Steroids 76 (2011) 558–563

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Conformational analysis of steroid hormone molecules in the lipid environment – A solid-state NMR approach

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ARTICLE INFO

Article history:
Received 16 October 2010
Received in revised form 8 February 2011
Accepted 9 February 2011
Available online 16 February 2011

Keywords:
SSNMR
DHEA
Conformation
Chemical shifts
Lipid

ABSTRACT

Solid-state $^1H/^{13}C$ cross-polarization/magic angle spinning (CP/MAS) NMR spectroscopy has been applied to two steroid compounds: dehydroepiandrosterone (DHEA) and spironolactone (SPI), to analyze their conformations at the atomic level. In the absence of lipid, the high-resolution ^{13}C CP/MAS NMR signals of DHEA and SPI in a powder form reveal multiple patterns, with splittings of 30–160 Hz, indicating the existence of multiple conformations. In the mimic lipid environment formed by mixing 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) in a molar ratio 3:1, the resulting DHEA and SPI spectra revealed mostly *singlet* patterns, suggesting that these steroids undergo a conformational change leading to a specific conformation in the lipid environment. Evidence from chemical shift isotropy and anisotropy analysis indicates that DHEA might adopt conformations subtly different from that seen in solution and in the powder form. In conclusion, we demonstrate by solid-state NMR that the structures of DHEA and SPI may adopt slightly different conformations in different chemical environments.

1. Introduction

Steroid hormones mediate a variety of biological activities in humans, including maturation, reproduction, development of gonads [1,2], maintenance of blood volume and electrolyte concentration [3], and synthesis of bone and muscle [4,5]. Among them, dehydroepiandrosterone (DHEA) is the most abundant steroid in human blood. It has been demonstrated that low levels of DHEA will induce hormonal deficiency-related health problems in menopausal women [6]. In men, around 40–50% of androgens are made in peripheral tissues from adrenal DHEA. It has been shown in humans afflicted with prostate cancer that adrenals secrete a large amount of the inactive precursor DHEA, which can be converted into active androgens in peripheral tissues; this is different from what is observed in other animals [7]. The synthetic steroidal compound spironolactone (SPI) has been used extensively for the treatment of hypertension, congestive heart failure, and hyperaldosteronism [8]. However, SPI has limited oral biomedical availability due to its low solubility. In this study, we applied solid-state NMR to study the conformations of these two steroid hormone compounds, DHEA and SPI, in the lipid environment at atomic resolution.

In spite of different biological functions, both DHEA (1a), and SPI (1b) have a similar fused-ring skeleton made of three six-member rings and one five-member ring, which are labeled as **A**-, **B**-, **C**- and **D**-rings, respectively (see Scheme 1). Double-bond modifications at different positions of the six-membered ring are found in these steroid compounds, in which a double bond is introduced between C5 and C6 of the **B**-ring in 1a, and between C4 and C5 residues of the **A**-ring in 1b. In addition, 1b has an extra side-chain attached to C7 and a five-member ring attached to C17, respectively.

Solid-state NMR spectroscopy can provide structural information on inherently water-insoluble biomolecules, such as amyloid fibrils, membrane proteins [9–11], and steroid compounds. In particular, a combination of the cross-polarization [12]/magic angle spinning [13] (CP/MAS) NMR techniques gives rise to high-resolution ^{13}C spectra comparable to solution spectra. Solid-state NMR has been applied by Yang et al. [14] to a variety of steroid compounds, including testosterone, hydrocortisone, *trans*-dehydroandrosterone, prednisolone, prednisone and estradiol. In these analyses, the high-resolution $^{13}C\{^1H\}$ CP/MAS spectra revealed a distinct *multiplet* pattern in the ^{13}C signal, with splittings of 0.2–1.5 ppm (15–110 Hz). The *multiplet* pattern was interpreted as indicating different steroid conformations. Similar phenomena have been observed previously in ^{13}C solid-state NMR spectra of vitamin D compounds [15] and other steroid compounds, such as deflazacort [16], prednisolone *tert*-butylacetate [17], estradiol [18], and testosterone [19]. And further supports of different steroid con-

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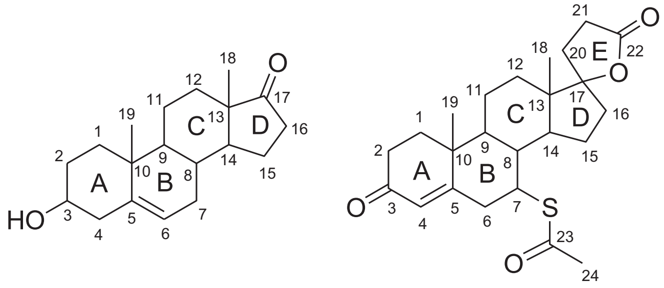
doi:10.1016/j.steroids.2011.02.005



1a 1b

Scheme 1.

formations by X-ray crystallography are reported in these steroidal compounds [16,17,19].

It is well known that the hydrophobic steroid hormones are not well dissolved in blood. To be transported to the tissues and to be able to pass through the plasma membrane, they must be bound to specific carrier proteins. Thus, it is intriguing to find out whether the steroid hormone compounds would adopt different conformations when they are associated with a lipid environment. To study this issue *in vitro*, we mixed DHEA and SPI with a mixture of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC), as a mimic of the lipid environment. The steroid/lipid mixture was examined by solid-state NMR spectroscopy. Interestingly, the resulting ^{13}C spectra revealed *singlet* patterns for DHEA and SPI in the presence of lipid molecules, implying that the lipid molecules interact with the steroid compounds to form a specific conformation. As shown by chemical shift and chemical shift anisotropy analyses, these conformations differed subtly from those observed in the solution phase and in the powder form. Thus, ^{13}C solid-state NMR spectroscopy provides a direct and sensitive measure of steroid conformation in the lipid environment.

