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CHROMATOGRAPHIC ANALYSIS OF THE EFFECTS OF FATTY ACIDS AND GLYCATION ON BINDING BY PROBES FOR SUDLOW SITES I AND II TO HUMAN SERUM ALBUMIN

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Abstract

The primary endogenous ligands of human serum albumin (HSA) are non-esterified fatty acids, with 0.1–2 moles of fatty acids normally being bound to HSA. In type II diabetes, fatty acid levels in serum are often elevated, and the presence of high glucose results in an increase in the non-enzymatic glycation of HSA. High-performance affinity chromatography (HPAC) was used to examine the combined effects of glycation and the presence of long chain fatty acids on the binding of HSA with *R*-warfarin and L-tryptophan (i.e., probes for Sudlow sites I and II, the major sites for drugs on this protein). Zonal elution competition studies were used to examine the interactions of myristic acid, palmitic acid and stearic acid with these probes on HSA. It was found that all these fatty acids had direct competition with *R*-warfarin at Sudlow site I of normal HSA and glycated HSA, with the glycated HSA typically having stronger binding for the fatty acids at this site. At Sudlow site II, direct competition was observed for all the fatty acids with L-tryptophan when using normal HSA, while glycated HSA gave no competition or positive allosteric interactions between these fatty acids and L-tryptophan. These data indicated that glycation can alter the interactions of drugs and fatty acids at specific binding sites on HSA. The results of this study should lead to a better understanding of how these interactions may change during diabetes and demonstrate how HPAC can be used to examine drug/solute-protein interactions in complex systems.

Keywords

Human serum albumin; Glycation; Fatty acids; Drug-protein binding; High-performance affinity chromatography

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1. Introduction

The primary endogenous ligands of human serum albumin (HSA) are non-esterified fatty acids [1–3]. HSA is the most prominent protein in human plasma and is an important carrier of fatty acids, as well as other endogenous solutes such as hemin, bilirubin and tryptophan [4–6]. In addition, HSA can bind many drugs [4–7]. The interactions of drugs with HSA typically occurs at two major sites, Sudlow sites I and II, which are located in subdomains IIA and IIIA, respectively [7–9]. Sudlow site I, which is also known as the warfarin-azapropazone site, tends to bind bulky heterocyclic anionic compounds. Examples of drugs that bind at this site include coumarin compounds like warfarin and non-steroid anti-inflammatory drugs like phenylbutazone [7–10]. Sudlow site II is preferred by aromatic carboxylates with an extended configuration, such as L-tryptophan and ibuprofen [7–11].

HSA can undergo non-enzymatic glycation in the circulation; this process initially involves the addition of glucose to lysine residues or the *N*-terminus to form a reversible Schiff base, which can then rearrange to form a stable Amadori product [12–16]. The extent of glycation is known to increase during diabetes, with the amount of glycated HSA increasing by 2- to 5-fold for diabetic patients versus normal individuals [17]. It has been suggested that this type of modification can alter the binding of some drugs or solutes to HSA [18–23]. Another change that can occur during diabetes is an elevation in the levels of fatty acids in serum. Between 0.1 and 2 moles of fatty acids per mole of protein are bound to HSA under normal conditions; however, in type II diabetes up to 6 moles of fatty acids may be bound per mole of HSA [24–26].

Some fatty acids can have direct competition with drugs or lead to allosteric effects during the binding of drugs and other solutes with HSA [19,27,28]. There are many binding regions for fatty acids on HSA, with the strongest of these interactions having association equilibrium constants in the range of 10^5 to 10^8 M⁻¹ [1,27–32]. The possible locations for several such regions are shown in Fig. 1, using the complex of HSA with myristic acid as an example (Note: other fatty acids, such as stearic acid and palmitic acid, have similar binding regions) [33]. The main drug binding sites on HSA (i.e., Sudlow sites I and II), as well as various lysine residues that are known to be common sites for glycation-related modifications [34], are also shown in Fig. 1. This structural information indicates that a number of glycation sites and fatty acid binding regions are at or near Sudlow sites I and II [33,34].

