IDENTITY OF STRUCTURE OF OVINE AND BOVINE ACTH: CORRECTION OF REVISED STRUCTURE OF THE OVINE HORMONE

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1. Introduction

The amino acid sequences of the pituitary hormone corticotropin (ACTH) from four different mammalian species (porcine [1], ovine [2,3], bovine [4,5], and human [6]) have been elucidated, some of them almost twenty years ago. The proposed structures, however, contained a few errors which remained undetected until the amino acid sequences of porcine and human ACTH were recently corrected by two different groups [7,8]. As a consequence of these studies C. H. Li was prompted to re-examine his earlier proposals for the ovine and bovine hormones [9]. In both cases, Edman degradation of the tryptic fragments 22–39 showed that the amino acid sequence 25–32 was not correct and therefore had to be revised. According to these new findings, the hormones of the two species should differ in position 25, with aspartic acid in ovine and asparagine in bovine ACTH. Glutamine has been proven to be present in both species in position 33 [3,5].

It is known from the early work on sheep ACTH that this compound is unstable under alkaline conditions and that it easily undergoes deamidation [10]. The same applies to porcine and human ACTH, since both contain the very labile Asn^{25}–Gly^{26} sequence. The revised structure postulated by Li [9] for sheep ACTH, however, could not explain this lability. It seemed therefore most probable to us that ACTH from this species should also contain asparagine in position 25 instead of aspartic acid and thus should be identical with beef ACTH.

The deamidation of asparagine peptides in alkaline conditions leads, via the succinimide derivatives as intermediates, to a mixture of α- and β-aspartic peptides, in which the latter predominate. The difficulties

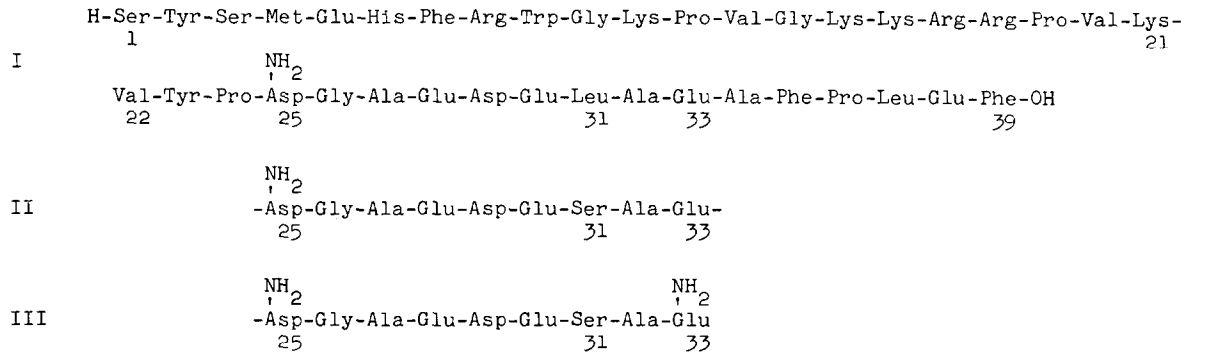

Fig. 1. Amino acid sequences of the known corticotropins from mammalian species. I: Porcine; II: Human; III: Ovine and bovine. (Sequences 1–24 and 34–39 of II and III as in I.)

in the sequence studies with ACTH peptides had quite clearly been caused by this deamidation, which had given rise to wrong results in the Edman degradation process from asparagine[25] onwards. In order finally to settle the question of the amide group, we decided to isolate and compare both ovine and bovine ACTH, carefully avoiding alkaline conditions during the preparation and in the analytical procedures. The C-terminal tryptic fragments comprising the sequence 22–39 were used as key compounds for this work. In addition, the corresponding synthetic human fragment [11] with its known sequence (fig. 1, II) served as a reference compound.

By means of electrophoretic and deamidation experiments, as well as by Edman degradation, we were able to prove that bovine and ovine ACTH are identical, both having asparagine in position 25. The only structural difference between human ACTH and the ovine and bovine hormone is at position 33, which is occupied by glutamic acid in human, and by glutamine in ovine and bovine ACTH.

2. Experimental

2.1. *Isolation procedures*

Ovine ACTH was isolated and converted to the fragment 22–39 in the following manner: Sheep pituitary glands were homogenized in 0.25 M saccharose and extracted with HCl–acetone at 0°C [12]. After removal of the residue by centrifugation, acetone was added to the supernate and the precipitate collected by centrifugation. Sodium chloride fractionation was performed as described by Papkoff and Li [13]. The resulting precipitate was dissolved in 1 M AcOH, desalted by Sephadex G-50, and freeze-dried. This material was further purified by absorption on CM-cellulose (Whatman CM 23) according to Homan and Ederzeel [14]. Final purification of the crude ACTH was achieved by gel filtration on Sephadex G-50 using 1 M acetic acid as eluant, followed by countercurrent distribution in the solvent system t-butanol–2% trifluoroacetic acid–chloroform–toluene (10:10:1:1) [11].