2. Experimental

2.1. Samples

The steroid compounds dehydroepiandrosterone (DHEA) and spironolactone (SPI) were purchased from Sigma–Aldrich (St. Louis, MO). A total of five different lipid molecules, including 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). These samples were used without further purification. In this conformational study, we mixed the steroid compounds with DMPC:DHPC at a molar ratio of 3:1, or with POPC, DOPG and DOPC, respectively. The lipids (15 mg) were dissolved in chloroform (0.3 ml). An equal amount of steroid compound was added into the lipid/chloroform solution, and then incubated at room temperature for 2 h. After lyophilization overnight in a freeze-drier to remove chloroform, the dried DHEA/lipid and SPI/lipid mixtures were packed into a 4 mm MAS rotor for solid-state NMR measurements.

2.2. NMR spectroscopy

The ^{13}C CP/MAS NMR spectra were acquired with a Bruker (Spectrospin, Rheinstetten, Germany) Avance 300 MHz NMR spectrometer, equipped with a 4 mm double resonance probe operating at ^{1}H and ^{13}C Larmor frequencies of 300.13 and 75.47 MHz, respectively. The ^{1}H to ^{13}C polarization transfer was optimized to fulfill the Hartman–Hahn matching condition [12]. For the polarization transfer, the contact-time was set to 1 ms, and *rf* field strengths of 41.0 kHz were chosen for both the ^{1}H and ^{13}C channels. In ^{1}H-filtered experiments, a 50 μs delay was added before data acquisition. This allows the ^{1}H-coupled ^{13}C signal to be attenuated under strong ^{1}H dipolar coupling, so that only the ^{13}C signal of the non-protonated carbons is retained. Complementarily, in the short CP experiment, a contact time of 10 μs was used for detection of protonated carbons only. During data acquisition, ^{1}H decoupling by two pulse phase modulation (TPPM) [20] was applied with B_1 field strengths of 79.3 kHz. Powder samples were packed in double-bearing 4-mm zirconium oxide MAS rotors. If not specified, a sample spinning frequency of 7 kHz was used and regulated by a spinning controller within ±1 Hz. All CP/MAS experiments were carried out at ambient temperature. The ^{13}C CP/MAS spectra were processed with line broadening of 15 Hz and the ^{13}C chemical shifts were referenced to the glycine carboxyl carbon signal at 176.4 ppm.

3. Results and discussion

3.1. ^{13}C chemical shift assignments

The ^{13}C CP/MAS NMR spectra of steroid compounds DHEA and SPI are shown in Figs. 1a and 2a. In terms of spectral resolution of the steroid compounds, a common feature is that most of the ^{13}C resonances are distributed in two distinct zones. Those in the upfield zone (10–50 ppm) are crowded and less discernible. In contrast, the ^{13}C resonances in the downfield zone (90–200 ppm) are generally well resolved, and free from signal overlap. These findings are consistent with previously reported solid-state NMR spectra of a series of steroid compounds including DHEA [14]. To further simplify the ^{13}C spectrum, we carried out two experiments: a ^{1}H-filtered NMR spectroscopy and a normal $\{^1H\}/^{13}C$ CP/MAS experiment, using a short contact time of 50 μs, respectively. These two experiments are complementary, the former for acquiring "non-protonated" signals and the latter for "protonated" signals. The ^{13}C spectra of DHEA and SPI compounds are shown in Figs. 1b and c and 2b and c. Note that the overlap of ^{13}C resonances of C16 and C10, and of C11 and C19 in DHEA were differentiable in this way. On the other hand, the overlap of C9 and C13 and, of C14 and C10, were resolved in SPI. Given the improved spectral resolution, the ^{13}C chemical shift assignments could be determined readily, in accordance with the solution NMR assignments reported in the literature [21–24]. For example, in the case of DHEA, the carbonyl, methine, and tertiary ^{13}C residues presented an upfield ordering of C17, C5, C13, C10, C19 and C18, were assigned in a straightforward manner in the ^{1}H-filtered spectrum (see Fig. 1b). The protonated resonances in the order C6, C3, C14, C9, C4, C1, etc. were assigned in the short contact time CP spectrum. We were able to assign the ^{13}C chemical shifts with better spectral resolution, in particular in the region of 30–52 ppm. The ^{13}C chemical shift assignments of DHEA and SPI are shown in Table 1. Although the ^{1}H-filtered experiment was utilized to suppress the ^{1}H-coupled ^{13}C signals via gated decoupling, methyl carbons C18 and C19 were unexpectedly present in the "unprotonated" spectrum of DHEA. It is possible that the methyl protons undergo fast uniaxial rotation that averages out the ^{1}H-coupling effect so that the methyl carbons behave as "unprotonated" carbons. A similar phenomenon was also observed for C18 and C19 in the SPI spectrum.

Notably, in the spectra of DHEA and SPI recorded in solid, most ^{13}C resonances appeared as a *doublet* or *triplet* pattern, with splittings of 0.4–2.2 ppm (30–160 Hz), while only a few signals were obtained as *singlets*, namely C4, C5, C9, and C11 in DHEA and C9, C12, C20, and C21 in SPI (see Table 1). Given that ^{13}C chemical shifts