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Analysis of Hormone-Protein Binding in Solution by Ultrafast Affinity Extraction: Interactions of Testosterone with Human Serum Albumin and Sex Hormone Binding Globulin

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Abstract

Ultrafast affinity extraction was used to study hormone-protein interactions in solution, using testosterone and its transport proteins human serum albumin (HSA) and sex hormone binding globulin (SHBG) as models. Both single column and two-dimensional systems based on HSA microcolumns were utilized to measure the free fraction of testosterone in hormone/protein mixtures at equilibrium or that were allowed to dissociate for various lengths of time. These data were used to determine the association equilibrium constants (K_a) or global affinities (nK_a') and dissociation rate constants (k_d) for testosterone with soluble HSA and SHBG. This method was also used to simultaneously measure the free fraction of testosterone and its equilibrium constants with both these proteins in physiological mixtures of these agents. The k_d and K_a values obtained for HSA were $2.1\text{--}2.2\text{ s}^{-1}$ and $3.2\text{--}3.5 \times 10^4\text{ M}^{-1}$ at pH 7.4 and 37 °C. The corresponding constants for SHBG were $0.053\text{--}0.058\text{ s}^{-1}$ and $0.7\text{--}1.2 \times 10^9\text{ M}^{-1}$. All of these results gave good agreement with literature values, indicating that this approach could provide information on a wide range of rate constants and binding strengths for hormone-protein interactions in solution and at clinically-relevant concentrations. The same method could be extended to alternative hormone-protein systems or other solutes and binding agents.

Keywords

Ultrafast affinity extraction; Hormone-protein binding; Testosterone; Human serum albumin; Sex hormone binding globulin

INTRODUCTION

A number of low mass hormones are present in the bloodstream in both a free, non-bound form and a protein-bound form.^{1–3} One example is testosterone, which is a steroid hormone that has a normal plasma concentration of 10–42 nM in adult males.^{1,4} Two important binding proteins for testosterone in blood are human serum albumin (HSA) and sex hormone binding globulin (SHBG, also known as testosterone binding globulin).^{2,5} HSA is the most abundant serum protein, accounting for 60% of the total serum protein content and having a normal concentration of 30–50 g/L (or roughly 450–750 μM).^{1,3} Testosterone binds to HSA

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at a single site on domain IIA^{4,6} and with low-to-moderate affinity (i.e., an association equilibrium constant of $2.0\text{--}4.1 \times 10^4 \text{ M}^{-1}$ at 37°C).^{2,7–9} SHBG is a homodimeric glycoprotein that acts as a transporting agent for steroid hormones such as testosterone, dihydrotestosterone and estradiol.^{2,5} The concentration of SHBG in adult males is 10–60 nM.^{1,5} Testosterone has an overall affinity for SHBG that has been reported to be as high as 10^9 M^{-1} , with this binding occurring at one or two sites per homodimeric unit.^{2,8,10–13} Only about 1–3% of testosterone is normally present in its free form in serum, with approximately half of this hormone being bound to HSA and most of the remaining half being bound to SHBG.^{2,4,9}

The free form of testosterone is generally considered to play an important role in the tissue uptake and biological activity of this hormone.^{2,8,9,14} However, some studies have shown that the form of testosterone that is bound to HSA may also be available to tissues.¹⁴ To better understand how each fraction of testosterone affects the activity of this hormone, it is important to have information regarding the interactions of testosterone with proteins such as HSA or SHBG, and of how these interactions affect the free fraction of this hormone in blood. Both the overall binding and rates of these interactions are of great interest in describing these processes.^{14,15}

Various methods have been used to investigate the interactions between steroid hormones and HSA or SHBG. These methods have included rapid filtration assays,^{15,16} equilibrium dialysis,^{7,17–20} two-phase equilibrium partitioning,^{2,14,20,21} affinity capillary electrophoresis,²² and electron spin resonance spectroscopy.²³ However, some of these methods require relatively large sample volumes or amounts of protein, or may have long analysis times (e.g., equilibrium dialysis, gel filtration and equilibrium partitioning).^{7,9,14,18} In addition, prior binding studies with testosterone in many of these methods have used highly diluted serum or samples that were not prepared at typical physiological concentrations.^{2,15,16} It would also be desirable in such studies to use a single method that can provide both thermodynamic and kinetic parameters, but previous reports have instead examined steroid interactions with plasma proteins by employing separate approaches or experimental conditions to obtain such information.^{15–23} Some methods based on capillary electrophoresis (CE) and size exclusion chromatography have used dissociation profiles to obtain both binding constants and rate constants for solute-protein interactions;^{24,25} however, the equations used in these methods can be relatively complex and these techniques have not yet been applied to hormones such as testosterone or to solutes that have physiological concentrations similar to those expected for this hormone (i.e., levels in the nM range).²⁵

