NMR STUDIES OF THE HISTIDINE RESIDUES OF THE α-SUBUNIT OF PORCINE LUTROPIN

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1. Introduction

The glycoprotein hormones of the adenohypophysis, lutropin (LH), follitropin (FSH) and thyrotropin (TSH) each consist of two non-identical subunits, α and β, associated by non-covalent bonds. The α-subunit has the same amino acid sequence in the three hormones, while the β-subunit differs and once combined to the α chain specifies the type of hormonal activity of the active complex (reviewed [1,2]). As the biological activity is dependent on the quaternary structure of the hormone, an important question is: how does the association of the two subunits confer biological properties, i.e., stimulation of target cells, to the complex? This question could be solved in three steps:

1. Identification of the interaction sites between the subunits within the native hormone.
2. Study of the conformational changes caused by the interaction between the subunits.
3. Definition of the hormone sites interacting with the cellular receptors.

The first point has received the greatest attention. Spectrophotometric methods [3–8], chemical modification [9–14] and amino acid sequence comparison [2] have shown that tyrosyl residues are located in the region of interaction between subunit surfaces.

The second question has been approached by immunochemical studies [15] and circular dichroism spectrometry [16]. The study of ovine lutropin [16] indicates that both subunits are involved in the unfolding of the hormone, when the active complex is dissociated at low pH. A nuclear magnetic resonance study of the subunit conformations in the isolated and in the associated active state can help to identify the portions of the subunit molecules which participate to the folding during the formation of the active hormonal complex.

2. Materials and methods

Porcine lutropin and its α and β subunits were prepared as in [17]. Biological potency of porcine LH was 1.2-times NIH-LH-S18 standard. NMR samples were prepared by lyophilisation of the protein from D_2O (98.5%) containing 50 mM potassium phosphate, pH* 6.9, in order to replace exchangeable protons by deuterium, and then redissolved in D_2O. Protein concentration were 2.7 mM for lutropin and the α subunit. The pH of the solution was adjusted by addition of microliter volumes of 0.5 M NaOD or DCl. pH measurements were performed at 40°C; the readings (given as pH*) were not corrected for the isotope effect on the glass electrode. ^1H NMR spectra, 270 MHz, were recorded using a Bruker WH 270 spectrometer equipped with Fourier transform facilities. All spectra were run at 40°C.

Sample volumes at the beginning of the titration

experiment were 0.5 ml in 5 mm tubes; 1500–2000 transients were accumulated with a pulse interval of 0.5 s. When the water proton peak became too large, it was minimised by saturation with a selective 0.4 s pulse immediately before the non-selective observing pulse. Dioxan was present in the samples at a concentration of 2 mM as a chemical shift reference.

After the NMR experiments, size homogeneity of the samples was checked by gel filtration on Sephadex G-75 (column 2.5 X 95 cm) in 0.05 M ammonium bicarbonate buffer. Subunit samples were lyophilised and their ability to recombine with a fresh sample of the complemental subunit was assayed in 0.5 M sodium acetate pH 5.3 during 5 days at 4°C, protein concentrations being 0.3 mM in each subunit. Binding activity of native and recombined lutropin to particulate receptor from immature porcine testis [18] was measured by radioligand receptor assay using porcine [^{125}I] lutropin. After the NMR experiments, all samples of the α subunit were able to combine with the lutropin β subunit to give a recombined lutropin with full receptor binding activity.

3. Results and discussion

Porcine lutropin contains four histidine residues, three in the α-subunit and one in the β-subunit [19,20]. Spectra of the histidine C2-H resonances of the isolated α-subunit are shown in fig.1. Both at pH* 2.3 and at pH* 7.9, relatively sharp resonances can be seen for all three histidines (at high pH, two of the resonances are superimposed). At intermediate pH* values, however, the histidine resonances show two contrasting types of behaviour, as indicated by the representative spectra shown in fig.1.