High-performance affinity chromatography (HPAC) and columns containing normal HSA or glycated HSA were recently used to compare the overall effects of glycation and fatty acids on the binding of HSA with several sulfonylurea drugs [18,19]. Each of these drugs is known to have multisite binding to both normal HSA and glycated HSA and to interact at both Sudlow sites I and II [10,18,19,21,22]. The analysis of these systems by HPAC was simplified in Refs. [18,19] by using fatty acid concentrations that were up to 100-fold below the normal levels of the same fatty acids in serum, thus emphasizing the initial, high affinity interactions for these fatty acids with HSA.

This report will use HPAC columns containing normal HSA or glycated HSA to examine the effects of glycation and fatty acids at the specific regions of Sudlow sites I and II. These experiments will be used to look at the interactions that occur between three long chain fatty acids (i.e., myristic acid, palmitic acid and stearic acid) [18] with *R*-warfarin and L-tryptophan, which will be used as site-specific probes for Sudlow sites I and II, respectively [8,9,21,22]. The results of this study should lead to a better understanding of how the interactions of drugs and fatty acids with HSA may change during diabetes. The same data should illustrate how HPAC can be used to examine drug/solute-protein interactions in complex systems, such as those that combine drug/fatty acid binding and modifications due to glycation.

2. Experimental

2.1. Materials

The HSA (Cohn fraction V, essentially fatty acid free, lot no. 018K7540), glycated HSA (2.1 mol hexose/mol HSA, essentially fatty acid free, lot no. 039K6023), *R*-warfarin (> 99% pure), L-tryptophan (> 99%), D-(+)-glucose (99.5%), sodium azide (> 95%), myristic acid, palmitic acid, and stearic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Nucleosil Si-300 (particle size, 7 μ m; pore size, 300 Å) was from Macherey-Nagel (Düren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). The fructosamine assay kit was from Diazyme Laboratories (Poway, CA, USA). The sterilized culture tubes (17 $\times$ 100 mm) and siliconized polypropylene microcentrifuge tubes (13 $\times$ 40 mm) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Slide-A-Lyzer dialysis cassettes (7 kDa MW cutoff; 12–30 mL volume) were purchased from Thermo Scientific (Rockford, IL, USA). Econo-Pac 10 DG disposable desalting columns were acquired from Bio-Rad Laboratories (Hercules, CA, USA). All buffers and aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and were filtered using 0.2 μ m GNWP nylon filters from Millipore (Billerica, MA, USA).

2.2. Apparatus

The chromatographic experiments were carried out by using a Jasco 2000 HPLC system (Easton, MD, USA) that contained a DG-2080-53 three solvent degasser, two PU-2080 isocratic pumps, an AS-2057 autosampler equipped with a 100 μ L sample loop (operated in the partial loop injection mode), and a UV-2075 absorbance detector. The columns were kept at 37°C by using a Jasco CO-2060 column oven. A Rheodyne Advantage PF six-port switching valve (Cotati, CA, USA) was used to alternate the passage of solutions containing fatty acids or only buffers through the columns. The chromatographic system was controlled by Jasco LCNet II/ADC and EZChrom Elite software v3.2.1 (Scientific Software, Pleasanton, CA, USA). The central moments of the chromatographic peaks were determined by using PeakFit 4.12 (SeaSolve Software, San Jose, CA, USA). The columns were packed by using a model CP constant high pressure column packing system from Scientific Systems (State College, PA, USA).

2.3. Preparation of *in vitro* glycated HSA

The chromatographic studies were performed by using two samples of glycated HSA with similar characteristics but from different sources (see Table 1). The first preparation of glycated HSA (gHSA1) was made through *in vitro* glycation by using a modified version of a previous method [21,22]; this preparation was made from the same batch of normal HSA that was used in the chromatographic binding studies. The other preparation of *in vitro* glycated HSA was purchased from a commercial source (Sigma-Aldrich) and will be referred to as gHSA2.

To avoid microbial growth during the glycation process, only autoclaved glassware and sterile materials were used during the preparation of gHSA1. The solutions used to make gHSA1 were prepared using sterile pH 7.4, 0.2 M phosphate buffer that contained 1 mM sodium azide to retard bacterial growth. The final glycation mixture for this preparation contained 42 g/L HSA and 15 mM glucose that were dissolved in 100 mL of the pH 7.4 buffer. These levels of HSA and glucose, as well as the temperature and pH that were employed for glycation, closely mimic the conditions that occur during the glycation of HSA in blood during diabetes [21,22].