Recently, a method based on ultrafast affinity extraction was developed for estimating both the equilibrium constants and rate constants for drugs with HSA in solution.²⁶ In this technique, a single affinity microcolumn that contained an immobilized binding agent with relatively fast and strong binding for the target of interest was used to extract the free form of drugs from their protein-bound form in drug/protein samples (see Figure 1). By varying the flow rate and column size, which altered the time for passage of the sample through the microcolumn, it was possible to obtain information on both the dissociation rate constant for a drug-protein complex and the binding constant for this process, with values being obtained

that ranged from roughly $0.35\text{--}4\text{ s}^{-1}$ and $10^4\text{--}10^5\text{ M}^{-1}$, respectively.²⁶ Advantages of this approach included its ability to be used as a label-free method and to directly examine interactions in solution. Other potential advantages were the speed of this method, its ease of automation, and its ability to be used with various HPLC detectors, as well as its good correlation with reference methods and need for only small amounts of drugs and proteins.²⁶ It has also been shown that this method can be modified to measure the free fractions of many drugs at clinically-relevant concentrations.^{27,28}

In this study, ultrafast affinity extraction will be adapted and tested for use in examining hormone-protein interactions in solution, using the binding of testosterone with HSA and SHBG as models. The conditions needed in this method will be examined and optimized to allow both dissociation rate constants and equilibrium constants to be obtained for these systems. The adaptation of this work for use at clinically-relevant concentrations will further be considered, such as by employing a two-dimensional affinity system. This approach will also be examined as a means for measuring the free fraction of testosterone in mixtures of HSA and SHBG that mimic the conditions found in serum and to simultaneously examine the binding of testosterone with both of these proteins in such samples. The results should provide valuable information on the relative advantages or limitations of this approach and on how this technique could be extended to the analysis of other hormone- or solute-protein interactions.

EXPERIMENTAL SECTION

Materials and Reagents

The HSA (Cohn fraction V, 96% pure, essentially fatty acid free,) and testosterone were from Sigma (St. Louis, MO, USA). The purified human SHBG (90%, batch number 060613) was obtained from AbD Serotec (Raleigh, NC, USA). The reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). The Nucleosil Si-300 silica (7 μm particle diameter, 300 Å pore size) was from Macherey Nagel (Düren, Germany). All buffers and aqueous solutions were prepared using water from a NANOpure system (Barnstead, Dubuque, IA, USA) and were passed through Osmonics 0.22 μm nylon filters from Fisher Scientific (Pittsburgh, PA, USA) to sterilize these solutions by removing microorganisms and particulate matter (Note: these conditions have been found to be suitable in previous work using HSA columns for drug-protein binding studies,^{26–30} although the use of an autoclave to sterilize glassware and buffers or aqueous solutions has also been employed in related studies).^{27,31}

Apparatus

The microcolumns used in this study were packed using a Prep 24 pump from ChromTech (Apple Valley, MN, USA). The HPLC system consisted of a PU-2080 Plus pump, an AS-2057 autosampler, and a UV-2075 absorbance detector from Jasco (Tokyo, Japan), plus a six-port LabPro valve (Rheodyne, Cotati, CA, USA). A CHM column heater and a TCM column heater controller from Waters (Milford, MA, USA) were used to maintain a temperature of $37.0 (\pm 0.1)^\circ\text{C}$ for the columns during all experiments. ChromNAV v1.8.04

software and LCNet from Jasco were used to control the HPLC system. The chromatograms were analyzed through the use of PeakFit v4.12 (Jandel Scientific, San Rafael, CA, USA).

Column Preparation

HSA was immobilized to diol-bonded Nucleosil Si-300 by using the Schiff base method, as described previously (see Supporting Information);^{29,30} the same material and immobilization method, but with no HSA being added, was used to prepare a control support. A BCA assay was utilized to determine the protein content of the final support; this assay was done in triplicate by using HSA as the standard and the control support as the blank.^{29,30} The affinity support was found by this method to contain 66 ($\pm$ 1) mg HSA/g silica, where the value in parentheses represents $\pm$ 1 S.D.