Two of the resonances shift progressively downfield with decreasing pH, as the imidazole ring is titrated. In the middle of the titration range, these two resonances become very broad (see spectra at pH* 5.4 and pH* 5.2 in fig.1). Fortunately, there is only one resonance from a slowly exchanging NH proton in this region of the spectrum (at approx. –5.0 ppm) and this is only clearly visible at low pH. As a result, it is possible to follow the broad histidine C2-H resonances fairly readily, particularly in difference spectra, and to obtain estimates of the pK values of 5.4 and 6.0 (± 0.2). The behaviour of the linewidth as a function of pH is typical of that expected for the situation where the exchange between protonated and unprotonated histidine (i.e., the proton exchange) is no longer fast enough to completely fulfill the fast exchange condition (see [21]). Estimating the line-

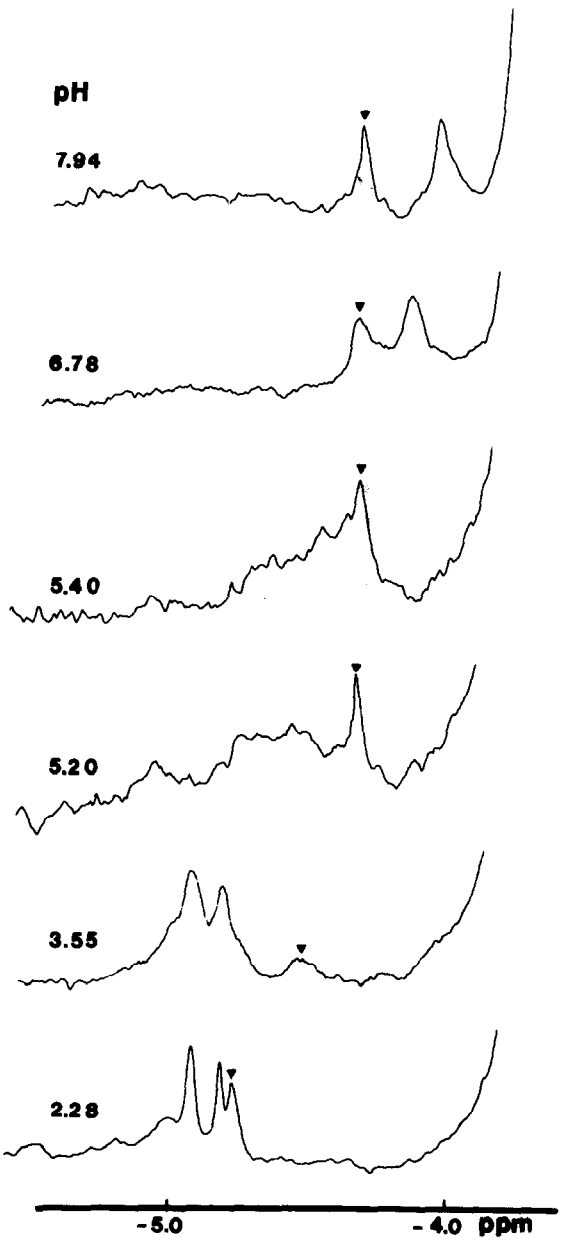

Fig.1. Histidine C2-H resonances (at 270 MHz) of porcine lutropin α-subunit in 50 mM potassium phosphate at the indicated pH* values.

width at the midpoint of the titration curve as 50 Hz, and taking that at pH* 2.3 (7.5 Hz) to be the linewidth in the absence of exchange, we calculate the rate of proton exchange to be roughly 3×10^3 s^{-1}.