The glycation mixture was placed into sterile culture tubes and incubated for 4 weeks at 37°C. After incubation, the glycated HSA solution was passed through an Econo-Pac 10 DG size-exclusion desalting column to remove any excess glucose and sodium azide. The collected protein fraction was placed into sterile dialysis cassettes and dialyzed against water while gently stirring for 2 h at room temperature, followed by a second dialysis cycle against water for another 2 h under the same conditions. This sample was dialyzed against water a third time without stirring at 4°C for 14–18 h, with the protein then being lyophilized and stored at –80°C. The level of glycation in this sample was determined by using a modified fructosamine assay, as described previously [21,22,35].

2.4. Chromatographic studies

The normal HSA and glycated HSA columns were prepared by converting Nucleosil Si-300 silica into a diol form and then using the Schiff base method for immobilization, as described previously [22,36]. As noted in prior work, the immobilization of HSA by this approach mainly occurs through the *N*-terminus or lysines that are not located at Sudlow sites I and II [37] and has been shown to provide columns with normal HSA or glycated HSA that are good models for the soluble forms of these proteins [10,18,21,22]. Control supports were prepared in the same manner but with no protein being added during the immobilization step.

Each protein support was downward slurry-packed into a separate 1.0 cm × 2.1 mm I.D. column at 3500 psi (24.1 MPa). A pH 7.4, 0.067 M potassium phosphate buffer was used as the packing solution, and the columns were stored in this buffer at 4°C when not in use. All columns were used over a period of a few months and each column was employed with only one fatty acid. Similar columns have been shown to provide stable retention factors under comparable operating conditions [10,18,21,22].

To mimic physiological conditions, all chromatographic experiments were performed at 37°C and using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. The solutions of *R*-warfarin were prepared in this mobile phase and filtered through a 0.2 µm nylon filter, followed by a 15 min degassing step prior to use. All solutions and samples containing *R*-warfarin were used within two weeks of preparation to ensure stable conditions for drug-protein binding measurements [21,22,38]. The L-tryptophan solutions were prepared fresh daily in the same pH 7.4 buffer, as described previously [21,22,39]. These samples were applied or injected at a typical flow rate of 0.50 mL/min, as shown in earlier studies to provide reproducible retention factors and binding capacities for these drugs and related solutes on similar HSA columns [10,18,21,22].

The zonal elution competition studies that probed interactions at Sudlow sites I or II were performed by injecting 20 µL of either 5 µM *R*-warfarin or 5 µM L-tryptophan in the presence of a mobile phase that contained pH 7.4, 0.067 M phosphate buffer, or the same buffer plus a known concentration of the desired fatty acid. Fig. 2 shows the structures and concentrations of the fatty acids that were used in this study. The elution of *R*-warfarin was monitored at 308 nm, and the elution of L-tryptophan was followed at 280 nm. No significant changes in retention occurred when using slightly higher sample concentrations of these drugs, indicating that linear elution conditions were present, or when using slower flow rates. The void time of each column was measured by injecting 5 µM sodium nitrate as a non-retained solute, which was monitored at 205 nm. Studies with the control column indicated that the injected solutes had no significant non-specific binding to the support (i.e., less than 3% of the total retention noted on the normal HSA or glycated HSA columns) [10,18].

3. Results and discussion

3.1. General properties of normal HSA and glycated HSA columns

Table 1 summarizes the properties of the normal HSA and glycated HSA supports that were prepared in this study. The normal HSA had only a small amount of glycation-related modification, with less than 0.1 mol hexose per mol of HSA being measured for this protein preparation when using a fructosamine assay. The gHSA1 sample, which was prepared using 15 mM of glucose and 4 weeks of incubation, contained 1.93 ($\pm$ 0.10) mol hexose per mol HSA. The commercial preparation of gHSA2 had a similar glycation level of 2.07 ($\pm$ 0.23) mol hexose per mol HSA. These levels of glycation are consistent with the amounts of modification that are typically present in patients with diabetes (i.e., 0.5–3 mol hexose per mol HSA) [35]. The supports made with these preparations each contained 62–63 µg protein per mg silica. This amount corresponded to a protein content of approximately 15 nmol of normal HSA or glycated HSA when these supports were packed into 1.0 cm $\times$ 2.1 mm I.D. columns.