The HSA support and control support were packed into stainless steel columns with 5–25 mm lengths and 2.1 mm i.d. The packing solution was pH 7.4, 0.067 M potassium phosphate buffer. The packing pressure was 4000 psi (28 MPa) for the 10–25 mm long columns and 3000 psi (20 MPa) for the 5 mm long columns. Each of these columns was stored in sterile pH 7.4, 0.067 M phosphate buffer at 4 °C when not in use (i.e., storage conditions that have been used in prior work with similar columns).^{26,27} The affinity microcolumns were each used for up to 40–200 injections over the course of six months to provide optimum retention and peak resolution; however, the same types of columns have been found to be stable for at least 300–400 injections under comparable conditions to those used in this report.^{26,27}

Chromatographic Studies

The mobile phase used for sample preparation and for the chromatographic studies was pH 7.4, 0.067 M potassium phosphate buffer, which was degassed for 30 min prior to use. Aqueous solutions and samples of testosterone were prepared as described in the Supporting Information. All testosterone/protein mixtures were incubated for at least 30 min at 37 °C prior to injection or analysis to allow equilibrium to be established between the free and protein-bound fractions of the hormone.^{26–29} Replicate injections (n = 4) were made for all samples and standards. Components eluting from the chromatographic system were monitored at 249 nm.

To measure association equilibrium constants or apparent (global) affinities, relatively fast flow rates and short column residence times were used for sample injection to minimize the time allowed for dissociation of testosterone from its complex with a protein such as HSA or SHBG.²⁶ Dissociation rate constants were obtained by using low-to-moderate flow rates and longer residence times as the sample passed through an affinity microcolumn. The flow rates used for these experiments were selected and optimized based on previous guidelines that have reported for the analysis of other systems by ultrafast affinity extraction.^{26–28} The apparent free fraction of testosterone in a testosterone/protein mixture, as obtained at a given flow rate, was found by dividing the peak area for the free testosterone fraction by the peak area measured for a standard solution containing the same total concentration of testosterone but no soluble protein. The relative precision of these peak areas was better than $\pm$ 10% (n = 4) throughout this study.

When using only a single column system for the binding and dissociation studies, 1 μL samples that contained 20 μM testosterone or 20 μM testosterone/40 μM HSA were injected onto a 10 mm $\times$ 2.1 mm i.d. HSA microcolumn at 0.1–5.0 mL/min. Similar experiments were performed on a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn by making 1 μL injections of 10 μM testosterone or 10 μM testosterone/20 μM HSA at 0.5–2.5 mL/min. The interactions between testosterone and SHBG were examined by injecting 50 μL of 42 nM testosterone or 42 nM testosterone/20 nM SHBG onto a 20 mm $\times$ 2.1 mm i.d. HSA microcolumn at 0.25–2.00 mL/min, along with similar injections of 25 nM testosterone or 25 nM testosterone/35 nM SHBG.

A two-dimensional affinity system (see right portion of Figure 1) was used to measure the association equilibrium constant for testosterone with HSA at physiological concentrations, as well as the free fractions and binding constants for testosterone in mixtures of HSA and SHBG at physiological concentrations. This approach was employed in these situations because of the large excess of HSA that was present versus testosterone (i.e., over a 10^4 fold mole excess),^{1,3} which made it impractical to measure the free fraction of testosterone when using only the single column system. Work on the two-dimensional system with HSA was carried out by injecting 50 μL samples of 25 nM testosterone or 25 nM testosterone/600 μM HSA onto a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn at 2.0 mL/min. A 25 mm $\times$ 2.1 mm i.d. HSA column was then placed on-line with the first column to further separate the extracted free hormone fraction from other sample components (e.g., HSA or testosterone that had dissociated from soluble HSA). This second column was placed on-line with the first column at 0.35–0.40 min after sample injection, with the flow rate being changed at the same time to 0.50 mL/min. The free testosterone concentration was determined by comparing the peak area that was obtained on the second column to the peak areas that were obtained on the same system for testosterone standards.^{27,28} The same conditions were used to examine samples containing 25 nM testosterone or 25 nM testosterone in combination with 450–750 μM HSA and 10–60 nM SHBG.

RESULTS AND DISCUSSION

Optimization of Ultrafast Affinity Extraction for Testosterone-Protein Binding Studies

The flow rate and column size are two important factors to consider in ultrafast affinity extraction, as they determine the time allowed for dissociation of the protein-bound form of a solute as a sample passes through the column.^{26–30} For instance, a short column or a high flow rate can be used to decrease the time allowed for passage through the column, which helps minimize dissociation of the solute from its complexes with proteins in the sample. This effect is illustrated in Figure 2 for the injection of mixtures containing testosterone and HSA or SHBG that were made at various flow rates onto 5 or 20 mm $\times$ 2.1 mm i.d. HSA microcolumns.

