INFLUENCE OF pH ON THE INTERACTION BETWEEN HUMAN SERUM ALBUMIN AND SERUM TRANSFERRIN WITH CYCLOPHOSPHAMIDE: SPECTROSCOPIC, ZETA POTENTIAL AND MOLECULAR DYNAMIC INVESTIGATION

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For the first time, the interaction between cyclophosphamide (Cyc) with human serum transferrin (hTf) and human serum albumin (HSA) was studied at different pH by fluorescence quenching, three-dimensional fluorescence, circular dichroism (CD), zeta potential and molecular dynamic techniques. The fluorescence spectroscopy experiments were performed in order to determine conformational changes, possibly due to a discrete reorganization of Trp residues during hTf-Cyc and HSA-Cyc binding at different ligand concentrations. Also, the quenching properties of the drug-serum transferrin and serum albumin complexes were studied and a differentiation was carried out between static and dynamic quenching. The binding affinity and number of binding sites were obtained for the interaction between Cyc with HSA and hTf at different pH. The variation of the Ksv values suggested that hydrophobic and electrostatic interactions were the predominant inter-molecular forces stabilizing the complex. Moreover, the CD data revealed that the presence of Cyc decreased the α-helix content of hTf and HSA and induced a remarkable unfolding of the proteins. This confirmed certain micro-environmental and conformational changes of the hTf and HSA molecules. The binding distance (r) between Cyc and the Trp residues of hTf and HSA was obtained according to Förster’s theory of non-radiative energy transfer.

This study on the interaction of drugs with proteins should be helpful for realizing the distribution and transportation of drugs in vivo, elucidating the action mechanism and dynamics of a drug at the molecular level. It should moreover contribute to understanding the pharmacokinetic and pharmacodynamic mechanisms of the drug.

Key words: human serum albumin, serum transferrin, spectroscopy, cyclophosphamide, molecular dynamics.

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INTRODUCTION

Serum albumins are the most widely studied proteins in the plasma. A three-dimensional structure of HSA has been determined through X-ray crystallographic measurements (1). HSA plays a key role in the transport, distribution and metabolism of many endogenous and exogenous ligands, such as fatty acids, metabolites and drugs (2, 3). Crystal structure analysis has demonstrated that HSA has two drug binding sites: IIA and IIIA. A large hydrophobic cavity is present in the IIA sub-domain (4).

Transferrins are a family of glycoproteins whose members (serum transferrin, ovo-transferrin and lactoferrin) are responsible for transporting iron and/or preventing bacterial growth by sequestering iron. hTf is a 80-kDa glycoprotein with two homologous lobes, termed the N- and C-lobes, each of them folded to form a cleft in which ferric iron (Fe$^{3+}$) binds (5). All of the transferrins consist of a single polypeptide chain of about 680 amino acids (6), which can be divided into two homologous regions: the N-terminal domain (residues 1–336) and the C-terminal domain (residues 337–679). The latter contains a deep cleft capable of binding a metal ion. In turn, each lobe consists of two areas (N$_1$ and N$_2$ and C$_1$ and C$_2$) that are connected by a flexible hinge, comprising a series of α-helices, which overlay a central β-sheet backbone. When the binding sites are empty, the protein is described as apo-hTf, as opposed to holo-hTf, when the sites are occupied. Iron binding by apo-hTf is very strong at an extra-cellular pH of about 5.9 in combination with a low endosomal pH of 5.5 and the binding of holo-hTf as its receptor (7, 8).

Cyc (Scheme 1) is an alkylating agent related to mustard gas. It causes cell death by interfering with the way the genetic material (DNA) in the cell multiplies. Cyc is a white crystalline powder with the molecular formula C$_7$H$_{15}$Cl$_2$N$_2$O$_2$P$\cdot$H$_2$O and a molecular weight of 279 Da. The chemical name of Cyc is 2-(bis (2-chloroethyl) amino) tetrahydro-2H-1, 3, 2-oxazaphosphorine 2-oxide monohydrate. Cyc is used to treat many forms of cancer. The type and extent of the disease, as well as the body size in question determine the exact dose and administration schedule of this medicine. Up to now, several extensive investigations (9) on interactions between

![Scheme 1. The chemical structure of Cyc.](image-url)
proteins and components of living systems or pharmaceutical molecules have been carried out, since such studies can provide information on the features that affect the therapeutic effect of the drugs.

The HSA-Cyc and hTf-Cyc binding process plays a very important part in drug pharmacology and pharmacokinetics and strongly influences the absorption, distribution, metabolism and excretion properties of typical drugs. Moreover, the therapeutic drug effect is related to the balance between the bound and unbound fraction of the drug. Therefore, studies on this aspect can provide information of the structural features that determine the therapeutic influence of drugs, and have become an interesting research field in life science, chemistry, and clinical medicine. In addition, protein quantitative determination has received increasing attention both within the fields of biochemistry, chemistry, clinical medicine, and immunodiagnostics, and as a reference for the measurement of other components in biological samples.

This study on the interaction of Cyc to HSA and hTf should prove helpful for realizing the distribution and transportation of drugs \textit{in vivo}, as well as for elucidating the action mechanism and dynamics of drugs at the molecular level. Therefore, the present work describes a systematic investigation by spectroscopic methods and molecular dynamics of the mechanisms, characters and binding parameters, as well as the transfer efficiency of the energy of interaction between Cyc and either HSA or hTf.

\section*{MATERIALS AND METHODS}

\section*{MATERIALS AND SOLUTIONS}

All reagents were of analytical grade and purchased from Sigma. HSA and hTf solutions (4.52 \times 10^{-6} \text{ mM} and 3.79 \times 10^{-6} \text{ mM}) were prepared in 50-mM phosphate buffer with pH= 6.4, 7.4, 8.4. A Cyc solution (5.0 \times 10^{-5} \text{ mM}) in phosphate buffer was also prepared. The solutions were stored in a refrigerator at 4 \degree C in the dark. All pH measurements were performed with a Metrohm digital pH-meter (Metrohm, Germany).