3.2. General approach for site-specific competition studies

It is known that fatty acids can have many binding sites on HSA and may exhibit direct competition or allosteric interactions with drugs on this protein [18,20–26]. In prior work using HPAC, it was found that there was a significant increase in retention (i.e., stronger

binding) for drugs such as acetohexamide, tolbutamide and gliclazide when comparing glycated HSA with normal HSA. It was also noted that the addition of long chain fatty acids to the mobile phase led to lower binding and weaker retention for these drugs on both the normal HSA and glycated HSA columns. Most long chain fatty acids (e.g., myristic acid and stearic acid) produced similar overall shifts in the retention of sulfonylurea drugs on these two types of columns, while other fatty acids (i.e., palmitic acid) had a much larger effect on retention in the presence of glycated HSA versus normal HSA [18].

In this current report, zonal elution competition studies were used to examine the interactions of columns containing normal HSA or glycated HSA with myristic acid, palmitic acid and stearic acid. These particular solutes were chosen for use in this work as a few model and representative fatty acids that are found in serum (i.e., based on the results that were obtained by HPAC with a broader range of such compounds in Ref. [18]). These studies were performed by injecting a small amount of a site-specific probe for HSA while the fatty acid was placed into the mobile phase as a potential competing agent (e.g., see Fig. 3). These experiments were carried out by using *R*-warfarin and L-tryptophan as site-specific probes for Sudlow sites I and II, respectively [8,9,40,41]. In addition, fatty acid concentrations that were up to 100-fold below their normal serum levels were used to investigate the initial, high affinity interactions for HSA with these fatty acids [18,19].

In these zonal elution competition studies, the retention time (t_R) of the site-specific probe (or analyte, A) was measured as a small amount of this probe was injected onto a given column and in the presence of mobile phases that contained various concentrations of a competing agent (I), which in this case was a fatty acid. The resulting retention times for the probe and the elution time of a non-retained solute (t_M) were then used to calculate the retention factor (k) for the probe, where $k = (t_R - t_M)/t_M$. A plot of $1/k$ versus the competing agent concentration, $[I]$, was then made, as is illustrated in Figs. 4 and 5. According to Eq. (1), a linear relationship with a positive slope should be obtained for this type of plot if direct competition between the probe and competing agent is present at a single group of common binding regions on the immobilized protein [36,42].

$$\frac{1}{k} = \frac{K_{aI} V_M [I]}{K_{aA} m_L} + \frac{V_M}{K_{aA} m_L} \quad (1)$$

In this equation, K_{aA} and K_{aI} represent the association equilibrium constants for the site-specific probe and competing agent, respectively, V_M is the column void volume, and m_L is the moles of common binding sites in the column.

If a linear fit to Eq. (1) with a positive slope is obtained for a zonal elution competition study, as occurs in Figs. 4 and 5(a), the value of K_{aI} can be found by taking the ratio of the slope over the intercept [42,43]. Deviations from this type of linear response, as noted in Fig. 5(c), are an indication that a more complex scenario is present, such as allosteric interactions or a mixture of allosteric interactions and direct competition. In addition, if only random variations are seen in the data, as occurs in Fig. 5(b), this indicates that there is no competition between the probe and competing agent. These features make such plots useful in providing both qualitative and quantitative information on solute interactions that are

occurring at a particular site on a protein. This information can include the nature of the competition between the probe and competing agent and the value of the association equilibrium constants at the binding sites that are involved in the interaction [36,42,43].

3.3. Site-specific competition studies at Sudlow site I

All of the fatty acids that were utilized in this study gave a decrease in the binding of *R*-warfarin to both normal HSA and glycosylated HSA as the fatty acid concentration in the mobile phase was increased. An example of this behavior is shown in Fig. 3 for the competition of myristic acid with *R*-warfarin on the normal HSA and gHSA2 columns. This weaker binding is represented in Fig. 3 by a decrease in the observed retention time for *R*-warfarin as the concentration of myristic acid in the mobile phase was raised, and in Fig. 4 by an increase in the values of $1/k$ for *R*-warfarin that were obtained under the same experimental conditions.

