Involvement of lysine residues in the binding of ovine chorionic somatomammotropin to lactogenic and somatotropic receptors

N. Chêne, J. Martal* and P. de la Llosa+

Laboratoire de Physiologie de la Lactation, INRA, 78350 Jouy-en-Josas and *+Laboratoire des Hormones Polypeptidiques, CNRS, 91190 Gif-sur-Yvette, France*

Received 17 October 1983; revised version received 25 November 1983

The biological activities of several ovine chorionic somatomammotropin (oCS) derivatives obtained by chemical modification of the lysine residues were studied by radioreceptor assays using rabbit mammary homogenates (lactogenic activity, L.A.) and liver homogenates (somatotropic activity, S.A.). Even if the control treatment with BH_4^- markedly decreased the L.A., it was clear that methylation mainly affected the S.A. and that ethylation reduced both activities. Guanidination inactivated almost completely both activities and acetimidination at a very low degree (3 of 14 lysines) led to less than 50% of both activities. These results show the involvement of lysine residues in the interaction of oCS with lactogenic and somatotropic receptors.

Ovine chorionic somatomammotropin *Prolactin* *Growth hormone* *Lysine residue* *Receptor*

1. INTRODUCTION

Structurally, placental lactogenic hormones [placental lactogen (PL) or chorionic somatomammotropin (CS)] are located between prolactins and growth hormones and exhibit special physiological characteristics. In ruminants, these hormones have both lactogenic and growth hormone activities [1–3]. In other species, e.g. rat and mouse, only lactogenic activity has been demonstrated [2,4]. In the human female, human chorionic somatomammotropin (hCS) is almost unable to bind to growth hormone receptors [5], which raises some questions concerning the physiological role of this hormone. Furthermore, some mammals seem to lack any CS-like hormone [6–8]. The oCS, endowed with both lactogenic and somatotropic action, is a very interesting molecule for studying relationships between chemical structure and biological activity. Purification and physicochemical characterization of oCS have recently been performed [9,12]. The amino acid sequence of this molecule is still unknown. The only known sequence in the family of CS is that of hCS, but this molecule shows exclusively prolactin activity despite a very close homology of its chemical structure with hGH [13,14].

It was of interest to investigate the effect of chemical modification of oCS on its binding to somatotropic and lactogenic receptors. As the basic residues might be involved in specific binding of hormones to receptors, we first studied the effect of modification of the hormonal lysine residues on their binding to liver somatotropic receptors and to mammary prolactin receptors. Four different modifications were performed: methylation of the amino groups which is accompanied by a slight change in the pK; ethylation which introduces a longer alkyl chain and the possibility of considerable steric hindrance at the interface between the hormone and the receptor; guanidination which modifies the pK and the distance between the charge and the polypeptide backbone; and acetimidination with the same effect, but more marked.

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

2. MATERIALS AND METHODS

2.1. *Purification of oCS*

Purification was performed as in [10,12]. oCS was purified about 1000-fold and was not contaminated by either prolactin or growth hormone. Its activity estimated by radioreceptor assay was equivalent to 20 IU/mg NIH ovine prolactin standard and to 0.8 IU/mg NIH bovine growth hormone.

The amino acid composition was established using a Technicon Autoanalyzer. The content of lysine for an M_r of 22000 was 14 residues.

2.2. *Chemical modifications*

2.2.1. Reductive alkylation

Reductive methylation and ethylation were carried out by addition of sodium borohydride and formaldehyde or acetaldehyde to oCS (0.5–1.0 mg) dissolved in borate buffer (pH 9) as in [15]. The solutions were dialyzed against a pyridine solution (1%) and freeze-dried. Determinations of lysine, methyl- and ethyllysine were made using a Technicon autoanalyzer and elution gradients containing isopropanol [15,16]. A blank for biological assays was prepared by treating hormone with borohydride and borate buffer (pH 9, no added aldehyde).

2.2.2. Guanidination

This reaction was performed using 0.3 M *O*-methylisourea sulfate (Aldrich, Europe) at pH 10.3 and 5°C (0.7 mg oCS/0.15 ml) for two periods, 24 h and 5 days. At the end of the reaction, the solutions were dialyzed against pyridine solutions. After 1 day of reaction, the solution became slightly opalescent. In both cases, the solutions were centrifuged and the precipitate discarded. The degree of guanidination was measured by amino acid analysis. A blank was prepared by treatment of the hormone at pH 10.4 for 5 days at 5°C.