\section*{Apparatus}

\section*{Absorption Measurements}

UV–vis spectroscopy absorption spectra were obtained with a Jasco V-630 spectrophotometer. The optical system was based on a slit beam with a grating bandwidth of 5 nm. The light source was a xenon lamp. The absorption measurements of all samples were carried out using quartz cells with a 1-cm optical path. All measurements were performed at room temperature.
Fluorescence measurements

Fluorescence measurements were performed on a spectrofluorometer Model F-2500 (Hitachi, Japan) with a 150-W xenon lamp, a 1.0-cm quartz cell and a thermostat bath. The width of the excitation and emission slit was set at 5.0 nm. The excitation wavelength was set at 280 nm, and the emission wavelength was recorded between 300 nm and 500 nm. The operation software automatically corrected the spectral scan for photomultiplier characteristics. Moreover, the fluorescence intensities were corrected for inner filter and dilution effects before analysis of the binding and quenching data. An appropriate buffer was taken as a blank and subtracted from the experimental spectra to correct for the background of fluorescence. All measurements were performed at room temperature.

Synchronous fluorescence spectroscopy

Synchronous fluorescence spectroscopy was carried out by simultaneously scanning the excitation and emission monochromators. The spectra only showed the Tyr and Trp residues of hTf and HSA when the wavelength interval (∆λ) was 15 nm and 60 nm, respectively.

Circular dichroism spectroscopy

Far-UV CD experiments were performed on a Jasco-815 spectropolarimeter equipped with a Jasco 2-syringe titrator. Spectra were recorded on samples (with HSA and hTf concentrations of 4.52×10⁻⁶ mM and 3.79×10⁻⁶ mM, respectively) in a 1-mm path length quartz cuvette. A bandwidth of 1 nm and a response of 2 s were used, with a scanning rate at 50 nm min⁻¹ to obtain final spectra as an average of three scans. The induced ellipticity was obtained by subtracting the ellipticity of the drug from that of the drug-hTf mixture at the same wavelength. The obtained value was given in degrees. The results are expressed as the mean residue ellipticity (θ), defined as (θ) = 100×θ_{obsd}(LC), where θ_{obsd} is the observed ellipticity in degrees, C is the concentration in residue mol cm⁻³, and L is the length of the light path in cm.

The CD results were given as MRE (Mean Residue Ellipticity) in deg cm² dmol⁻¹, which is defined as (10):

\[ MRE = \theta_{obs} / C_p n l \times 10 \]

Here, \( \theta_{obs} \) is the CD in milli-degree, \( C_p \) is the mole fraction; \( n \) is the number of amino acid residues and \( l \) is the path length of the cell. The α-helical contents could be calculated from the MRE values at 208 nm using the following equation:

\[ \alpha - \text{helical}(\%) = (–MRE_{208} – 4000 / 33000 – 4000) \times 100 \]

In this case, MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE where the form and random coil conformations cross at 208 nm, and 33000 is the MRE value of the α-helix at 208 nm.
Zeta potential

Colloidal particles accumulate charge at their surface which can be expressed as a surface potential. The latter is an important factor for determining the magnitude of charge based colloidal interactions of a particle, most crucially electrostatic repulsion of other like-charged particles.

The surface charge perturbs the ionic distribution in the medium surrounding it. First, a layer of tightly bound counter-ions (i.e., of opposing charge) accumulates at the particle surface, the so-called stern layer, and beyond this a region of decaying excess concentration, the so-called diffuse layer, extends a considerable distance (~ nm) into the surrounding aqueous media. Measuring the colloidal charge typically involves applying an electrical voltage to the particle and measuring the speed of movement induced. In practice, one or more layers of hydrated ions move with the particle and thus the determined potential is not that at the surface but rather at a short, undefined distance into the diffuse layer. It is known as the zeta potential.

Surface charge in protein particles is due to the partial ionization of various amino acid residues. The effective charge on a protein particle is affected by pH, ionic strength and the accumulation of ligands or surface charge at the interface. Zeta-potential measurements were performed by a Zeta-sizer (Nano-ZS) from Malvern Instruments and a Dispersion Technology Software (DTS). The Zetamaster 5002 took the average of five measurements at the stationary level. The cell used a 5 mm×2 mm rectangular quartz capillary. The temperature of the experiments was 298 ± 0.01 K. All measurements were carried out using HSA, hTf and Cyc with concentrations of 4.52×10⁻⁶ mM, 3.79×10⁻⁶ mM and 5.0×10⁻⁵ mM, respectively.

Three-dimensional fluorescence spectra

Three-dimensional fluorescence measurements were performed on a Jasco FP-6200 spectrofluorometer (Japan) with a thermostat bath. Three-dimensional fluorescence spectra were obtained under the following conditions: the emission wavelength was recorded between 220 and 500 nm, and the initial excitation wavelength was set to 220 nm with increments of 500 nm.

General procedure

The HSA and hTf solution was added to a 1.0-cm quartz cell to make up 2 ml and the drug solution was gradually titrated manually into the cell using a micro-injector. The HSA and hTf volume in the cell was 2 ml. An amount of 5 ml of a 5×10⁻⁵ mM stock solution of Cyc was added to HSA and hTf in each injection. The solution was mixed thoroughly and equilibrated for 5 min at room temperature. The volume values of HSA-Cyc and hTf-Cyc after each injection were corrected
by a dilution factor. The fluorescence spectra were then measured (excitation at 280 nm and 295 nm and emission wavelengths of 300–600 nm) at room temperature. Both the entrance and exit slit widths were 5 nm and the scanning speed was 240 nm/min. Fluorescence quenching spectra and synchronous fluorescence spectra were obtained. The UV/vis absorbance spectra of HSA, hTf and Cyc were recorded at 291 nm at room temperature. Moreover, far-UV CD spectra of HSA, hTf and Cyc were recorded and spectral scanning curves were obtained under the same conditions. The HSA, hTf and Cyc solutions were freshly prepared for each experiment.