The plots that were made according to Eq. (1) for *R*-warfarin gave a linear increase in $1/k$ with an increase in the mobile phase concentration of the fatty acids under the conditions used in this study. Examples of such plots are shown in Fig. 4. The best-fit parameters that were obtained are summarized in Table 2. All sets of fatty acid data for the normal HSA columns were found to have linear behavior in this type of plot, with correlation coefficients that ranged from 0.932 to 0.956 ($n = 5-7$) and with only random variations being seen in the residuals about the best-fit lines. The same type of behavior was noted for the glycosylated HSA columns, where a linear fit was acquired for all combinations of the glycosylated HSA columns and fatty acids that were tested (correlation coefficients, 0.936 to 0.986; $n = 6-7$). The linearity of these plots indicated that these fatty acids had direct competition with *R*-warfarin at Sudlow site I on both normal HSA and the tested samples of glycosylated HSA. This result was consistent with the fact that a binding region for all of these fatty acids overlaps with Sudlow site I (e.g., see Fig. 1) [30].

The linear behavior of these plots also made it possible to use the best-fit parameters for these lines to estimate the value of K_{dI} for each fatty acid at Sudlow site I on the normal HSA and glycosylated HSA columns. The resulting values are included in Table 2. The K_{dI} values that were obtained at Sudlow site I for these fatty acids were in the range of 10^5 to 10^7 M⁻¹. These results fell within the approximate range of 10^5 to 10^8 M⁻¹ that has been reported for fatty acids with normal HSA [1,19,27-32]. These values in Table 2 represent regions with moderate-to-strong interactions and rank in the top 3-5 of the strongest sites that have been reported for these fatty acids on HSA [27]. These K_{dI} values also agree with binding constants of 10^5 to 10^7 M⁻¹ that have been estimated for the same fatty acids during their competition with sulfonylurea drugs on normal HSA and glycosylated HSA, which probably occurred at a combination of both Sudlow sites I and II [19]. Myristic acid gave the highest affinity at Sudlow site I on each type of HSA column, followed by stearic acid and palmitic acid. The same trend in the affinities for these fatty acids has been noted in their competition with sulfonylurea drugs for normal HSA and glycosylated HSA [19].

The results in Table 2 were also used to compare the K_{dI} values that were measured for each fatty acid at Sudlow site I for glycosylated HSA versus normal HSA. When this was done, a number of significant changes in these association equilibrium constants were found at the 95% confidence level. For example, in going from normal HSA to gHSA1, the value of K_{dI}

for myristic acid at Sudlow site I increased by 1.5-fold, and there was an increase of 2.0-fold when going from normal HSA to gHSA2 (Note: the differences in K_{dI} between gHSA1 and gHSA2 may reflect differences in the conditions that were used to make these glycosylated proteins) [19,21,22,34]. An increase of 1.9- or 2.2-fold in the K_{dI} for stearic acid at Sudlow site I was found when comparing gHSA1 and gHSA2 with normal HSA. A 2.7-fold increase in affinity was present for palmitic acid in going from normal HSA to gHSA1; however, a smaller apparent change of 1.3-fold, which was not significant at the 95% confidence level, was seen when comparing gHSA2 with normal HSA. Overall, these results indicated that glycation can have an appreciable effect on the binding strength of fatty acids at Sudlow site I. This observation fits with the fact that a number of lysine residues known to take part in glycation-related modifications are located at or near this region of HSA (see Fig. 1) [20,34].

3.4. Site-specific competition studies at Sudlow site II

Competition studies based on zonal elution were also performed using L-tryptophan as a site-specific probe for Sudlow site II. The normal HSA columns gave linear behavior with a positive slope for all three fatty acids when using plots of $1/k$ versus $[I]$, as prepared according to Eq. (1). An example of such a graph is provided in Fig. 5(a). The correlation coefficients for these plots ranged from 0.946–0.979 ($n = 7-8$), with only random variations being seen in the residuals about the best-fit line. This behavior indicated that each of these fatty acids had direct competition with L-tryptophan at Sudlow site II of normal HSA. Such a result agreed with the fact that a binding region for all of these fatty acids is also present within the general location of Sudlow site II (see Fig. 1) [30].