2.2.3. Acetimidination

To obtain a low degree of chemical modification, this reaction was performed at a low concentration of reagent (ethylacetimidate hydrochloride, Aldrich) for 24 h, at pH 10 and 5°C. Determination of the ϵ-acetimidyllysine was performed by amino acid analysis as in [17].

2.3. *Radioreceptor assay of lactogenic and growth activities*

2.3.1. Lactogenic activity

This activity was measured as in [10].

Mammary gland membranes were obtained from rabbits treated on day 10 of lactation with 2-α-bromocryptine (CB 154, Sandoz, 2 mg twice daily for 2 days) to desaturate their receptors. The standard curve was established by incubation of membrane receptors, radioiodinated prolactin and different concentrations of unlabelled ovine prolactin (NIH-PS-7, 24 IU/mg) for 5 h at 21°C. Lactogenic activity of modified hormones was determined by adding these hormone derivatives to the incubation medium instead of the unlabelled prolactin. The specificity of the assay was checked: only hormones with lactogenic activity in the rabbit (prolactins of different species, placental lactogens and human growth hormone) are able to compete with ovine prolactin at the rabbit mammary receptor sites. The rabbit receptor exhibits less strict specificity than the ovine receptor.

2.3.2. Growth hormone activity

This activity was determined by radioreceptor assay [5] with membranes of rabbit liver on day 17 of pregnancy in the presence of ^{125}I-labelled Pentex bovine growth hormone (bGH) and standard unlabelled bGH (Pentex, 1 IU/mg). In rabbit, the liver is particularly rich in GH receptors and poor in prolactin receptors. Each sample was tested several times in duplicate at various dilutions in the following buffer: 25 mM Tris (pH 7.6), 10 mM $MgCl_2$, bovine serum albumin 0.1% (w/v).

3. RESULTS AND DISCUSSION

Table 1 lists the relative potencies expressed in terms of native oCS (percentage). These values were calculated by comparison of the hormone concentration needed for 50% inhibition of specific binding. Amino acid analysis of oCS prepared in our laboratory showed a lysine content similar to that reported in [18]. The results obtained in the radioreceptor assay for lactogenic activity are reported in fig.1 and those from a similar assay for somatotropic activity in fig.2.

Treatment of oCS with borohydride at 0°C and pH 9 (conditions necessary for alkylation of amino groups) caused a slight loss in the binding activity

Table 1

Relative potencies of oCS derivatives in terms of native oCS in the lactogenic and somatotropic radioreceptor assays

Treatments	Native oCS	Control (pH 9, BH_4^-, 1 h) (Red-oCS)	Methylation (Met-oCS)	Ethylation (Et-oCS)	Control (pH 10, 4.5 days, 4°C)	Guanidination (Gu-oCS) (24 h)	Acetimidination (Am-oCS)
Modified lysine residues (%)	0	0	75 (10)[a]	55 (8)[a]	0	81 (11)[a]	20 (3)[a]
Lactogenic binding activity (%)	100	45.5	38.5	10	100	1	29.5
Somatotropic binding activity (%)	100	94	7.5	36.5	100	1.5	64

[a] Number of modified lysine residues out of the 14 residues found

Relative potencies expressed as a percentage of the binding activity of native oCS calculated by comparison of the hormone concentration needed for 50% inhibition of specific binding

of the molecule to somatotropic receptors (table 1). The loss was much larger for lactogenic receptors. We observed that this treatment also drastically reduced the biological activity of ovine prolactin as a consequence of the reduction of some disulfide bridges and subsequent intermolecular rearrangements (unpublished).

3.1. *Methylation*

The least disturbing chemical modification which can be introduced into the amino groups only slightly reduced the lactogenic activity below the level observed in the blank (treated at pH 9 with borohydride) despite the high degree of modification (75% of lysine residues) (table 1). However,

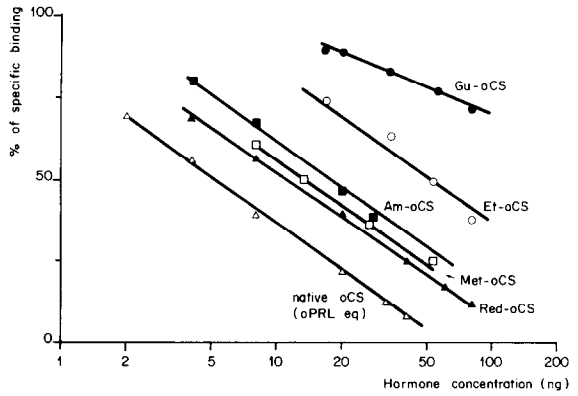

Fig.1. Specific binding of native and modified oCS in a radioreceptor assay for lactogenic activity with lactating rabbit mammary membranes. oCS activity is expressed in ovine prolactin equivalents (NIH PS 7, 24 IU/mg) (Δ—Δ); Red-oCS, reduced oCS (▲—▲); Met-oCS, methylated oCS (□—□); Am-oCS, acetiminidylated oCS (■—■); Et-oCS, ethylated oCS (○—○); Gu-oCS, guanidinated oCS (●—●).