**Molecular modeling**

The three-dimensional coordinates of HSA were obtained from the Protein Data Bank (PDB entry code 1AO6). The crystal structure of hTf was modeled based on a homology modeling (11) and was fitted to the hTf from the Protein Data Bank (PDB entry code 1SUV). The RMS was 0.29. HSA and hTf were prepared and the energy was minimized at three pH-values of 6.4, 7.4 and 8.4, with the MOE software 2008.10. The Auto Docked4 program package was used to locate possible Cyc binding sites on the HSA and hTf molecules. With the ADT (Auto Dock Tools) software (12), the torsion angles of the ligands were identified, polar hydrogen was added to the macromolecules, bond distances were edited and solvent parameters were added. The docking parameter files were generated using the Genetic Algorithm and local Search Parameters (GALS), and the number of generations was set to 100 for each protein. Following each docking procedure, results were submitted to the WebLab Viewer Lite software (13) and the Swiss-PDB viewer 4 (14) for further evaluation.

**RESULTS AND DISCUSSION**

**FLUORESCENCE STUDIES OF HTF AND HSA UPON INTERACTION WITH CYC**

The effect of different Cyc concentrations on the hTf and HSA fluorescence intensity at various pH (6.4, 7.4, and 8.4) are shown in Fig. 1 (A, B and C). Fig. 1A displays the fluorescence spectrum of HSA at pH=6.4. It was obvious that HSA had a strong fluorescence emission at 343 nm after being excited with a wavelength of 280 nm. The addition of different concentrations of Cyc caused a noticeable decrease in the fluorescence intensity of HSA. The maximum emission wavelength produced a blue shift. The strong quenching of the Trp214 fluorescence indicated that the HSA conformation may have become changed and that an inter-molecular energy transfer occurred between Cyc and HSA. This was moreover an indication of the chromospheres of the protein being transferred to a
more hydrophobic environment and the conformation of the protein becoming altered (14, 15).
Fig. 1. – (A) Fluorescence emission spectra of the HSA-Cyc system. The concentration of HSA was $4.5 \times 10^{-6}$ mM and the cyclophosphamide concentration was increased from 0 to $4.9 \times 10^{-6}$ mM; pH= 6.4, 298 K. Inset: Fluorescence emission spectra of the hTf-Cyc system. The concentration of hTf was $3.79 \times 10^{-6}$ mM and the cyclophosphamide concentration was increased from 0 to $4.9 \times 10^{-6}$ mM; pH= 6.4, 298 K; (B) Fluorescence emission spectra of the HSA-Cyc system. The concentration of HSA was $4.5 \times 10^{-6}$ mM and the Cyc concentration was increased from 0 to $4.9 \times 10^{-6}$ mM; pH= 7.4, 298 K. Inset: Fluorescence emission spectra of the hTf-Cyc system. The concentration of hTf was $3.79 \times 10^{-6}$ mM and the Cyc concentration was increased from 0 to $4.9 \times 10^{-6}$ mM; pH= 7.4, 298 K; (C) Fluorescence emission spectra of the HSA-Cyc system. The concentration of HSA was $4.5 \times 10^{-6}$ mM and the Cyc concentration was increased from 0 to $4.9 \times 10^{-6}$ mM; pH= 8.4, 298 K. Inset: Fluorescence emission spectra of the hTf-Cyc system. The concentration of hTf was $3.79 \times 10^{-6}$ mM and the Cyc concentration was increased from 0 to $4.9 \times 10^{-6}$ mM; pH= 8.4, 298 K.

The inset in the figure shows the fluorescence spectrum of hTf at pH=6.4. As can be seen, hTf had a significant fluorescence emission at 328 nm after being excited with a wavelength of 280 nm. The addition of Cyc to the hTf solution resulted in the decrease of the hTf fluorescence intensity and the maximum emission wavelength produced a red shift. It can be noted that a complex was formed between Cyc and hTf, which was responsible for the quenching of hTf (16). Fig. 1B and 1C present the fluorescence spectrum of HSA at pH=7.4 and 8.4 and the insets show the fluorescence spectra of hTf at these pH-values. As can be
Effects of cyclophosphamide to HSA and holo-transferrin

seen, the addition of Cyc to the protein solution resulted in a diminution of the protein’s fluorescence intensity.

It is well known that quenching occurs through either a static or a dynamic process, both of which can result in a linear Stern-Volmer plot. To analyze the data from the quenching experiments, the Stern-Volmer equation was employed (17):

\[ \frac{F_0}{F} = 1 + k_q \tau_0 (Q) = 1 + K_{sv}(Q) \]  

(3)

where \( F \) and \( F_0 \) are the fluorescence intensities in the presence and absence of quencher, respectively. \( K_{sv} \), \( k_q \), \( \tau_0 \) and \( (Q) \) denote the Stern-Volmer constant, the quenching rate constant, the original lifetime of HSA and the concentration of quencher, respectively. This extent and the degree of accessibility of the fluorophores to the quencher showed a dependence on its size and charge (18).

Fig. 2A illustrates the Stern-Volmer plots of HSA-Cyc at three different pH values (6.4, 7.4, and 8.4). At pH=7.4, the plots had two classes of apparent binding constants. The \( K_{SV1} \) and \( K_{SV2} \) values were \( 8.6 \times 10^8 \) and \( 6.34 \times 10^8 \) L mol\(^{-1}\), respectively (Table 1). It can be seen that the affinity of the binding constant decreased with an increasing drug concentration. At pH=6.4 and 8.4, the Stern-Volmer plots exhibited only one class of apparent binding constant. The \( K_{SV} \) values of HSA-Cyc were \( 10^6 \) and \( 4.1 \times 10^8 \) L mol\(^{-1}\) for pH=6.4 and pH=8.4, respectively. According to Fig. 2A, when the pH was either lower or higher than 7.4, the binding of Cyc to the proteins showed only one region and the Stern-Volmer plots were linear. Figure 2B presents the Stern-Volmer plot of hTf-Cyc at the three pH-values (6.4, 7.4, and 8.4). According to the plots, there was one class of apparent binding constant, and the fluorescence quenching was mainly static. For the three pH values of 6.4, 7.4 and 8.4, the \( K_{SV} \) results were \( 1.3 \times 10^6 \), \( 6.08 \times 10^7 \) and \( 10^8 \) mol L\(^{-1}\), respectively.