Using Eq. (1), it was possible to estimate the value of K_{dI} for each of the fatty acids at Sudlow site II of normal HSA. These association equilibrium constants are provided in Table 3. These equilibrium constants varied from 10^5 to 10^7 M^{-1} , which again agreed with the general range of 10^5 to 10^8 M^{-1} that has been reported for fatty acids with normal HSA [1,19,27–32], as well as with binding constants of 10^5 to 10^7 M^{-1} that have been measured for myristic acid, stearic acid and palmitic acid during their competition with sulfonylurea drugs for normal HSA and glycosylated HSA [19]. Myristic acid had the strongest binding at Sudlow site II, followed by stearic acid and palmitic acid. This is the same relative order of binding strengths that was observed at Sudlow I (see Table 2) and in previous studies looking at the competition of these fatty acids with sulfonylurea drugs on normal HSA and glycosylated HSA [19]. It was also found that the association equilibrium constants of these fatty acids at Sudlow site II were consistently higher than those for the same fatty acids at Sudlow site I, with the values at Sudlow site II being 2.2- to 3.5-fold greater. The association equilibrium constants that were measured here at Sudlow site II fit the values for the strongest 1–2 sites that have been observed for myristic acid with HSA and the top 4–5 sites that have been detected for stearic acid and palmitic acid on this protein [27].

Another difference that was found in comparing the results at Sudlow sites I and II was in the effects of glycation on the interactions of fatty acids at these sites. While glycation changed the binding strength of each fatty acid at Sudlow site I, direct competition was still observed for these fatty acids with *R*-warfarin at this site. However, the data generated through the competition studies with glycosylated HSA at Sudlow site II indicated glycation

could produce additional changes in the types of interactions that were occurring for fatty acids at this region. For example, two of the fatty acids (i.e., myristic acid and stearic acid) no longer showed any significant competition with L-tryptophan when using columns that contained gHSA1. Such behavior is illustrated in Fig. 5(b). The other combinations of fatty acids and glycated HSA that were examined gave a non-linear response with a negative slope for plots that were made according to Eq. (1). An example of this response is given in Fig. 5(c). This latter behavior indicated that positive allosteric interactions were now present between the fatty acids and L-tryptophan. Both sets of changes showed that glycation can affect the types of interactions that occur between fatty acids and drugs at Sudlow site II. This result is also consistent with the fact that some lysine residues that are known to take part in glycation-related modifications can be found at or near Sudlow site II (see Fig. 1) [20,34].

4. Conclusions

This study demonstrated how HPAC and columns containing HSA can be utilized to examine the binding of fatty acids at specific sites on this protein, along with the qualitative and quantitative changes that may occur in this binding due to glycation. For instance, it was possible to classify the interactions of each fatty acid with probes for Sudlow sites I and II as involving direct competition, allosteric interactions or no competition. In the case where direct competition was observed, it was further possible to measure the association equilibrium constants for the fatty acids at these specific sites on HSA.

The effects of glycation varied with the binding site that was being examined and the type of glycated HSA or fatty acid that was being considered. At Sudlow site I, direct competition occurred for each fatty acid with *R*-warfarin when using either normal HSA or glycated HSA. However, an increase in the association equilibrium constants for myristic acid, stearic acid and palmitic acid at Sudlow site I was seen when the HSA became glycated. Additional changes in the types of interactions that occurred for these fatty acids at Sudlow site II were found when comparing normal HSA and glycated HSA. For instance, the competition of these fatty acids with L-tryptophan for normal HSA followed a direct competition model, while the use of glycated HSA resulted in either allosteric interactions or no competition between these solutes. The results of this report indicate that glycation, as occurs during diabetes, can alter the binding of fatty acids and their interactions with drugs at Sudlow sites I and II of HSA. These experiments also demonstrate how HPAC can be used to study changes in biological systems that may involve modified proteins or complex solute-protein interactions.