For the data that were acquired at low-to-moderate flow rates in Figure 2, the apparent free fraction for testosterone increased as the injection flow rate was decreased. This effect occurred below a flow rate of 2.00 mL/min for the testosterone/HSA data and below a flow rate of 1.25 mL/min for the testosterone/SHBG results. This behavior was caused by an increase in dissociation of testosterone from its complex with soluble HSA or SHBG as the

samples passed through the affinity microcolumns over longer periods of time. The use of larger affinity columns gave comparable trends (see Supporting Information); however, these columns required higher flow rates, but similar sample residence times, to provide free hormone fractions that did not vary significantly with the flow rate.^{26,27}

Figure 3 shows some typical chromatograms that were obtained for injections of testosterone and HSA onto a 5 mm × 2.1 mm i.d. HSA microcolumn. The non-retained peak, which was due to HSA and testosterone's complex with soluble HSA in the sample, eluted within 10 to 50 s for the data shown in Figure 3. The elution time for the peak due to the retained, free fraction of testosterone occurred between 0.3 min (at 2.5 mL/min) and 1.5 min (at 0.5 mL/min), with this peak being separated from the protein-bound fraction of testosterone in the sample. Similar separations were obtained on 10 mm × 2.1 mm i.d. HSA microcolumns, but with the peaks shifting to proportionately longer elution times when the same flow rates were employed. These elution times made it possible to measure the actual or apparent free fractions for testosterone in as little as 0.2–0.3 min and in less than 6–9 min when using the single column or two-dimensional affinity systems that were employed in this report.

Estimation of Dissociation Rate Constants and Equilibrium Constants

The apparent free hormone fractions that were measured at low-to-moderate flow rates were used to estimate the dissociation rate constants for testosterone from its complex with soluble HSA or SHBG. This was accomplished by plotting these results according to a first-order integrated rate expression, as described by either eq 1 or 2.²⁶

$$\ln \frac{(1-F_0)}{(1-F_t)} = k_d t \quad (1)$$

$$\ln \frac{1}{(1-F_t)} = k_d t - \ln(1-F_0) \quad (2)$$

In these equivalent equations, F_t is the apparent free hormone fraction that was measured at a given flow rate and column residence time t , and F_0 is the free fraction of the hormone in the original sample. The value of t can be measured by using the elution time of a non-retained solute or calculated by employing the flow rate and column void volume.²⁶

Eqs 1 and 2 predict that a hormone-protein system which follows first-order dissociation during ultrafast affinity extraction should provide a plot for either $\ln[(1-F_0)/(1-F_t)]$ or $\ln[1/(1-F_t)]$ versus t that gives a linear relationship.²⁶ Examples of such plots are provided in Figure 4. In either type of plot, the dissociation rate constant k_d can be determined from the slope of the best-fit line. However, the use of each equation has its own advantages. When eq 1 is used to examine the data, a separate measurement for F_0 is required (i.e., providing a reference point under conditions of equilibrium), and the resulting plot should have an intercept at or near zero. Plots constructed according to eq 2 do not require prior

information on F_0 (i.e., giving them one less piece of information for the fit), with the intercept being a non-zero and positive value that can instead be used to estimate F_0 .²⁶

Data acquired at high flow rates or small column residence times (i.e., conditions under which hormone-protein dissociation is negligible) were employed to determine the original free fraction (F_0) of the hormone. If the sample was initially at equilibrium and a hormone such as testosterone (T) has a single binding site on a protein (P), the association equilibrium constant (K_a) for this system can be calculated by using F_0 along with the known total concentrations of the hormone and the soluble protein, $[T]_0$ and $[P]_0$, as shown in eq 3.^{26–30}

$$K_a = \frac{1 - F_0}{F_0([P]_0 - [T]_0 + [T]_0 F_0)} \quad (3)$$

If multiple but independent binding sites are present for the hormone on the protein, the value obtained by eq 3 will give the apparent or global affinity constant for this system, nK_a' , where n represents the number of binding sites per protein that are involved in the interaction (see Supporting Information).^{26–29}

Determination of Equilibrium Constant for Binding of Testosterone with HSA

The association equilibrium constant for testosterone with HSA was initially determined by using a 10 mm × 2.1 mm i.d. HSA microcolumn for ultrafast affinity extraction. This type of microcolumn and column size have previously been used for studying the interactions of HSA with drugs that have binding constants in the range of 10^4 – 10^5 M⁻¹, which are comparable to the affinities that have been reported for testosterone with HSA.^{2,7–9,26,27} The experiments with this particular microcolumn were carried out by injecting 1.0 µL samples that contained 20 µM testosterone and 40 µM HSA at flow rates of 0.1–5.0 mL/min (see Supporting Information). A consistent free hormone fraction was obtained when the flow rate was at or above 4.5 mL/min, or when the column residence time for the sample was less than or equal to about 370 ms.