Table 1

<table>
<thead>
<tr>
<th>system</th>
<th>pH</th>
<th>( K_{SV1} / \text{M}^{-1} )</th>
<th>( K_{SV2} / \text{M}^{-1} )</th>
<th>( k_{q1} / \text{M}^{-1}\text{s}^{-1} )</th>
<th>( k_{q2} / \text{M}^{-1}\text{s}^{-1} )</th>
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</thead>
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<tr>
<td>HSA</td>
<td>6.4</td>
<td>( 1.0 \pm 0.01 \times 10^6 )</td>
<td>( ... )</td>
<td>( 1.0 \pm 0.01 \times 10^14 )</td>
<td>( ... )</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>( 8.6 \pm 0.01 \times 10^6 )</td>
<td>( 6.3 \pm 0.01 \times 10^6 )</td>
<td>( 8.6 \pm 0.01 \times 10^{16} )</td>
<td>( 6.3 \pm 0.01 \times 10^{16} )</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>( 4.1 \pm 0.01 \times 10^6 )</td>
<td>( ... )</td>
<td>( 4.1 \pm 0.01 \times 10^{16} )</td>
<td>( ... )</td>
</tr>
<tr>
<td>hTf</td>
<td>6.4</td>
<td>( 1.3 \pm 0.02 \times 10^7 )</td>
<td>( ... )</td>
<td>( 1.3 \pm 0.02 \times 10^{15} )</td>
<td>( ... )</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>( 6.1 \pm 0.01 \times 10^7 )</td>
<td>( ... )</td>
<td>( 6.8 \pm 0.01 \times 10^{15} )</td>
<td>( ... )</td>
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<tr>
<td></td>
<td>8.4</td>
<td>( 1.0 \pm 0.01 \times 10^8 )</td>
<td>( ... )</td>
<td>( 1.0 \pm 0.01 \times 10^{16} )</td>
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</tr>
</tbody>
</table>
Fig. 2. – (A) Stern-Volmer plots of HSA fluorescence quenching treated with different concentrations of Cyc; [HSA] = 4.25×10^{-6} mM. pH= 6.4 (∆), 7.4 (Ο) and 8.4 (□), 298 K; (B) Stern-Volmer plots of hTf fluorescence quenching treated with different concentrations of Cyc; [hTf] = 3.79×10^{-6} mM. pH = 6.4 (∆), 7.4 (Ο) and 8.4 (□), 298 K.

The accessible surface of the fluorophore to the quencher can be calculated by Lehrer’s expression (19):

\[
\frac{F_0}{F} = 1 + K_Q (Q)/(1 + K_Q (Q)) (1 - f_B) + f_B
\]

(4)

Here,

\[
f_B = \frac{F_{0,B}}{F_0}
\]

(5)
and $F_{0, B}$ is the fluorescence intensity of the fluorophore population accessible to the quencher. When both sub-populations (accessible and non-accessible) have identical quantum yields, the $f_0$ value represents the mole fraction of the accessible population (Table 2). The values of $K_{SV}$ were calculated from the slope of $F_0/F$ versus $(Q)$ when a linear behavior was observed (Eq. (4)).

Table 2

<table>
<thead>
<tr>
<th>System</th>
<th>pH</th>
<th>$K_{A1}$ / $M^{-1}$</th>
<th>$K_{A2}$ / $M^{-1}$</th>
<th>$n_1$</th>
<th>$n_2$</th>
<th>$f_1$</th>
<th>$f_2$</th>
</tr>
</thead>
<tbody>
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<td>6.4</td>
<td>$(5.2\pm0.01)\times10^3$</td>
<td>.....</td>
<td>0.8</td>
<td>.....</td>
<td>0.96</td>
<td>.....</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>$(4.6\pm0.01)\times10^3$</td>
<td>$(4.9\pm0.01)\times10^3$</td>
<td>0.7</td>
<td>0.8</td>
<td>0.96</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>$(6.1\pm0.02)\times10^3$</td>
<td>.....</td>
<td>1.1</td>
<td>.....</td>
<td>2.08</td>
<td>.....</td>
</tr>
<tr>
<td>hTf</td>
<td>6.4</td>
<td>$(6.4\pm0.02)\times10^3$</td>
<td>.....</td>
<td>1.2</td>
<td>.....</td>
<td>4.1</td>
<td>.....</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>$(2.6\pm0.01)\times10^3$</td>
<td>.....</td>
<td>1.3</td>
<td>.....</td>
<td>0.25</td>
<td>.....</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>$(2.6\pm0.01)\times10^3$</td>
<td>.....</td>
<td>0.7</td>
<td>.....</td>
<td>0.37</td>
<td>.....</td>
</tr>
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</table>

In a number of situations, the Stern-Volmer plots have been found to be linear, and in these cases the quenching mechanism is mainly due to the static process, dominated by diffusion. In other cases, the experimental results show a positive deviation from the linear Stern-Volmer relation (20-22) possibly due to one of the above processes other than or along with a diffusion process. Apart from this, the polarity of the solvent medium and the range of quencher concentration were also expected to play a part in this medium.

Table 1 shows the $K_a$ and $k_q$ of HSA-Cyc and hTf-Cyc complexes at the three pH. We can see for HSA at pH=7.4 that Cyc bound to a larger extent than at the other pH. Moreover, for hTf at pH=8.4, the affinity of binding was higher than at the other pH.

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant ($K_b$) and the number of binding sites (n) can be determined by the following equation:

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_b + n \log[Q]$$  \hspace{1cm} (6)

Here, $F_0$ and $F$ are the fluorescence intensity of the protein in respectively the absence and presence of a quencher, and $(Q)$ is the quencher concentration (23). The values of $K_b$ and n for HSA-Cyc and hTf-Cyc are calculated and listed in Table 2. According to Eq. (4), the Hill plot of HSA-Cyc and hTf-Cyc at pH=7.4 can be fitted with two lines, which would indicate the presence of two binding sites with different $K_b$ values.
The synchronous fluorescence spectra present information about the molecular micro-environment in the vicinity of the fluorophore functional groups, and have several advantages, such as sensitivity, spectral simplification, spectral bandwidth reduction and the possibility of avoiding several perturbing effects (24). When the D-value (Δλ) between excitation and emission wavelengths was set to 15 or 60 nm, the synchronous fluorescence could provide the characteristic information of Tyr and Trp residues in the protein (25).

