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A multi-step, dynamic allosteric model of testosterone's binding to sex hormone binding globulin

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ABSTRACT

Purpose: Circulating free testosterone (FT) levels have been used widely in the diagnosis and treatment of hypogonadism in men. Due to experimental complexities in FT measurements, the Endocrine Society has recommended the use of calculated FT (cFT) as an appropriate approach for estimating FT. We show here that the prevailing model of testosterone's binding to SHBG, which assumes that each SHBG dimer binds two testosterone molecules and that the two binding sites on SHBG have similar binding affinity is erroneous and provides FT values that differ substantially from those obtained using equilibrium dialysis.

Methods: We characterized testosterone's binding to SHBG using binding isotherms, ligand depletion curves, and isothermal titration calorimetry (ITC). We derived a new model of testosterone's binding to SHBG from these experimental data and used this model to determine FT concentrations and compare these values with those derived from equilibrium dialysis.

Results: Experimental data on testosterone's association with SHBG generated using binding isotherms including equilibrium binding, ligand depletion experiments, and ITC provide evidence of a multi-step dynamic process, encompassing at least two inter-converting microstates in unliganded SHBG, readjustment of equilibria between unliganded states upon binding of the first ligand molecule, and allosteric interaction between two binding sites of SHBG dimer. FT concentrations in men determined using the new multistep dynamic model with complex allostery did not differ from those measured using equilibrium dialysis. Systematic error in calculated FT vales in females using Vermeulen's model was also significantly reduced. In European Male Aging Study, the men deemed to have low FT (<2.5th percentile) by the new model were at increased risk of sexual symptoms and elevated LH.

Conclusion: Testosterone's binding to SHBG is a multi-step dynamic process that involves complex allostery within SHBG dimer. FT values obtained using the new model have close correspondence with those measured using equilibrium dialysis.

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1. Introduction

Testosterone, the major androgen in humans, circulates in blood bound largely to sex hormone binding globulin (SHBG) and albumin (Bhasin et al., 2010; Hammond and Bocchinfuso, 1996; Mendel, 1989; Rosner, 1991; Rosner et al., 2007). Testosterone can also bind to orosomucoid and transcortin proteins, but the amount of testosterone bound to these proteins in human plasma is negligible. In many conditions that affect SHBG concentrations, such as obesity, diabetes, aging, hyperthyroidism, liver disease, and HIV-infection, total testosterone concentrations are altered because of changes in SHBG concentrations; in these conditions, expert panels have recommended the determination of free testosterone (FT) concentration to obtain an accurate assessment of androgen status (Bhasin et al., 2010; Hammond and Bocchinfuso, 1996; Mendel, 1989; Rosner, 1991; Rosner et al., 2007).

Reflecting the growing interest in men's health and the success of pharmaceutical advertising, testosterone sales have grown from 23 million dollars in 1993 to 70 million in 2000 to 1.7 billion dollars in 2012 (Spitzer et al., 2012). Testosterone is the second most frequently ordered test, next only to 25-hydroxyvitamin D. In 2012, nearly 4 million free testosterone tests were performed in the USA alone. A number of direct and indirect methods – equilibrium dialysis, ultrafiltration, tracer analog methods, and calculations based on simple law-of-mass action equations – have been developed for the determination of FT levels (Adachi et al., 1991; Mazer, 2009; Rosner, 1997; Rosner et al., 2007; Sinha-Hikim et al., 1998; Sodergard et al., 1982; Van Uytfanghe et al., 2004; Vermeulen et al., 1971, 1999; Winters et al., 1998). Expert panels have expressed concern about the accuracy and methodological complexity of the available assays for FT (Rosner et al., 2007; Sodergard et al., 1982; Vermeulen et al., 1971). Recognizing these methodological difficulties in the measurement of free testosterone, the Endocrine Society's Expert Panel suggested that "*the calculation of free testosterone from reliably measured total testosterone and SHBG using mass action equations provides the best approach for the estimation of free testosterone…*" (Rosner et al., 2007). Therefore, algorithms for calculating FT from total testosterone, SHBG and albumin concentrations using the extant linear binding model (also referred to as law-of-mass-action equations) (Adachi et al., 1991; Bhasin et al., 2011; Ly and Handelsman, 2005; Ly et al., 2010; Mazer, 2009; Morales et al., 2012; Morley et al., 2002; Rosner et al., 2007; Sartorius et al., 2009; Sodergard et al., 1982; Vermeulen et al., 1999) or empirically-derived equations (Ly and Handelsman, 2005; Ly et al., 2010; Nanjee and Wheeler, 1985; Sartorius et al., 2009) have been published and used widely (Adachi et al., 1991; Bhasin et al., 2011; Ly and Handelsman, 2005; Ly et al., 2010; Mazer, 2009; Morales et al., 2012; Morley et al., 2002; Rosner et al., 2007; Sartorius et al., 2009).