These data were next plotted according to eqs 1 and 2. Linear relationships were obtained for both types of plots (see Supporting Information), with correlation coefficients that ranged from 0.998 to 0.999 ($n = 5$ – 6). The value of K_a for testosterone with HSA was determined by utilizing eq 3 and a measured value of F_0 or by using eq 3 and a value of F_0 that was obtained from the intercept of a plot made according to eq 2. As is shown in Table 1, the K_a values obtained by both methods were consistently in the range of 3.2 – 3.5×10^4 M⁻¹ at pH 7.4 and 37 °C. These results gave excellent agreement with values of 2.0 – 4.1×10^4 M⁻¹ that have been previously determined for the same system when using other methods.^{2,7,9}

To verify these K_a values, ultrafast affinity extraction was repeated by using two-fold lower concentrations of testosterone and HSA, as well as a shorter HSA microcolumn (i.e., 5 mm × 2.1 mm i.d.). As shown in Figure 2, a minimum flow rate of only 2 mL/min was now required to obtain a consistent free hormone fraction on this shorter column; however, the column residence time obtained at this flow rate (416 ms) was comparable to the maximum

allowable residence time that was observed when using the longer 10 mm $\times$ 2.1 mm i.d. HSA microcolumn. The data obtained with the 5 mm $\times$ 2.1 mm i.d. HSA microcolumn gave linear relationships when they were plotted according to either eqs 1 or 2 (see Supporting Information). The correlation coefficients ranged from 0.998 to 0.999 ($n = 3-4$), and the values of K_a that were acquired from these plots were $3.2-3.3 \times 10^4 \text{ M}^{-1}$. These values agreed with those measured on the 10 mm $\times$ 2.1 mm i.d. HSA microcolumn and again fell within the range of values that have been reported in the literature.^{2,7,9} The relative precision for the K_a values that were measured on these single column systems varied from ± 6.3 to 18%.

Further experiments were conducted by using a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn as part of a two-dimensional affinity system to examine samples that contained physiological levels of both testosterone and HSA (i.e., 25 nM testosterone and 600 μM HSA). These experiments gave a K_a of $3.2 (\pm 0.1) \times 10^4 \text{ M}^{-1}$ (relative precision, $\pm 3.1\%$), which was consistent with the literature values and those measured earlier on the single column system at concentrations for testosterone and HSA in the mid- μM range. The consistency of these results confirmed that the association equilibrium constant for testosterone with HSA was independent of concentration, as would be expected for a system involving saturable sites.²⁶⁻²⁹

Determination of Rate Constants for Interactions of Testosterone with HSA

The dissociation rate constant for testosterone and HSA was first found by fitting the data for this system (as obtained on a 10 mm $\times$ 2.1 mm i.d. HSA microcolumn) directly to eq 2 or to eq 1 with a point included at the origin (see Supporting Information). These plots gave values for k_d of $2.17-2.20 \text{ s}^{-1}$ at pH 7.4 and 37 $^\circ\text{C}$. This set of values was consistent with a k_d of $3.5 (\pm 0.4) \text{ s}^{-1}$ that has been previously determined by a rapid filtration assay at the same temperature and pH when using radiolabeled testosterone and a Krebs-tricine buffer.¹⁶ Similar k_d values for testosterone with HSA were obtained when using a shorter HSA microcolumn (5 mm $\times$ 2.1 mm i.d.). The relative precision of the k_d values that were determined by using either type of HSA microcolumn was in the range of $\pm 1.4-5.8\%$.

The K_a and k_d values that were measured in this study were next used to calculate the second-order association rate constant (k_a) for testosterone with soluble HSA, based on the relationship $k_a = k_d K_a$. The estimates of k_a that were obtained ranged from $6.7-7.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. These values were in good agreement with association rate constants that have been reported for HSA with other solutes (e.g., gliclazide and chlorpromazine) that have similar association equilibrium constants and/or dissociation rate constants for this protein.²⁶

Determination of Equilibrium Constant for Binding of Testosterone with SHBG

The next set of experiments used the same HSA microcolumns to examine the binding of testosterone with a second protein (SHBG) and at nM concentrations for both of these agents. The equilibrium constant for this system was determined by using a similar procedure to the one employed for studying the interaction of testosterone with HSA. As shown earlier in Figure 2, injections of 42 nM testosterone and 20 nM SHBG onto a single column system gave a consistent free fraction for testosterone at a flow rate that was above

1.25–1.50 mL/min. This measured free fraction was used with eq 3 to calculate the global affinity constant for testosterone with SHBG. A nK_a' value of $1.1 (\pm 0.1) \times 10^9 \text{ M}^{-1}$ at pH 7.4 and 37 °C was provided by this method (see Table 2); a similar value of $0.7 (\pm 0.1) \times 10^9 \text{ M}^{-1}$ was obtained when the concentrations were changed to 25 nM testosterone and 35 nM SHBG. A two-dimensional affinity system was used to further examine this second sample (see Supporting Information) and gave a value for nK_a' of $0.8 (\pm 0.1) \times 10^9 \text{ M}^{-1}$, which agreed with the results acquired on the single column system.