Figure 3(A-C) shows the synchronous fluorescence intensity of the quenching of HSA and hTf by addition of Cyc. These indicate a higher slope when λ= 60 nm, suggesting that the main contribution to the fluorescence intensities of HSA and hTf was the Trp residues. Indeed, Cyc was closer to the Trp residues as compared to the Tyr residues in the protein.
Figure 3D shows the synchronous fluorescence intensity of the quenching of HSA and hTf (inset) by Cyc for $\Delta \lambda=60$ nm. The results point at a higher slope at pH=8.4 than at the other pH values for both HSA and hTf.

Figure 3E shows the synchronous fluorescence intensity of the quenching of HSA and hTf by addition of Cyc for $\Delta \lambda=15$ nm. According to the results, the slope was higher for pH=7.4 than for the other pH values for both HSA and hTf. This signifies that the Tyr residues in HSA and hTf participated in the interaction with Cyc at pH=7.4 to a larger extent than at the other pH-values.
Fig. 3. – (A) Synchronous fluorescence spectra of the quenching of HSA by Cyc. The concentration of HSA was 4.25 x 10^{-6} mM. pH= 6.4. ∆λ = 15(Δ) and ∆λ= 60(▲). Inset: Synchronous fluorescence spectra of the quenching of hTf by cyclophosphamide. The concentration of hTf was 3.79 x 10^{-6} mM. pH= 6.4. ∆λ = 15(Δ) and ∆λ= 60(▲); (B) Synchronous fluorescence spectra of the quenching of HSA by cyclophosphamide. The concentration of HSA was 4.25 x 10^{-6} mM. pH= 7.4. ∆λ = 15(Ο) and ∆λ= 60(●). Inset: Synchronous fluorescence spectra of the quenching of hTf by cyclophosphamide. The concentration of hTf was 3.79 x 10^{-6} mM. pH= 7.4. ∆λ = 15(Ο) and ∆λ= 60(●); (C) Synchronous fluorescence spectra of the quenching of HSA by cyclophosphamide. The concentration of HSA was 4.25 x 10^{-6} mM. pH= 8.4. ∆λ = 15(□) and ∆λ= 60(■). Inset: Synchronous fluorescence spectra of the quenching of hTf by cyclophosphamide. The concentration of hTf was 3.79 x 10^{-6} mM. pH= 8.4. ∆λ = 15(□) and ∆λ= 60(■); (D) Synchronous fluorescence spectra of the quenching of HSA by cyclophosphamide. The concentration of HSA was 4.25 x 10^{-6} mM. pH= 6.4. ∆λ = 15(Δ), 7.4 (Ο) and 8.4(□). Inset: Synchronous fluorescence spectra of the quenching of hTf by cyclophosphamide. The concentration of hTf was 3.79 x 10^{-6} mM. pH= 6.4 (Δ), 7.4 (Ο) and 8.4 (□); (E) Synchronous fluorescence spectra of the quenching of hTf by cyclophosphamide. The concentration of hTf was 3.79 x 10^{-6} mM. ∆λ= 15. pH= 6.4 (Δ), 7.4 (Ο) and 8.4 (□). Inset: Synchronous fluorescence spectra of the quenching of hTf by cyclophosphamide. The concentration of hTf was 3.79 x 10^{-6} mM. ∆λ= 15. pH= 6.4 (Δ), 7.4 (Ο) and 8.4 (□).

Fluorescence resonance energy transfer (FRET)

The overlapping of the UV absorption spectrum of Cyc with the fluorescence emission spectrum of HSA and hTf at pH= 7.4 is given in Fig. 4A and B (11), respectively. According to Förster’s non-radiative energy transfer theory (26), energy transfer occurs under the following conditions: (1) when the donor can produce fluorescent light; (2) when the fluorescence emission spectrum of the donor and UV absorbance spectrum of the acceptor overlap; and (3) when the distance of approach between the donor and the acceptor is shorter than 7 nm.
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Fig. 4. – (A) Spectral overlap of the fluorescence spectrum of HSA (a) with the absorption spectrum of cyclophosphamide (b); pH=6.4, 298 K, $C_{\text{drug}}/C_{\text{HSA}} = 1:1$; (B) Spectral overlap of the fluorescence spectrum of hTf (a) with the absorption spectrum of cyclophosphamide (b); pH=6.4, 298 K, $C_{\text{drug}}/C_{\text{hTf}} = 1:1$.

The energy transfer effect is related not only to the distance between the acceptor and donor ($r_0$), but also to the critical energy transfer distance ($R_0$), i.e.,:

$$ E = 1 - \frac{F}{F_0} = \frac{R_0^6}{(R_0^6 + r^6)} $$

(7)

Here, $F$ and $F_0$ are the fluorescence intensities of HSA or hTf in the presence and absence of Cyc, respectively; $r$ is the distance between the acceptor and donor, and $R_0$ is the critical distance when the transfer efficiency is 50%.
\[ R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J \] (8)

where \( K^2 \) is the spatial orientation factor of the dipole, \( N \) is the refractive index of the medium, \( \Phi \) is the fluorescence quantum yield of the donor, and \( J \) is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor (Fig. 4A and B). The latter can be calculated by the equation:

\[ J = \frac{\int \int F(\lambda) \varepsilon(\lambda) \Delta \lambda}{\int \int F(\lambda) d(\lambda)} = \frac{\sum F(\lambda) \varepsilon(\lambda) \Delta \lambda}{\sum F(\lambda) \Delta \lambda} \] (9)

where \( F(\lambda) \) is the fluorescence intensity of the fluorescence donor at wavelength \( \lambda \), and \( \varepsilon(\lambda) \) is the molar absorption coefficient of the acceptor at wavelength \( \lambda \). The distance between Cyc and fluorophore at pH=7.4 for HSA was 1.84 nm, whereas it was 1.73 nm in hTf. Obviously, these values were lower than 7 nm after interaction between Cyc and HSA or hTf, which is in accord with the conditions of Förster’s non-radiative energy transfer theory.