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Highlights

- Binding was examined by fatty acids to the major drug sites of human serum albumin.
- High-performance affinity chromatography was used to study these interactions.
- The effect of non-enzymatic glycation on this site-specific binding was determined.
- Glycation altered both the strength and types of binding that occurred at these sites.

Figure 1. Crystal structure of HSA, including the regions on this protein that bind to myristic acid (shown in red) and the locations of Sudlow sites I and II. This image was generated using file PDB 1H9Z from the Protein Data Bank [33]. *R*-Warfarin, which is a common site-specific probe for Sudlow site I, is shown in green. The lysine residues on HSA that are most likely to undergo glycation are labeled and indicated in blue, as based on data from Ref. [34]; a list of other possible modification sites is also provided in this reference.

Figure 2.

(a) Structures of *R*-warfarin and *L*-tryptophan, which were used in this report as site-specific probes for Sudlow sites I and II, respectively; and (b) structures and concentrations of the fatty acids that were examined in this study.

Figure 3. Zonal elution competition studies carried out at 0.50 mL/min for the injection of 20 μ L of 5 μ M *R*-warfarin onto 1.0 cm $\times$ 2.1 mm I.D. columns containing normal HSA or glycated HSA (gHSA2) in the presence of pH 7.4, 0.067 M phosphate buffer or the same buffer containing 43 nM myristic acid.

Figure 4.

Plots of the inverse of the observed retention factor ($1/k$) for *R*-warfarin versus the concentration of stearic acid in the mobile phase during the injection of 5 μ M *R*-warfarin at 0.50 mL/min onto 1.0 cm $\times$ 2.1 mm I.D. columns containing (●) normal HSA, (■) gHSA1 or (▼) gHSA2. The error bars for each mean result, when expressed as a range of ± 1 S.D. ($n = 3$), are comparable in size to the data markers in this plot.

Figure 5. Plots of the inverse of the observed retention factor ($1/k$) for L-tryptophan versus the concentration of palmitic acid in the mobile phase during the injection of 5 μ M L-tryptophan at 0.50 mL/min onto 1.0 cm $\times$ 2.1 mm I.D. columns containing (a) normal HSA, (b) gHSA1 or (c) gHSA2. The error bars for each mean result, when expressed as a range of ± 1 S.D. ($n = 3$), are comparable in size to the data markers in these plots.

Table 1Properties of the normal HSA and glycated HSA supports^a

	Type of support		
	<i>Normal HSA</i>	<i>gHSA1</i>	<i>gHSA2</i>
Protein content (mg HSA/g silica)	63 ($\pm$ 7)	62 ($\pm$ 4)	62 ($\pm$ 4)
Glycation level (mol hexose/mol HSA)	< 0.1	1.93 ($\pm$ 0.10)	2.07 ($\pm$ 0.23)

^aThe values shown in parentheses represent a range of $\pm$ 1 S.D.

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Table 2

Best-fit parameters and association equilibrium constants (K_{ad}) obtained at Sudlow site I for various fatty acids using supports that contained normal HSA, glycated HSA1 (gHSA1) or glycated HSA2 (gHSA2).