The apparent free hormone fractions that were measured for these samples at low-to-moderate flow rates on the single column system were next used with eqs 2–3 to estimate nK_a' . The binding constants obtained with this second method ranged from $0.9\text{--}1.0 \times 10^9 \text{ M}^{-1}$. Both these values and those determined when using the free fractions measured at higher flow rates showed good agreement with binding constants of $0.3\text{--}1.9 \times 10^9 \text{ M}^{-1}$ that have been measured by other methods for the interaction of testosterone with SHBG at pH 7.4 and 37 °C.^{2,7,9,32} In addition, the consistency of the nK_a' values that were obtained at various concentrations of testosterone and SHBG indicated that this binding constant was essentially independent of concentration under the conditions that were used in these experiments.^{26–29}

Determination of Rate Constants for the Interactions of Testosterone with SHBG

The rate constants for testosterone with SHBG were found by using eq 1 or 2 and the apparent free fractions that were measured for this interaction at low-to-moderate flow rates. Figure 4 shows some typical plots that were obtained, which gave linear responses and correlation coefficients that ranged from 0.991 to 0.997 ($n = 5\text{--}6$). The overall dissociation rate constants that were determined in this study were $0.053\text{--}0.058 \text{ s}^{-1}$, with relative precisions of $\pm 3.4\text{--}8.9\%$. These dissociation rate constants agreed with a value of $0.056 (\pm 0.02) \text{ s}^{-1}$ that has been measured at the same pH and temperature by a rapid filtration assay,¹⁵ and with values of $0.032\text{--}0.053 \text{ s}^{-1}$ that have been reported at pH 7.2 or 8 and 37–37.5 °C.^{33,34}

The overall second-order association rate constant for testosterone with soluble SHBG was calculated from the measured values for k_d and nK_a' . The estimated k_a for this interaction was $3.9\text{--}6.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4 and 37 °C. This result agreed with a k_a range of $1.7\text{--}10.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ that was predicted from the literature values for k_d and nK_a' in Table 2.^{2,7,9,15,32} This value was higher than an association rate constant of $1.75 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ that has been reported for the same system at a temperature of only 4 °C;³⁴ however, it was similar to a value of $2.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ that has been noted for a related system with a comparable binding strength, as based on the interaction of testosterone with progesterone-binding globulin (nK_a' , $2.4 \times 10^9 \text{ M}^{-1}$ at 4 °C).³⁵

Analysis of Testosterone Mixtures with HSA and SHBG at Physiological Concentrations

The next series of experiments used the two-dimensional affinity system to measure the free fractions and binding constants for testosterone in mixtures of this hormone with HSA and SHBG that mimicked conditions found in serum. These samples contained 25 nM testosterone and concentrations for SHBG and HSA that ranged from 10–60 nM and 450–

750 μM , respectively (i.e., typical serum levels for each of these agents in adult males).^{1,3–5} The free testosterone fractions that were measured in these mixtures ranged from 1.46 to 3.87% (i.e., free testosterone concentrations of 0.36–0.97 nM), which gave good agreement with the normal range expected in males.^{2,4,9} These free fractions were measured with an absolute precision of ± 0.20 – 0.82% . It was also observed that these free fractions decreased in value (i.e., a change significant at the 95% confidence level) as the concentration of either SHBG or HSA was increased, as would be expected in clinical samples with similar levels of these proteins.¹⁴

It was possible to use the data for these mixtures to simultaneously estimate the equilibrium constants for testosterone with both HSA and SHBG. This was done by using the free fractions that were measured in two samples with difference concentrations of HSA and/or SHBG and using this data with expressions based on eq 3 to solve for the values of K_a and nK_a' (see Supporting Information for further details). Table 3 summarizes the results that were obtained. The estimated value for the global affinity constant of testosterone with SHBG ranged from 1.0 – $1.2 \times 10^9 \text{ M}^{-1}$, with an overall average of $1.1 (\pm 0.1) \times 10^9 \text{ M}^{-1}$. The association equilibrium constant estimated for HSA with testosterone ranged from 3.2 – $3.4 \times 10^4 \text{ M}^{-1}$, with an overall average of $3.3 (\pm 0.3) \times 10^9 \text{ M}^{-1}$. Both set of results gave good agreement with the literature values for these interactions^{2,7,9,15,32} and with the binding constants that had been measured earlier in this report for testosterone in the presence of only HSA or SHBG. This was the case even though these measurements were carried out in mixtures in which HSA was present at 1 – 6×10^4 -fold or 1 – 3×10^4 -fold higher concentrations than SHBG or testosterone.