Table 3 shows the \( r \) values for both HSA and hTf upon interaction with Cyc at three different pH. One can see that the distance between HSA and Cyc is larger than in the case of hTf and Cyc. This was due to HSA containing one Trp unit positioned on the surface of HSA whereas hTf has several Trp units in the protein interior. Consequently, hTf presented an interaction at a shorter distance in comparison to HSA.

<table>
<thead>
<tr>
<th>pH</th>
<th>HSA-Cyc (r / nm)</th>
<th>hTf-Cyc (r / nm)</th>
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<tbody>
<tr>
<td>6.4</td>
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<td>1.71</td>
</tr>
<tr>
<td>7.4</td>
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<td>8.4</td>
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<td>1.78</td>
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</table>

**Circular dichroism (CD) analysis**

CD spectroscopy is an optical technique that allows the detection and quantization of the chirality of molecular structures. It also provides information about the secondary and tertiary structures of proteins. The optical activity of an \( \alpha \)-helix in the far-UV region permits the use of CD spectroscopy for investigating conformational changes in proteins. CD band positions for various structures, such
as the α-helix, the inter-chain hydrogen bonded β-structure and a fully extended parallel or anti-parallel arrangement of peptide chains, have been reported. Developments include empirical methods utilizing reference databases consisting of spectra of proteins with known structures, allowing the decomposition of the CD spectrum of an unknown protein, and providing information on secondary structural features. Such data are often used to complement the more detailed structural information available from other techniques.

Figure 5 and Fig. 6 show the CD spectra of respectively HSA and hTf at different pH values. Figure 5 (A-D) and Fig. 6 (A-D) illustrate the α-helix, beta sheet, turn and unordered contents as the drug concentration is increased. One can see a greater conformational change of HSA as opposed to hTf. The contents of α-helix and turn became decreased after increasing the drug concentration whereas the amounts of β-sheet and unordered structures increased. These CD data suggest that not only conformational changes at the tertiary structure level, as was seen by the fluorescence data, but also structural changes at the secondary structure level were involved in the aggregation process. Owing to the conformational change of the protein, a number of hydrophobic groups became exposed by the solvents. This may be an important factor leading to the aggregation of protein.
Fig. 5. – Far-UV CD spectra of HSA in the presence of cyclophosphamide. A= α-helix, B= β-sheet, C= Turn and D= Unordered contents. [HSA]= 4.25×10^{-6} Mm. The cyclophosphamide concentration for the HSA-cyclophosphamide system ranged from 0 to 4.5×10^{-6} mM; pH= 6.4 (▲), pH=7.4 (●), 7.4 and 8.4 (○). T= 298 K.
Fig. 6. – Far-UV CD spectra of hTf in the presence of cyclophosphamide. A= α-helix, B= β-sheet, C= Turn and D= Unordered contents. [hTf] = 3.79×10^-6 Mm. The cyclophosphamide concentration for the hTf-cyclophosphamide system ranged from 0 to 4.5×10^-6 mM; pH= 6.4 (▲), pH= 7.4 (●), and 8.4(○). T= 298 K.

Zeta-potential measurements

The measurement of the zeta-potential is a way to probe a characteristic colloidal property in a complex mixture of particles and is a useful technique for explaining their behavior. The zeta-potential is generated when a liquid is forced with pressure to flow directly through a small gap formed by two sample surfaces. Charge carriers bound in the double layer are removed, and the potential can be then measured between two electrodes. The biomaterial’s zeta-potential thus demonstrates the electric surface properties. The surface charge in protein particles is due to the partial ionization of various amino acid residues, and the effective charge on a protein particle is affected by pH, ionic strength and the accumulation of ligands or surfactant at the interface (27, 28). The zeta potential was calculated from the electrophoretic mobilities, \( \mu_E \), using the Henry equation (29):

\[
\zeta = \left( \frac{3\mu_E \eta}{2\varepsilon_0 \varepsilon_r} \right) \left( 1 / \int f(\kappa a) \right)
\]  

(10)
where $\varepsilon_0$ is the permittivity of vacuum, $\varepsilon_r$ and $\eta$ are the relative permittivity and viscosity of water, and $\kappa$ is the Debye length. The function $K$ represents the particle radii, at the viscosity of water, and depends on the particle shape. For the system in question, it was expressed as:

$$f(\kappa a) = (2/3) - (9/2\kappa a) + (75/2\kappa^2 a^2) - (330/\kappa^3 a^3)$$

valid for $K_a > 1$

The interaction between the adsorbed molecules may either be attractive or repulsive, depending on the type and magnitude of electric charge of the residues. These complicated processes involving protein adsorption are also reflected in the zeta-potential changes. Figs. 7A and B show the effect of Cyc concentrations on the zeta-potential of HSA and hTf, respectively. At first, the adsorption of Cyc on the HSA and hTf surfaces increased with an increasing zeta-potential and then abruptly changed, giving lower values of zeta-potential. The higher zeta-potential values confirmed that electrostatic forces were the primary mode of interaction of Cyc with HSA and hTf. The decrease in zeta-potential confirmed the existence of hydrophobic interactions between Cyc with HSA and hTf, or in other words, a critical induced aggregation concentration of drugs on both proteins. The rise in surface charge on colloidal particles increased the magnitude of inter-particle electrostatic repulsion, which tended to disrupt existing protein aggregates and discourage further aggregation (28, 30). Consequently, the Cyc molecules could bind to HSA and hTf through a combination of electrostatic and hydrophobic interactions and form micelle-like clusters.
Fig. 7. – (A) Zeta-potential spectra of HSA at varying concentration of Cyc. [HSA] = 4.25 × 10^{-6} mM. pH= 6.4 (▲), pH= 7.4 (●), and 8.4 (○). T= 298 K; (B) Zeta-potential spectra of hTf at varying concentration of Cyc. [hTf] = 3.79 × 10^{-6} mM. pH= 6.4 (▲), pH= 7.4 (●), and 8.4 (○). T= 298 K.