Fatty acid ^a	Best-fit line and correlation coefficient (r) ^b	Type of competition with <i>R</i> -warfarin	K_{ad} ($\times 10^6$ M ⁻¹)
<i>Normal HSA</i>			
Myristic acid	$y = 3.1 (\pm 0.6) \pm 10^4 x + 9.0 (\pm 0.1) \times 10^{-3}$, $r = 0.932$ ($n = 7$)	Direct	3.5 (± 0.6)
Stearic acid	$y = 2.0 (\pm 0.3) \pm 10^4 x + 1.5 (\pm 0.01) \times 10^{-2}$, $r = 0.956$ ($n = 6$)	Direct	1.3 (± 0.2)
Palmitic acid	$y = 6.1 (\pm 1.1) \pm 10^3 x + 1.9 (\pm 0.01) \times 10^{-2}$, $r = 0.956$ ($n = 5$)	Direct	0.3 (± 0.1)
<i>gHSA1</i>			
Myristic acid	$y = 4.0 (\pm 0.6) \pm 10^4 x + 7.5 (\pm 0.2) \times 10^{-3}$, $r = 0.936$ ($n = 8$)	Direct	5.3 (± 0.8)
Stearic acid	$y = 2.9 (\pm 0.4) \pm 10^4 x + 1.2 (\pm 0.02) \times 10^{-2}$, $r = 0.961$ ($n = 7$)	Direct	2.5 (± 0.3)
Palmitic acid	$y = 8.3 (\pm 0.7) \pm 10^3 x + 1.1 (\pm 0.01) \times 10^{-2}$, $r = 0.986$ ($n = 6$)	Direct	0.8 (± 0.1)
<i>gHSA2</i>			
Myristic acid	$y = 3.4 (\pm 0.3) \pm 10^4 x + 4.8 (\pm 0.06) \times 10^{-3}$, $r = 0.982$ ($n = 7$)	Direct	7.1 (± 0.6)
Stearic acid	$y = 2.1 (\pm 0.3) \pm 10^4 x + 7.2 (\pm 0.1) \times 10^{-3}$, $r = 0.958$ ($n = 7$)	Direct	2.9 (± 0.4)
Palmitic acid	$y = 1.9 (\pm 0.2) \times 10^3 x + 6.6 (\pm 0.04) \times 10^{-3}$, $r = 0.972$ ($n = 7$)	Direct	0.4 (± 0.3)

^aThe fatty concentrations that were used in these experiments are given in Figure 1.

^bThese binding parameters were calculated from data obtained at pH 7.4 and 37°C and using the best-fit lines generated according to Eq. (1).

The values in parentheses represent a range of ± 1 S.D. and were determined by using error propagation with the standard deviations of the slopes and intercepts of the best-fit lines ($n = 5-8$).

Table 3

Best-fit line parameters and association equilibrium constants (K_{ad}) obtained at Sudlow site II for various fatty acids using supports that contained normal HSA, glycated HSA1 (gHSA1) or glycated HSA2 (gHSA2).

Fatty acid ^a	Best-fit line and correlation coefficient (r) ^b	Type of competition with L-tryptophan	K_{ad} ($\times 10^6 \text{ M}^{-1}$)
Normal HSA			
Myristic acid	$y = 1.52 (\pm 0.1) \times 10^6 x + 0.15 (\pm 0.03), r = 0.979 (n = 8)$	Direct	10.2 (± 0.1)
Stearic acid	$y = 5.8 (\pm 0.6) \times 10^5 x + 0.13 (\pm 0.03), r = 0.970 (n = 8)$	Direct	4.6 (± 0.5)
Palmitic acid	$y = 4.6 (\pm 0.7) \times 10^4 x + 0.08 (\pm 0.02), r = 0.946 (n = 7)$	Direct	0.6 (± 0.1)
gHSA1			
Myristic acid	$y = -1.1 (\pm 0.9) \times 10^5 x + 7.9 (\pm 0.2) \times 10^{-2}, r = 0.424 (n = 8)$	No competition	n/a
Stearic acid	$y = -8.8 (\pm 0.7) \times 10^4 x + 0.11 (\pm 0.01), r = 0.984 (n = 7)$	Allosteric	n/a
Palmitic acid	$y = -2.6 (\pm 2.5) \times 10^4 x + 0.11 (\pm 0.05), r = 0.425 (n = 7)$	No competition	n/a
gHSA2			
Myristic acid	$y = -6.7 (\pm 2.4) \times 10^5 x + 0.12 (\pm 0.06), r = 0.751 (n = 8)$	Allosteric	n/a
Stearic acid	$y = -4.5 (\pm 1.3) \times 10^5 x + 0.12 (\pm 0.08), r = 0.858 (n = 6)$	Allosteric	n/a
Palmitic acid	$y = -8.8 (\pm 0.7) \times 10^4 x + 0.11 (\pm 0.01), r = 0.984 (n = 7)$	Allosteric	n/a

^aThe fatty concentrations that were used in these experiments are given in Figure 1.

^bThese binding parameters were calculated from data obtained at pH 7.4 and 37°C using the best-fit lines generated according to Eq. (1).

The values in parentheses represent a range of ± 1 S.D. and were determined by using error propagation with the standard deviations of the slopes and intercepts of the best-fit lines ($n = 6-8$).