CONCLUSION

This study used ultrafast affinity extraction to examine hormone-protein interactions in solution, with testosterone and HSA or SHBG being employed as models for this work. Information on both the dissociation rate constants and equilibrium constants for these interactions were obtained, with results that gave good agreement with literature values. It was found that ultrafast affinity extraction based on a single type of microcolumn (i.e., one containing immobilized HSA) could be used to examine the interactions of testosterone with either soluble HSA or SHBG, as well as in mixtures of these components at physiological concentrations.

Both a single column method and a two-dimensional affinity system were used to investigate these interactions. The single column method made it possible to quickly examine the strength and rate of binding for most of these interactions, including those between testosterone and SHBG at physiological concentrations. The modification of this system to one based on a two-dimensional method provided additional resolution between the free hormone fraction and other sample components, as needed for samples that contained physiological levels of testosterone (i.e., concentrations in the mid-to-low nM range) and HSA (i.e., which was present at over a 10^4 fold mole excess versus testosterone). It was also possible to use this latter method to simultaneously examine the binding of testosterone with both HSA and SHBG in samples that were prepared at clinically-relevant levels for these agents.

Each of these approaches involved label-free measurements that directly examined the interactions of testosterone with its binding proteins in solution. Absorbance detection was used in this particular study, but other types of HPLC detectors or detection schemes (e.g., fluorescence or chemiluminescence) could be employed for such work.^{29,30,36,37} Only 1–50 μL of sample and as little as 1.25 pmol testosterone, 0.5 pmol SHBG, or 20 pmol HSA were needed per injection, which is less than the amounts of sample that have been used in many prior methods for such work.^{7,9,14,18,24} This feature was particularly useful in experiments that involved a relatively expensive binding agent such as SHBG. This approach only required peak area measurements and the use of a linear fit for a first-order dissociation process, which made the data analysis easier to carry out than in other recent dissociation-based methods (e.g., those based on CE or size-exclusion chromatography).^{24,25} In addition, this technique could measure free hormone fractions in less than 6–9 min, as opposed to analysis times ranging from 22 min to several hours in prior methods used to examine testosterone-protein interactions or related systems.^{7,9,14,18–20,23,24} The combined use of the single column and two-dimensional systems also made it possible to significantly increase the range of solute concentrations, rate constants and equilibrium constants that could be examined by ultrafast affinity extraction (e.g., detection at low nM levels, k_d values spanning from at least 10^{-2} to 10 s^{-1} , and K_a or nK_a' values ranging from at least 10^4 to 10^9 M^{-1}).

The results of this work indicated that ultrafast affinity extraction should be useful as a general approach in examining other hormone- or solute-protein interactions in solution,^{27–29} as well as the interactions of a hormone or solute with a mixture of binding agents. Future work will explore the use of microcolumns containing alternative binding agents to allow a similar approach to be used with hormones and solutes that have little or no retention on immobilized HSA. Further research will also be carried out in characterizing the conditions that are required to analyze solute-protein interactions by ultrafast affinity extraction. In addition, the modification of this approach to provide information on reaction stoichiometry for multivalent processes will be considered. Some anticipated applications for this method include its use in screening the binding of hormone analogs or mimics to proteins or receptors that bind the parent hormone, biological interaction studies aimed at modeling hormone distribution and transport in the body, and measurements of the free or bound fractions of hormones or other solutes in clinical samples for disease diagnosis or treatment.^{2,8,9,14,15,33,34}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

General scheme for examining hormone-protein binding by ultrafast affinity extraction. In this approach, a mixture of a hormone (○) and protein (◐) is injected onto an affinity microcolumn that contains an immobilized binding agent for the hormone, such as HSA (◑). A separation of the free and protein-bound forms of the hormone is obtained on this column and provides apparent free hormone fractions that can be used to estimate the dissociation rate constant for the system (k_d) at low-to-moderate injection flow rates, and the association equilibrium constant (K_a) or global affinity constant (nK_a') for the same system at higher flow rates. If further resolution of the retained free fraction is needed from other sample components, a portion of this retained fraction can be passed to a second column for further separation prior to measurement of the free fraction.

Figure 2.

Effect of injection flow rate on measurement of the apparent free fraction of testosterone in the presence of soluble HSA or SHBG. These results were acquired at pH 7.4 and 37 °C for 1 μ L injections of 10 μ M testosterone/20 μ M HSA made onto a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn (top), or 50 μ L injections of 42 nM testosterone/20 nM SHBG made onto a 20 mm $\times$ 2.1 mm i.d. HSA microcolumn (bottom). Other conditions are given in the text.

Figure 3. Chromatograms obtained at various flow rates for 1 μ L injections of 10 μ M testosterone and 20 μ M HSA onto a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn at pH 7.4 and 37 $^{\circ}$ C.

Figure 4.