Three-dimensional fluorescence spectroscopy

Three-dimensional fluorescence spectroscopy has become a popular technique in recent years. The excitation wavelength, the emission wavelength, and the fluorescence intensity are the three parameters used to investigate the synthetic information of the samples. The outstanding advantage of three-dimensional fluorescence spectroscopy is that information regarding the fluorescence characteristics can be entirely acquired by varying the excitation and emission wavelengths simultaneously. The maximum emission wavelength and the fluorescence intensity of the residues have a close relation to the polarity of their micro-environment (31).

The three-dimensional fluorescence spectra and the contour maps for both HSA-Cyc and hTf-Cyc in pH=7.4 are shown in Fig. 8 and 9, respectively. Fig. 8 (A and B) displays peak a as the Rayleigh scattering peak ($\lambda_{\text{ex}} = \lambda_{\text{em}}$) and peak b as the second-ordered scattering peak ($\lambda_{\text{em}} = 2\lambda_{\text{ex}}$). As can be seen, the fluorescence intensity of peaks a and b decreased with the addition of Cyc at three different pH. The contour map (Fig. 8 (A' and B')) gives a bird’s eye view of the fluorescence spectra. The results indicate that the three-dimensional fluorescence map for HSA and hTf-Cyc in pH=7.4 were different, obviously. At the same time, there were two “humps” in the three-dimensional spectra for both the HSA and hTf-Cyc...
complexes at pH=7.4, marked peaks 1 and 2. Peak 1 reveals the spectral behavior of the Trp and Tyr residues. When HSA and hTf were excited at 280 nm, the intrinsic fluorescence was mainly that of the Trp and Tyr residues, whereas the fluorescence of the Phe residues was negligible. The second fluorescence, i.e., peak 2, reflects the fluorescence spectral behavior of the polypeptide backbone structure of HSA and hTf, which was caused by the transition of $\pi \rightarrow \pi'$ of hTf’s characteristic polypeptide C=O backbone structure. The fluorescence intensity of peak 2 decreased after addition of Cyc, signifying that the peptide strand structure of HSA and hTf became altered (32, 33).
Fig. 8. – (A) Three-dimensional fluorescence spectra of HSA. [HSA] = $4.5 \times 10^{-6}$ mM; (A’) The contour spectra of HSA. [HSA] = $4.5 \times 10^{-6}$ mM; (B) the three-dimensional fluorescence spectra of HSA–Cyc system. [HSA] = $4.5 \times 10^{-6}$ mM, [Cyc] = $5 \times 10^{-5}$ mM; (B’) The contour spectra of HSA-Cyc system. [HSA] = $4.5 \times 10^{-6}$ mM, [Cyc] = $5 \times 10^{-5}$ mM.
Fig. 9. – (A) The three-dimensional fluorescence spectra of hTf. [hTf] = 3.79 × 10^{-6} mM; (A’) The contour spectra of hTf. [hTf] = 3.79 × 10^{-6} mM; (B) The three-dimensional fluorescence spectra of the hTf-Cyc system. [hTf] = 3.79 × 10^{-6} mM, [Cyc] = 5 × 10^{-5} mM; (B’) The contour spectra of hTf-Cyc. [hTf] = 3.79 × 10^{-6} mM, [Cyc] = 5 × 10^{-5} mM.

Similar results were also obtained for the other pH values. The decrease in intensity of peaks 1 and 2 in combination with results from the synchronous fluorescence spectra pointed at the binding of Cyc to HSA and hTf at three different pH inducing a slight unfolding of the polypeptides, which led to
conformational changes in HSA and hTf. These, in turn increased the exposure of some hydrophobic regions that were previously buried (34). All these phenomena and the analysis of the two peaks suggested that the binding of Cyc to HSA and hTf as well as the formation of complexes induced changes in the micro-environment and in the conformational structure of HSA and hTf.

Red edge excitation shift (REES)

The red edge excitation shift (REES) is a displacement of the emission maximum toward a higher wavelength caused by a shift in the excitation wavelength toward the red edge of the absorption band (35). The REES is due to the electronic coupling between Trp indole rings and neighboring dipoles and occurs when the solvent medium undergoes slow relaxations. Thus, REES is particularly useful for monitoring motions around the Trp residues in protein studies (36).

The Trp emissions in the HSA-Cyc and hTf–Cyc systems were further investigated by red edge excitation shift (REES) experiments (37). In our case, we chose to excite the Trp at both 280 and 295 nm to investigate the REES effect, and the results are listed in Table 4 (A and B) for HSA and hTf, respectively. The value of ∆λ_{em max} was defined as the difference of the emission maximum for an excitation at 280 nm and at 295 nm. As shown, for the HSA and hTf at three pHs, the Trp residues were in an environment where their movement was slightly restricted. In the presence of Cyc, the values all became altered. Changes of ∆λ_{em max} signified that the introduction of Cyc had an obvious impact on the mobility of the Trp microenvironments.

Table 4A

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Effects of cyclophosphamide to HSA and holo-transferrin

### Table B

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<th>System</th>
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<th>$\Delta \lambda_{em, max}$ (nm)</th>
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<td></td>
<td></td>
<td>$\lambda_{exc}$: 280 nm</td>
<td>$\lambda_{exc}$: 295 nm</td>
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<td>6.4</td>
<td>327</td>
<td>332</td>
</tr>
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</table>
| hTf-Cyc      | 1:1 | 328                      | 331                              | 5
| hTf-Cyc      | 1:5 | 327                      | 331                              | 3
| hTf          | 7.4 | 336                      | 334                              | 2
| hTf-Cyc      | 1:1 | 329                      | 332                              | 3
| hTf          | 1:10| 329                      | 332                              | 3
| hTf-Cyc      | 1:10| 330                      | 333                              | 3
| hTf-Cyc      | 1:5 | 330                      | 333                              | 3

