituents. The objective of the present study is to examine the structural and binding interactions between HbA and diadzein (DZN, structure shown in Scheme 1) by absorption, fluorescence and circular dichroism spectroscopy, along with docking and molecular dynamics (MD) simulations. To study drug transport using a fluorescence-based approach, the availability of a non-invasive and photo-stable fluorescent substrate is required [20]. The intrinsic fluorescence of

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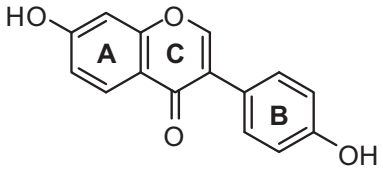

Scheme 1. Structure of the isoflavone diadzein (7-hydroxy-3(-4′-hydroxy-phenyl)-chromen-4-one).

DZN, which can probe the binding microenvironment, combined with its high therapeutic potency and low systemic toxicity fulfils these requirements. The results obtained for DZN in the protein matrix are useful for the identification of its location in HbA at the molecular level. The strong binding interactions between ligand and protein are evident from the high fluorescence anisotropy as well as the computational studies which show the presence of hydrophobic interactions between DZN and the surrounding amino acids in the HbA matrix.

The significance of the present work lies in the fact that HbA is the natural drug carrier in physiological systems and its binding studies with the therapeutically potent fluorescent isoflavone DZN provide insights into the underlying mechanisms of interactions between the drug and its delivery vehicle. This approach can be well extrapolated to drugs of similar nature and helps in understanding the pharmacokinetics and biological activities of plant flavonoids.

2. Experimental

2.1. Materials

Lyophilized powder of human hemoglobin (molecular weight 64,500 Da), diadzein, and phosphate buffer were purchased from Sigma Chemicals, USA, and used without further purification after confirming their purity by comparing their electronic absorption and emission spectra with published data [11]. Solvents used were of spectroscopic grade and obtained from Sigma. Purity of DZN was further confirmed by thin layer chromatography which showed only one spot under UV light. Absorption and fluorescence spectroscopic measurements were performed with DZN concentrations of 1×10^{-5} M. HbA was dissolved in pH 7.4 phosphate buffer solution (1×10^{-2} M) and the HbA stock solution (2×10^{-4} M) was kept in the dark at 277 K. The protein concentration was determined spectrophotometrically using the molar extinction coefficient of HbA at 276 nm (120,808 M^{-1} cm^{-1}) [21].

For fluorescence quenching studies, the HbA concentration was kept constant at 10^{-5} M. Varying aliquots of concentrated methanolic solution of DZN were added to obtain final concentrations ranging from 0 to 2.5×10^{-5} M. The concentrations of methanol were always kept <1% (by volume) in all samples.

2.2. Spectroscopic measurements

Steady state absorption spectra were recorded with Shimadzu UV2550 spectrophotometers. Steady state fluorescence measurements were carried out with Shimadzu RF5301 (equipped with a Fisher temperature controlled accessory) spectrofluorometers. The fluorescence anisotropy (r) measurements were performed on a Fluoromax-3 (Jobin Yvon Horiba) spectrometer. The values were obtained using the expression $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} are the vertically and horizontally polarized components of probe emission with excitation by vertically polarized light at the respective wavelength and G is the sensitivity factor of the detection system [21]. Each intensity value used in this expression represents the computer-averaged values of five successive measurements. All spectral measurements were carried out at room temperature (298 K) with freshly prepared solutions. The emission spectra were taken by exciting the samples and measuring the emissions at relevant wavelengths; appropriate blanks were subtracted for respective measurements. The bandwidths were 3 nm for both the excitation and emission with integration times of 1 s. All spectra were collected using quartz cuvettes with 1 cm pathlengths.

Circular dichroism spectra were acquired with a J-710 spectropolarimeter (Jasco). The scan rate was 100 nm/min, and three consecutive spectra were averaged to produce the final spectrum.

2.3. Molecular docking

AutoDock4 [22] was employed to gain an insight into the DZN binding with HbA. 3D atomic coordinates of HbA were obtained from the Brookhaven Protein Data Bank (PDB ID 2D60) and prepared for docking. Hemoglobin was considered as a tetramer. All hetero atoms were deleted and non-polar hydrogens were merged. The Kollman united-atom charge model was applied to the protein. Particular attention was given to the parameterization of the porphyrin rings. Partial atomic charges for the porphyrin ring were assigned using the Gasteiger–Marsili method while the state of the iron (Fe) was added manually. Atomic solvation parameters and fragmental volumes were added to the protein. Grid maps used by the empirical free-energy scoring function in AutoDock were generated. A grid box of $100 \times 100 \times 100$ grid points in size with a grid-point spacing of 0.375 Å was considered for docking. The map was centered such that it covered the entire protein including all possible binding sites.

The 3D structure of DZN was built using the HYPERCHEM [23] molecular builder module and optimized using the AM1 semi-empirical method to an RMS convergence of 0.001 kcal/(Å mol) with the Polak–Ribiere conjugate gradient algorithm implemented in the HYPERCHEM 7.5 package. Rotatable bonds were assigned for the ligand and partial atomic charges were calculated using the Gasteiger–Marsili method after merging non-polar hydrogens. 100 Docking runs were performed 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 of 0.8, 0.02 and 1, respectively.

2.4. Molecular dynamics of the HbA–diadzein complex

GROMACS molecular dynamics code [24] with OPLS all atom force-field [25] were employed to carry out the MD simulations on free hemoglobin and its complex with DZN. The parameters for the ligand (DZN) were developed from OPLS force-field [25] defined atomic groups. The partial atomic charges, which were adjusted to keep the charge neutrality of the atomic groups and make the ligand charge neutral, were tested by comparing the Gromacs optimized structure with (i) the parameter-independent QM optimized structure of DZN in vacuum, and (ii) the QM/MM optimized structures of DZN in explicit water. For QM code, we employed CPMD [26a] and for QM/MM, we employed GROMACS–CPMD [26b]. For QM/MM, the DZN molecule was considered in the QM sub-system and the water was considered in MM sub-system and their interphase interaction was described by the QM/MM Hamiltonian [26].

The crystal structure coordinates of HbA obtained from the protein data bank (PDB ID: 2D60) and used in the docking study, were considered for the simulation of free HbA. For HbA–DZN complex, the lowest energy docked complex obtained from the docking study was used for MD simulation. The OPLS parameters for the heme prosthetic group were taken from the previously published parameter set [27]. The HbA structure was primarily subjected to