Measurement of the overall dissociation rate constant for testosterone and SHBG at pH 7.4 and 37 °C by using ultrafast affinity extraction. The sample contained 42 nM testosterone/20 nM SHBG and was injected onto a 20 mm $\times$ 2.1 mm i.d. HSA microcolumn. These results were analyzed by using eq 2 (top) or eq 1 (bottom), where the latter approach included an additional point at the origin. The equations for the best-fit lines were $y = 0.057 (\pm 0.004) x + 0.785 (\pm 0.027)$ and $y = 0.058 (\pm 0.002) x + 0.002 (\pm 0.012)$, respectively. The correlation coefficients for these plots ranged from 0.991 to 0.997 ($n = 5-6$). The error bars represent a range of ± 1 S.D. ($n = 4$). The slope of either plot was used to provide the dissociation rate constant for the hormone/protein interaction that was occurring in solution, and the intercept obtained for the plot made according to eq 2 was used to estimate the binding constant for this interaction.

Table 1Equilibrium constants and rate constants for the interactions of testosterone with HSA^a

Samples ^b	Association equilibrium constant, K_a ($\times 10^4$ M ⁻¹)		Dissociation rate constant, k_d (s ⁻¹)	
	eq 3 only	eqs 2–3	eq 1	eq 2
10 μ M Testosterone/20 μ M HSA	3.2 ($\pm$ 0.2)	3.3 ($\pm$ 0.6)	2.07 ($\pm$ 0.03)	2.08 ($\pm$ 0.12)
20 μ M Testosterone/40 μ M HSA	3.5 ($\pm$ 0.3)	3.2 ($\pm$ 0.5)	2.20 ($\pm$ 0.04)	2.17 ($\pm$ 0.08)
Literature values [Refs.]	2.02–4.06 [2,7,9]		3.5 ($\pm$ 0.4) [16]	

^aThese values were measured at pH 7.4 and 37 °C. The numbers in parentheses represent a range of $\pm$ 1 S.D. ($n = 4$). The samples containing 10 μ M testosterone/20 μ M HSA were analyzed using a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn, and the samples containing 20 μ M testosterone/40 μ M HSA were analyzed using a 10 mm $\times$ 2.1 mm i.d. HSA microcolumn.

Table 2Equilibrium constants and rate constants for the interactions of testosterone with SHBG^a

Samples	Global affinity constant, nK_a' ($\times 10^9 \text{ M}^{-1}$)		Overall dissociation rate constant, k_d (s^{-1})	
	eq 3	eqs 2–3	eq 1	eq 2
42 nM Testosterone/20 nM SHBG	1.1 (± 0.1) ^b	1.0 (± 0.6)	0.058 (± 0.002)	0.057 (± 0.004)
25 nM Testosterone/35 nM SHBG	0.7 (± 0.1) ^b	0.9 (± 0.3)	0.053 (± 0.002)	0.056 (± 0.005)
Literature values [Refs.]	0.3–1.9 [2,7,9,32]		0.056 (± 0.002) [15]	

^aThese values were measured at pH 7.4 and 37 °C. The ultrafast affinity extraction results were obtained on a 20 mm $\times$ 2.1 mm i.d. HSA microcolumn. The numbers in parentheses represent a range of ± 1 S.D. ($n = 4$), except where otherwise indicated.

^bThis nK_a' is the average of three values that were measured at 1.25–2.00 mL/min.

Table 3

Free fractions and equilibrium constants measured for testosterone in physiological mixtures of HSA and SHBG^a

Protein content in Sample	Free fraction of testosterone	Association equilibrium constant for HSA, K_a (M^{-1})	Global affinity constant for SHBG, nK_a' (M^{-1})
450 μ M HSA/35 nM SHBG	2.66 ($\pm$ 0.20)%	$3.3 (\pm 0.4) \times 10^4$	$1.0 (\pm 0.2) \times 10^9$
750 μ M HSA/ 35 nM SHBG	1.96 ($\pm$ 0.53)%	$3.4 (\pm 0.3) \times 10^4$	$1.1 (\pm 0.2) \times 10^9$
600 μ M HSA/10 nM SHBG	3.87 ($\pm$ 0.82)%	$3.2 (\pm 0.2) \times 10^4$	$1.1 (\pm 0.1) \times 10^9$
600 μ M HSA/60 nM SHBG	1.46 ($\pm$ 0.46)%	$3.2 (\pm 0.2) \times 10^4$	$1.2 (\pm 0.1) \times 10^9$

^aThese values were measured at pH 7.4 and 37 °C for samples containing 25 nM testosterone on a two-dimensional affinity system consisting of 5 mm $\times$ 2.1 mm i.d. and 25 mm $\times$ 2.1 mm i.d. HSA microcolumns. The K_a and nK_a' values are the averages for four possible combinations of the given result with the data from the other samples that were tested; the values in parentheses represent a range of $\pm$ 1 S.D.