### Binding site allocation of Cyc on HSA and hTf at three different pH

The experimental observations were followed by docking studies where Cyc was docked to HSA and hTf to determine the preferred binding sites on the proteins. Docking was performed for both proteins (HSA and hTf) at three different pH-values (8.4, 7.4 and 6.4). For HSA at pH=8.4 (Fig. 10A), Cyc was situated in domain III and formed two hydrogen bonds with Glu 465 and Tyr 478. The distance between Cyc and Trp was observed as 1.82 nm, which was also confirmed by experimental data. At pH=7.4 (Fig. 10B) and 6.4 (Fig. 10C), Cyc was situated in domain II, and at pH=7.4 it formed two hydrogen bonds with Ser 287 and Arg 257. The distance between Cyc and Trp was observed as 1.79 nm. At pH=6.4, on the other hand, Cyc formed two hydrogen bonds with Ala 213 and Arg 209. The distance between Cyc and Trp was reported to be 1.03 nm.
Fig. 10. – Cyclophosphamide in the active site of HSA. Hydrogen bonding (dashed lines) was the main interaction of cyclophosphamide with the protein. (A) HSA in pH=8.4, (B) pH=7.4, (C) pH= 6.4.

Cyc was situated in C-Lobe of hTf at the three pH-values in question. At 8.4 and 7.4, the active sites were the same; at pH = 8.4 (Fig. 11A) the compound
formed two hydrogen bonds with Arg 453 and Tyr 512. The distance between Cyc and Trp 341 was 1.12 nm. At pH=7.4 (Fig. 11B), it formed three hydrogen bonds, one of which was with Asp 625 and the two others with Arg 453. The distance between the drug and Trp 341 was reported to be 1.22 nm. At pH = 6.4 (Fig. 11 C), Cyc formed one hydrogen bond with Glu 354, and the distance to Trp 355 was found to be 1.18 nm. Figures 12 (A-C) and 13(A-C) illustrate the whole proteins and the changes in active sites at pH= 6.4, 7.4 and 8.4.
Fig. 11. - Cyclophosphamide in the active site of hTf. Hydrogen bonding (green dashed lines) was the main interaction of cyclophosphamide with the protein. (A) hTf in pH= 8.4, (B) pH= 7.4, (C) pH= 6.4.
Fig. 12. – Changes in the active sites in HSA, at different pH values. (A) pH= 6.4, (B) pH= 7.4 and (C) pH= 8.4.
Fig. 13. – Changes in the active sites in hTf, at different pH values. (A) pH= 6.4, (B) pH= 7.4 and (C) pH=8.4.
Figure 14 (A and B) exhibits the distance between Cyc and Trp in HSA and hTf, respectively. At the three different pH-values, in the case of HSA, the number of H-bonds was the same. This was not the case for hTf however. For HSA, the inhibition constant ($K_i$) at pH =7.4 was lower than at the other pH values as was also found with the experimental data. Moreover, the affinity of Cyc to HSA at this pH was higher than the other pH values. On the other hand, the value of $K_i$ for hTf at pH=8.4 was lower than at the other pH values, which was also confirmed by the experimental results. Table 5 (A and B) shows the $K_i$ values and distances between Cyc and Trp for HSA and hTf at the three pH.

Fig. 14. – (A), Distances between Cyc and Trp 214 in HSA, pH= 7.4. (B), Distances between Cyc and the nearest Trp in hTf, pH= 7.4.
Table 5

(A) $K_i$ values and distances between Cyc and Trp for HSA. (A) pH= 6.4; (B) pH= 7.4; and (C) pH= 8.4. (B), $K_i$ values and distances between Cyc and the nearest Trp for hTf. (A) pH= 6.4; (B) pH= 7.4; and (C) pH= 8.4

<table>
<thead>
<tr>
<th>System</th>
<th>pH</th>
<th>The best $K_i$ / uM</th>
<th>Distance between Cyc and Trp / nm</th>
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<tbody>
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<td></td>
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</tr>
<tr>
<td></td>
<td>8.4</td>
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<td>1.12</td>
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</tbody>
</table>

Fig. 15 (A and B) presents electrostatic bonds at pH=7.4 for HSA and hTf, respectively. The red region shows the negative charges and the blue region displays the positive charges. For other pH values, the regions become altered since each residue had a different isoelectric pH (pI). When the pH-value decreased or increased, the electrostatic bonds were altered.
To confirm and support our docking data, we used multiple alignments to investigate the region in which Cyc might be found. Since we have a signal peptide in the sequences that we obtained from Uniprot, the number of amino acids in the docking and alignments is not the same. The results obtained from multiple alignments were confirmed by results of docking, i.e., a conserved area fit with the binding sites by docking (Table 6). Figure 16 (A and B) shows the multiple alignments of HSA and hTf at pH=7.4.

<table>
<thead>
<tr>
<th>System</th>
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<th>Amino acid in docking</th>
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<tbody>
<tr>
<td>HSA</td>
<td>270-280, 309-314</td>
<td>Arg 257, Ser 287</td>
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<tr>
<td>hTf</td>
<td>441-450, 618-625</td>
<td>Arg 453, Asp 625</td>
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Fig. 16. (A), Multiple alignments of HSA pH=7.4. (B), Multiple alignments of hTf in pH=7.4.
CONCLUSIONS

This paper has described an investigation by means of various spectroscopic techniques of the interaction of Cyc with HSA and hTF. The changes of the protein conformation upon binding were determined by fluorescence and CD spectroscopies as a function of the amount of added drug. The results indicated that the structure of the environments of the Trp and Tyr residues became altered and that the physiological functions of HSA and hTF were affected by Cyc. It was further shown that the fluorescence of HSA and hTF became quenched when reacting with Cyc and that a certain kind of new compound was formed. Based on the results of this study, the fluorescence quenching technique is expected to be a promising tool for investigating the interaction of organic drugs and proteins. The binding reaction of Cyc with HSA and hTF is spontaneous and largely mediated by hydrophobic and electrostatic forces, and the present study on the interaction between Cyc with HSA and hTF should open new vistas for what is reasonable regarding drug design.

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