The current model of testosterone's binding to SHBG assumes that each SHBG dimer binds two testosterone molecules, and that each of the two binding sites on SHBG dimer has similar binding affinity irrespective of the occupancy of the adjacent binding site (no allostery). Equations to determine FT, using this model, have been proposed by Vermeulen, Sodergard, and Mazer (Mazer, 2009; Rosner et al., 2007; Sodergard et al., 1982; Vermeulen et al., 1971). In present work we characterized testosterone's binding to SHBG using equilibrium dialysis (varying ligand and SHBG concentrations) and isothermal titration calorimetry (ITC) to characterize testosterone's binding to SHBG. We considered several possible mechanistic models of molecular interactions, including the prevailing model of homogeneous binding of testosterone to SHBG as envisioned by Vermeulen (Vermeulen et al., 1999), Sodergard (Sodergard et al., 1982) and implemented in a spreadsheet by Mazer (Mazer, 2009), and various allosteric mechanisms, including positive and negative cooperativity (Koshland et al., 1966; Monod et al., 1965), and ensemble allostery (Freiburger et al., 2011; Hilser and Thompson, 2007) (Fig. 1). Based on our analyses of the experimental data of testosterone's binding to SHBG, we constructed a novel multistep binding model with complex intra-dimer allostery for the calculation of FT, which provided the best fit to the totality of experimental data. This new model was then utilized to determine FT concentrations in samples derived from randomized testosterone trials in men and women, and to compare the results with those obtained using equilibrium dialysis. Finally, we used the algorithm to examine the distribution of FT levels in community-dwelling men in the Framingham Heart Study (FHS) and related the deviations from the mean to the risk of sexual symptoms and elevated LH levels in an independent sample of men in the European Male Aging Study (EMAS).

2. Materials and methods

2.1. Biophysical characterization

Human SHBG purified from serum (Binding Site Group, Birmingham, UK, cat# BH089.X) was characterized by protein gel denaturation–renaturation experiments and by measuring its ability to bind testosterone. Testosterone concentration in the SHBG stock solution, measured using LC-MS/MS, was undetectable. Testosterone standard 1.0 mg/mL ± 2% (3.47 mM) was obtained from Cerilliant (Round Rock, TX).

Binding isotherms were established using equilibrium dialysis (varying either testosterone or SHBG concentrations) in 96-well dialysis plates containing dialysis chambers separated by membranes with 10 kDa cut-off (Harvard Apparatus, Holliston, MA). SHBG and testosterone were reconstituted in dialysis buffer (30 mM HEPES pH7.4, 90 mM NaCl, 1 mM $MgSO_4$, 187 μM $CaCl_2$), and mixed to a desired concentration. Two hundred microliters of SHBG-testosterone mixture was loaded on one side of the dialysis membrane and dialyzed overnight against equal volume of dialysis buffer (200 μL). The equilibrium was achieved by rotating the dialysis plate overnight at 22 °C. Preliminary experiments demonstrated that 16 hours were sufficient to achieve steady-state equilibrium. Each concentration/condition was tested in three different wells, and each titration was repeated at least two times.

Testosterone concentration was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) assay that has been certified by the Center for Disease Control and has a sensitivity of 2 ng/dL (Bhasin et al., 2011).

Isothermal calorimetry (ITC) was performed using automated Auto-ITC200 Calorimeter (MicroCal, Northampton, MA) at Biological Calorimetry Facility (Huck Institutes of Life Sciences, University Park, PA). SHBG was reconstituted in 30 mM HEPES buffer, pH7.4, to a final concentration of 5 μM. Testosterone standard was prepared in DMSO and diluted in protein buffer to 100 μM in 5% DMSO. DMSO was added to SHBG by weight to match DMSO content in testosterone solution. Samples were degassed prior to loading to the calorimeter. Testosterone was injected into protein solution in 15 equal steps 2 μL each. Total reaction volume was 203 μL. Isothermal titration calorimetry experiment was repeated twice. Heat produced by each injection was measured by the calorimeter. Interval between injections was set at 240 seconds so that the temperature could return to baseline. The heat generated after each injection (after subtracting the heat of dilution of ligand in buffer) was integrated to produce calorimetric isotherm depicting the relation of the total heat generated in the reaction to testosterone-to-SHBG molar ratio.

At time $t = 0$, we computed initial concentration of S_2 and S'_2 as defined by the SHBG concentration and K_{d11}. During the process of reaching chemical equilibria, the computer program recorded the total flux of SHBG molecules through each of the elementary reactions Φ_j. Total heat generated/consumed during the equilibration was calculated as $H_i = \sum_j \Phi_{ij} \Delta H_j$, for each injection number "i". Total