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Encapsulation of testosterone and its aliphatic and aromatic dimers by milk beta-lactoglobulin

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ABSTRACT

The encapsulation of testosterone and it aliphatic dimer (alip) and aromatic dimer (arom) with milk β-lactoglobulin (β-LG) was studied in aqueous solution at pH 7.4. Multiple spectroscopic methods, transmission electron microscopy (TEM) and molecular modeling were used to characterize testosterone-β-LG binding and protein aggregation process. Spectroscopic analysis showed that steroids bind β-LG via hydrophobic and H-bonding interactions with overall binding constants $K_{test\text{-}\beta\text{-}LG} = 5.6\ (\pm 0.6) \times 10^4\ M^{-1}$, $K_{test\text{-}dimer\ alip\text{-}\beta\text{-}LG} = 4.8\ (\pm 0.5) \times 10^3\ M^{-1}$ and $K_{test\text{-}dimer\text{-}arom\text{-}\beta\text{-}LG} = 2.9\ (\pm 0.4) \times 10^4\ M^{-1}$. The binding affinity was testosterone > testosterone dimer-aromatic > testosterone dimer-aliphatic. Transmission electron microscopy showed major changes in protein morphology as testosterone–protein complexation occurred with increase in the diameter of the protein aggregate indicating encapsulation of steroids by β-LG. Modeling showed the presence of H-bonding stabilized testosterone-β-LG complexes with the free binding energy of −9.82 Kcal/mol indicating that the interaction process is spontaneous at room temperature.

1. Introduction

Milk bovine β-lactoglobulin is a globular protein that contains162 amino acids and has a molecular weight of 18.3 kDa. It is composed of nine β-strands and one α-helix, in which the hydrophobic sequences are mostly buried. At room temperature and neutral pH, it exists in the form of a dimer, which dissociates into monomers at acidic pH [1,2]. It shows major binding affinity for hydrophobic and hydrophilic ligands [3–9]. Under denaturing conditions, β-LG is able to form gels and aggregates or precipitates, depending on the protein concentration and other conditions, such as pH and temperature [10,11]. Aggregation and fibrillation of β-LG are extensively studied under different physico-chemical conditions [10,11]. β-LG gels formed through heating treatment are widely used in the food industry. The applications for β-LG gels in both drug and food supplements are reported [12,13]. It was found that β-LG can form fibrils on heating treatment at low pH, and under high pressure, or in the presence of organic solvents [11]. Transmission electron microscopy and spectroscopic methods were often used to characterize the nature of β-LG aggregation and fibrillation [10,11,14].

Testosterone, the principal androgen is transported by serum proteins in the blood. It is reported to interact with serum proteins with a stoichiometric ratio of 1:1 [15,16]. Several studies have examined the association of testosterone with HSA and BSA and the effect of steroid on protein structure and function [15–17]. Additionally, it is well known that dimers of biologically important compounds possess enhanced biological activities in comparison to the corresponding monomeric units [18,19]. Hence, our interest in studying the testosterone dimers (Scheme 1) described herein and to compare their physico–chemical properties with their monomeric unit.

In this report we examined the binding of testosterone and two of its dimeric derivatives (Scheme 1) with milk β-lactoglobulin and the effect of steroid–protein complexation on β-LG aggregation, using multiple spectroscopic methods, transmission electron microscopy, and molecular modeling. This study was undertaken to verify the potential interactions of testosterone and its dimers with an exogenic protein such as β-LG in order to gain insight into this unusual (alternate) type hormone-dietary protein association. The significance of this study resides in the likely development of new testosterone delivery system with β-LG for the treatment of

Abbreviations: β-LG, beta-lactoglobulin; testosterone, 17 β-hydroxy-4-androsten-3-one; testosterone aliphatic dimer (alip), 4-(17 β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoic acid 4-(17 β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoyloxy-butyl ester; testosterone aromatic dimer (arom), 4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoic acid 4-[4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoyloxymethyl]-benzyl ester; FTIR, Fourier transform infrared; TEM, transmission electron microscopy.

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androgen deficiency condition in men [20,21]. β-LG has also been used as a drug delivery tool by oral administration [22,23].