Tryptic cleavage of the purified ACTH was performed with 2.5% trypsin in 0.2 M ammonium acetate at pH 8 for 40 min at 38°C, acidification, lyophilisation and fractionation by countercurrent distribution in the system *n*-butanol–acetic acid–water (4:1:5). After 200 steps the pure fragment 22–39 was obtained from tubes 105–124; K=1.5.

Bovine ACTH was isolated, purified and degraded to the fragment 22–39 as outlined for the ovine ACTH with the exception that acetone powder of beef anterior pituitary glands, purchased from Miles Laboratories (Kankakee, Ill., USA) was used as starting material.

Isolation and purification of ovine and bovine ACTH were monitored by the in vitro bioassay according to Saffran and Schally [15] and by thin-layer chromatography.

2.2. *Analytical methods*

Electrophoreses were performed with 10 μg samples of peptides 22–39 on cellulose-coated plates (20 × 20 cm) in 0.05 M phosphate buffer, pH 7.7, for 1 hr at 30 V/cm. The spots were stained by the chlorination method.

In order to determine the rate of deamidation, 0.2% peptide solutions in 1N NH_3 were left at 25°C for various time intervals, evaporated to dryness, dissolved in 10% pyridine and analysed by electrophoresis.

Manual Edman degradations were carried out in micro-centrifuge tubes. 300 μg of the peptide 22–39, dissolved in 40 μl pyridine–H_2O (1:1), were incubated under N_2 with 25% phenylisothiocyanate in pyridine for 1 hr at 40°C. The mixture was extracted twice, each time with 200 μl benzene, and the aqeous phase dried over P_2O_5 and KOH. The residue was treated for 30 min at 25°C* with 10 μl of trifluoroacetic acid and then evaporated by spreading it in vacuo as a thin film on the glass surface. This residue was twice extracted for 5 min with 100 μl of benzene–1,2-dichloroethane (2:1), the insoluble material dried over KOH and used for the next degradation step. The organic extract was evaporated, treated with 20 μl 1 N HCl under N_2 for 10 min at 80°C and dried. The PTH-amino acid residue was taken up in 10 μl of ethylacetate–acetone (1:1), and 1 to 4 μl thereof spotted on silica gel thin-layer plates (Antec SL 254) containing fluorescence indicator. The solvent systems chloroform–methanol (95:5) and chloroform–methanol–acetic acid (90:10:3) were used, the latter per-

* In the third step with C-terminal proline, the temperature was raised to 40°C.

mitting the differentiation of the PTH-derivatives of Asp, Asn, Glu, and Gln. The spots were visible by their UV-absorption at 254 nm.

3. Results and discussion

Comparison of the electrophoretic mobility of the C-terminal fragments 22–39 at pH 7.7 gave the following results: ovine 4.9 (5.9); bovine 4.9 (5.9); synthetic human 5.9 (6.9). (The figures indicate migration distances in cm towards the anode, and the values of the deamidated forms are given in brackets.) These relationships prove that the ovine and bovine fragments both have an identical number of free carboxylic groups, and that after deamidation this number is the same as in the non-deamidated human reference peptide, which contains six free carboxylic and one carboxamide group. The non-deamidated ovine and bovine fragments therefore contain two amide side chains. The deamidation experiments further showed identical half-lives (approx. 50 min) in 1 N ammonia at 25°C for all three compounds, indicating the presence of the extremely labile Asn^{25}–Gly^{26} sequence. In addition, this sequence was substantiated by Edman degradation of the ovine and bovine fragments 22–39, which was carried out in five steps. PTH-asparagine, accompanied by a small amount of PTH-aspartic acid, was the product of both peptides after the fourth step, followed by PTH-glycine in the next one.

These results confirmed our assumption, i.e. the structure of the recently revised sequence of ovine ACTH [9] has to be corrected by adding an extra amide group in position 25. Thus, ovine and bovine ACTH are identical (III) and differ from the human hormone (II) only in position 33, which is glutamic acid in human-, but glutamine in ovine- and bovine-ACTH. The porcine type (I) is similar to the human one with regard to the single amide group in position 25, but contains $leucine^{31}$ instead of $serine^{31}$. These minor differences are located in the C-terminal part of the molecule which is not essential for the corticosteroidogenic activity of the hormone.

It is interesting to consider also in this comparison the only non-mammalian ACTH known so far: in dogfish ACTH [16] we find the same amino acids in position 25 and 33 as in the human and porcine types, despite a large number of alterations which affect mainly the carboxyl end.

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