The third histidine C2-H resonance (arrowed in fig.1) remains sharp, and unchanged in chemical shift over the pH* range 4.5–8.0. Below pH* 4.5, this histidine resonance also shifts downfield. However, this downfield shift is complete in only a little more than 1 pH unit, and substantial changes occur in other regions of the spectrum over the same pH range, as shown in fig.2. Both in the aromatic and aliphatic regions of the spectrum there are changes suggestive of a partial unfolding of the protein. For example, the highest field resonance in the aromatic region (fig.2A), probably arising from tyrosine residues disappears, as do the bulk of the resonances at the extreme high-field end of the spectrum (~ 3.0–3.3 ppm; fig.2B) which arise from methyl groups close to the face of aromatic rings. The spectrum at pH* 2.3 is clearly not that of a random-coil protein, but at least

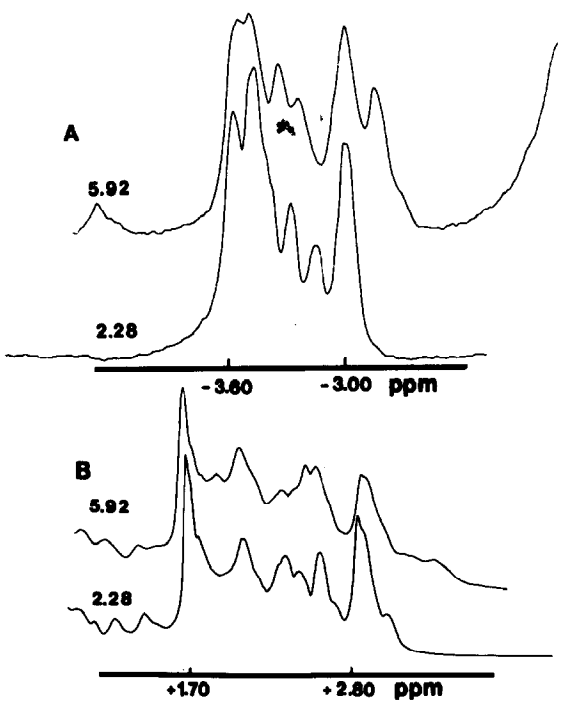

Fig.2. The aromatic (A) and aliphatic (B) regions of the 270 MHz ^{1}H NMR spectrum of porcine lutropin α-subunit at pH* 5.92 and pH* 2.28.

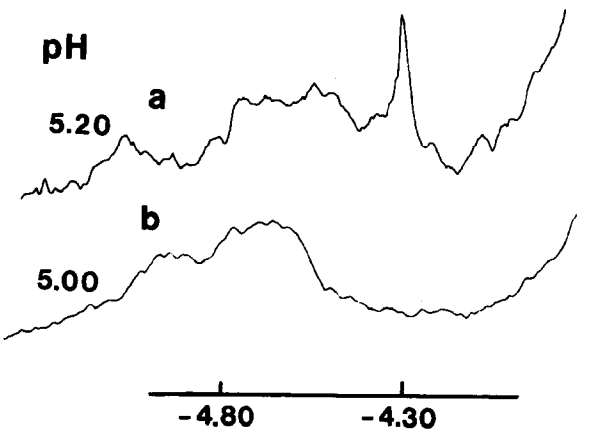

Fig.3. A comparison of the histidine C2-H resonances of porcine lutropin (b) and its isolated α-subunit (a) at similar pH* values.

a substantial loosening of the structure has occurred. It appears that the histidine residue whose resonance is arrowed in fig.1 has a pK below 4, and that its protonation is associated with (and perhaps requires) significant changes in the conformation of the protein; it must clearly be buried in a tightly-folded region of the subunit.

Comparison of the spectra of the histidine residues of the α-subunit with those of the whole hormone (α and β subunits) shows clearly that the sharp resonance has disappeared (fig.3) and that there is no histidine residue in the whole hormone with a pK below 5.0. A detailed comparison of the histidine residues in the whole hormone and its isolated subunits must await the results of experiments currently in progress to assign the histidine C2-H resonances to specific residues in the sequence. However, it is clear that the histidine residue which has such an abnormally low pK in the isolated α-subunit must have a much more nearly normal pK after combination with the β-subunit. Such a normalisation of pK clearly would not arise from occlusion of the histidine residue in the intersubunit interface. Rather, it must result from a change in the conformation of the α-subunit on combination with the β-subunit which transfers this histidine residue from a largely solvent-inaccessible and probably apolar environment to a more solvent-accessible one.

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