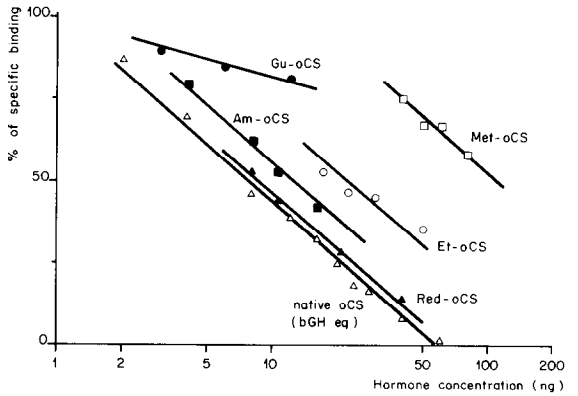

Fig.2. Specific binding of native and modified oCS in a radioreceptor assay for somatotropic activity with pregnant rabbit liver membranes. oCS activity is expressed in bovine growth hormone equivalents (bGH Pentex, 1 IU/mg) (Δ—Δ); Red-oCS, reduced oCS (▲—▲); Met-oCS, methylated oCS (□—□); Am-oCS, acetiminidylated oCS (■—■); Et-oCS, ethylated oCS (○—○); Gu-oCS, guanidinated oCS (●—●).

partial loss of the binding activity with regard to lactogenic receptors resulting from methylation of oCS might be masked by the considerable loss of activity due to disturbance of the conformation caused by reduction of disulfide bridges. In contrast, the somatotropic activity was clearly reduced suggesting more intimate involvement of the amino acid groups in binding of the hormone to its somatotropic receptors. That the binding to somatotropic receptors of oCS treated with borohydride is only slightly decreased suggests that the growth hormone activity is less dependent on the secondary and tertiary structure than is lactogenic activity. That may be due to the fact that the somatotropic activity is more strictly located. As methylation of the amino groups produced only a very slight change in pK [15], the drastic loss of binding activity of oCS to somatotropic receptors after methylation must be attributed to steric hindrance resulting from the introduction of methyl groups on ϵ-NH_2. Thus, some lysine residues appear to be essential for the growth hormone activity of oCS.

3.2. *Ethylation*

Ethylation, which may be the cause of steric hindrance around the amino groups, decreased the binding activity of the molecule to both lactogenic and somatotropic receptors (table 1; fig.1,2). The degree of ethylation was, as usual, lower than that of methylation which certainly explains the higher somatotropic activity of the ethylated derivative compared to the methylated one.

Treatment of oCS at pH 10.4 and 4°C (necessary for guanidination or acetimidination) even for 5 days is apparently without effect on the biological activity. The consequences of guanidination on the biological activity are thus more clearly shown: both lactogenic and somatotropic activities were almost completely abolished confirming the disturbing character of this chemical modification. Acetimidination, even at a very low degree (3 residues out of 14), caused a decrease of ~50% in both activities.

Accordingly, it may be concluded that the lysine residues are involved in the binding of oCS to both types of receptors. This involvement appears more marked in the case of somatotropic receptors since these receptors appear to be unable to recognise the methylated oCS which is active in regard to the lactogenic receptors. As yet, identification of these essential residues cannot be made as the amino sequence in oCS remains unknown. But, according to previous studies [19,20] on the relationship between structure and biological activity in other growth hormones, we may locate these essential lysine residues in the N-terminal segment containing two-thirds of the amino acid sequence. However, it may be assumed that the absence of somatotropic activity of certain placental lactogens results from a mutation in the nucleotide coding of some lysine residues.

Chemical modification of lysine residues of other hormones endowed with prolactin or GH activities also decreases their biological activity (unpublished). Mostly, the loss of activity is much larger for the somatotropic activity of these hormones.

A decrease in biological activity following chemical modification of the lysine residues has been reported for other hormones. However, the consequences of such modifications may vary considerably depending on the nature of the introduced modification or on the structure of the protein, even for phylogenetically related molecules such as LH and FSH. Methylated luteinizing hormone is quite active [16,21] but methylated FSH is inactive [22]. Ethylated and guanidinated luteinizing hormone are less active than the native hormone and the fully acetimidinated molecule is inactive [16,23,24]. Large losses of biological activity have also been observed after modification of amino groups in PMSG and glucagon [25,26]. A selective loss of activity has been observed occasionally: guanidinated LH is able to bind to the rat Leydig cell receptors without stimulation of steroidogenesis [27].