2. Experimental

2.1. Materials

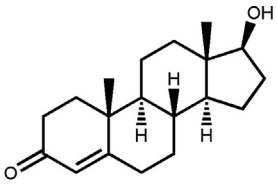
β-Lactoglobulin (A variant, purity > 90%) was purchased from Sigma Chemical Company and testosterone or 17β-hydroxy-4-androsten-3-one (**1**) was purchased from Steraloids Inc. and were used as supplied. Testosterone aliphatic dimer or 4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoic acid 4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoyloxy-butyl ester (**2**) and testosterone aromatic dimer or 4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoic acid 4-[4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoyloxymethyl]-benzyl ester (**3**) (Scheme 1) were synthesized in our laboratory using efficient modifications of testosterone skeleton that will be reported elsewhere. The novel dimers were fully characterized by IR, proton, and carbon NMR spectroscopy. The spectral data were in complete agreement with the dimers made for this study.

2.2. Preparation of stock solutions

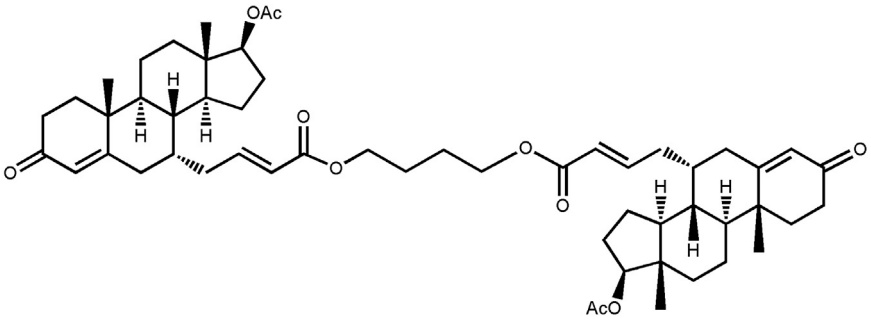
Testosterone and its dimeric compounds were dissolved in ethanolic solution (50/50%) and diluted in 10 mM Tris-HCl solution (pH 7.4). The β-lactoglobulin was dissolved in aqueous solution (4 mg/ml to obtain 0.25 mM protein content) containing Tris-HCl (pH 7.4). The protein concentration was determined spectrophotometrically using the extinction coefficient of 17,600 M^{-1} cm^{-1} at 280 nm [24]. After mixing the solution of steroid with protein solution, the ethanol content is reduced to 25%, which does not affect protein structure [6].

2.3. FTIR spectroscopic measurements

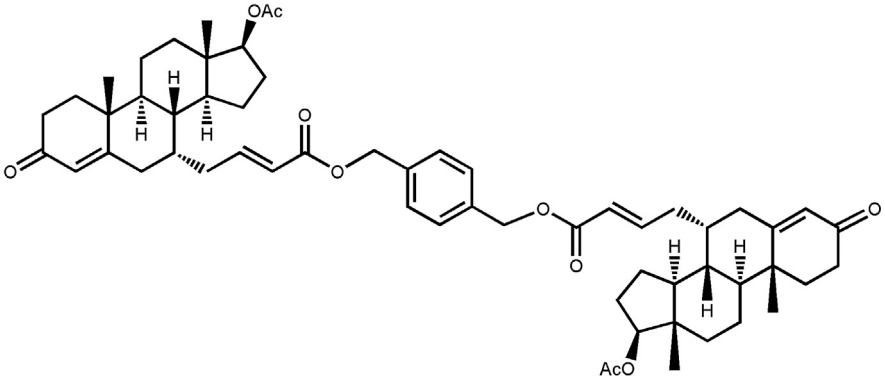
Infrared spectra were recorded on a FTIR spectrometer (Impact 420 model, Digilab), equipped with deuterated triglycine sulphate (DTGS) detector and KBr beam splitter, using AgBr windows. The



Testosterone or 17β-hydroxy-4-androsten-3-one (1)



Testosterone aliphatic dimer: 4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoic acid 4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoyloxy-butyl ester (2)



Testosterone aromatic dimer: 4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoic acid 4-[4-(17β-acetoxy-4-androsten-3-one-7α-yl)-but-2-enoyloxymethyl]-benzyl ester (3)

Scheme 1. Chemical structures of testosterone (**1**), the aliphatic dimer and aromatic dimer (**2**), and (**3**) and their respective chemical names.