ACKNOWLEDGEMENTS

We thank Mrs M. Roy for her expert technical assistance and Mrs J. Brugnolo for typing this manuscript. This work has been greatly facilitated by financial grants from CNRS, Paris (ATP no.54/82).

REFERENCES

[1] Forsyth, I.A. (1973) in: Le Corps Jaune (Denamur, R. and Netter, A. eds) pp.239–255, Masson, Paris.

[2] Kelly, P.A., Tsushima, T., Shiu, R.P.C. and Friesen, H.G. (1976) Endocrinology 99, 765–774.

[3] Martal, J. and Djiane, J. (1977) J. Steroid Biochem. 8, 415–417.

[4] Talamantes, F. (1975) Gen. Comp. Endocrinol. 27, 115–121.

[5] Tsushima, T. and Friesen, H.G. (1973) J. Clin. Endocrinol. Metab. 37, 334–336.

[6] Kelly, P.A., Shiu, R.P.C., Robertson, M.C. and Friesen, H.G. (1973) Endocrinology 92, suppl. abstr.370.

[7] Durand, P. and Djiane, J. (1977) J. Endocrinol. 75, 33–42.

[8] Martal, J. (1981) in: L'Utérus de la Vache (Constantin, A. and Messonnier, E. eds) pp.101–120, Sté Française de Buiatrie, E.N.V.A., Maisons-Alfort.

[9] Handwerger, S., Maurer, W., Barrett, J., Hurley, T. and Fellows, R.E. (1974) Endocrinol. Res. Commun. 1, 430–413.

[10] Martal, J. and Djiane, J. (1975) Biochem. Biophys. Res. Commun. 65, 770–778.

[11] Chan, J.S.D., Robertson, H.A. and Friesen, H.G. (1976) Endocrinology 98, 65–76.

[12] Martal, J. (1978) Ann. Biol. Anim. Biochem. Biophys. 18, 45–51.

[13] Li, C.H., Dixon, J.S. and Chung, D. (1971) Science 173, 56–58.

[14] Niall, H.D., Hogan, M.L., Sauer, R., Rosenblum, I.Y. and Greenwood, F.C. (1971) Proc. Natl. Acad. Sci. USA 68, 866–869.

[15] Means, G.E. and Feeney, R.E. (1968) Biochemistry 7, 2192–2201.

[16] De la Llosa, P., Durosay, M., Tertrin-Clary, C. and Jutisz, M. (1974) Biochim. Biophys. Acta 342, 97–104.

[17] Plapp, B.V. and Kim, J.C. (1974) Anal. Biochem. 62, 291–294.

[18] Fellows, R.E., Bolander, F.F., Hurley, T.W. and Handwerger, S. (1975) in: Growth Hormone and Related Peptides (Pecile, A. and Muller, E.E. eds) pp.315–326, Excerpta Medica, Amsterdam.

[19] Kostyo, J.L. (1976) in: Endocrinology, pp.186–190, Proc. 5th Congr. Endocrinol., Hamburg, Brühlsche Universitäts Druckerei, Giesen.

[20] Li, C.H. (1978) Proc. Natl. Acad. Sci. USA 74, 1016–1019.

[21] Liu, W.K., Yang, K.P., Nakagawa, Y. and Ward, D.N. (1974) J. Biol. Chem. 249, 5544–5550.

[22] De la Llosa, P. (1980) in: Synthesis and Release of Adenohypophyseal Hormones (Jutisz, M. and McKerns, K.W. eds) pp.404–414, Plenum, New York.

[23] Liu, W.K. and Ward, D.N. (1975) Biochim. Biophys. Acta 405, 522–528.

[24] Nureddin, A. and Atassi, M.Z. (1978) Biochim. Biophys. Acta 533, 257–262.

[25] Combarnous, Y. and De la Llosa, P. (1980) 13th FEBS Meet., Jerusalem, Aug.24–29, Comm. C10–P7.

[26] Epand, R.M., Rosselin, G., Hui Bon Hoa, D., Cote, T.E. and Laburthe, M. (1981) J. Biol. Chem. 256, 1128–1132.

[27] De la Llosa-Hermier, M.P., Tertrin-Clary, C., Evrard-Herouard, M., Colleaux, Y., Hermier, C. and De la Llosa, P. (1980) Acta Endocrinol. 93, 250–256.