# Biochemical analysis of bovine testicular anti-Müllerian hormone

Jean-Yves Picard, Chantal Goulut*, R. Bourrillon* and Nathalie Josso+

*Unité de Recherches sur l'Endocrinologie du Développement, INSERM, Hôpital des Enfants-Malades, 75743 Paris cedex 15* and **Département de Biochimie, UER des Saints-Pères, 45 rue des Saints-Pères, 75006 Paris, France*

Received 7 November 1985

Direct biochemical analysis has been applied to bovine testicular anti-Müllerian hormone (AMH), purified from incubation medium of bovine fetal testes by immunochromatography on a monoclonal antibody. The hormone contains a high proportion of hydrophobic amino acids and 13.5% carbohydrate. The oligosaccharide composition suggests that both *N*- and *O*-glycosidically linked chains are present. The molecular extinction coefficient is $3.27 \pm 0.06$. One RIA unit, defined as the amount of hormone released by 1 g fetal bovine testicular tissue incubated during 4 h, corresponds to $3.06 \pm 0.17$ μg protein.

*(Fetal testis)* *Anti-Müllerian hormone* *Müllerian inhibiting substance* *Glycoprotein*

## 1. INTRODUCTION

Anti-Müllerian hormone (AMH), a 145 kDa glycoprotein dimer responsible for the regression of Müllerian ducts in male fetuses, is produced by immature Sertoli [1] and adult granulosa cells [2]. Bovine testicular AMH has been purified recently, using affinity chromatography on a monoclonal antibody [3] but in insufficient amounts to allow a detailed biochemical analysis. Using a slightly modified technique, we have prepared enough purified bovine AMH to study its amino acid and sugar composition, determine its molecular extinction coefficient, and reliably establish the relationship between weight and RIA units.

## 2. MATERIALS AND METHODS

Bovine AMH was purified from incubation medium of bovine fetal testicular tissue using ammonium sulfate precipitation, anion-exchange chromatography and affinity chromatography on a monoclonal antibody as described [3], except that a single affinity chromatography was performed per batch of incubation medium, using protein-free elution buffer. A total of 9 l incubation medium were processed in 3 separate batches. In 2 instances, the immunochromatography fractions with the highest absorbance at 280 nm were pooled separately, therefore, a total of 5 independent AMH preparations were obtained. Aliquots of 2 of these preparations were reduced for 90 min at 37°C by 10 mM dithioerythritol, in 0.4 M Tris, 7 M guanidinium chloride, alkylated by 50 mM iodoacetic acid in the same buffer for 30 min at 0°C, dialyzed against distilled water and lyophilized.

AMH concentration was determined by a liquid-phase, competition-type radioimmunoassay [4] and expressed in RIA units. One AMH unit is the amount of hormone released by 1 g tissue during a 4 h incubation period, using a 1:5 tissue to medium ratio. Protein concentration of the various AMH preparations was determined by fluorometry [5], using a bovine serum albumin standard. $A_{280}$ was determined in a Gilford spectrophotometer.

The amino acid composition of 2 lyophilized

+ To whom correspondence should be addressed

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/86/$3.50 

samples (30 and 130 $\mu$g) was determined in a Jeol autoanalyzer, after a 7 h hydrolysis by 6 N HCl. The sugar composition of 2 (200 and 300 $\mu$g) lyophilized samples was determined by gas-liquid chromatography, as described by Chambers and Clamp [6].

## 3. RESULTS

The correlation between the AMH concentration determined by RIA and the protein concentration determined by fluorometry is shown in fig.1. Statistical evaluation of the results was computed using Abstat software, supplied by Anderson-Bell (Littleton, CO): the correlation coefficient was 0.972 and the variance for the slope of the curve was 1128. One RIA unit corresponds to 3.06 ± 0.17 $\mu$g.

The correlation between protein concentration, determined by fluorometry, and absorbance at 280 nm was also established ($r$ = 0.991), the molecular extinction coefficient being 3.27 ± 0.06.

The amino acid and sugar composition were obtained from 2 independent determinations, performed on different batches of reduced and alkylated lyophilized AMH. The results shown in table 1 are those obtained using the largest samples of lyophilized AMH. Acid and basic amino acids are more or less equally represented, and hydrophobic amino acids are relatively abundant, namely leucine, which accounts for 12.5% of the total weight of AMH. Carbohydrate accounts for 13.5% and is characterized by a relatively high amount of galactose and the presence of galactosamine, coexisting with mannose and glucosamine. Fucose is present, but in amounts too low to allow its quantitation.

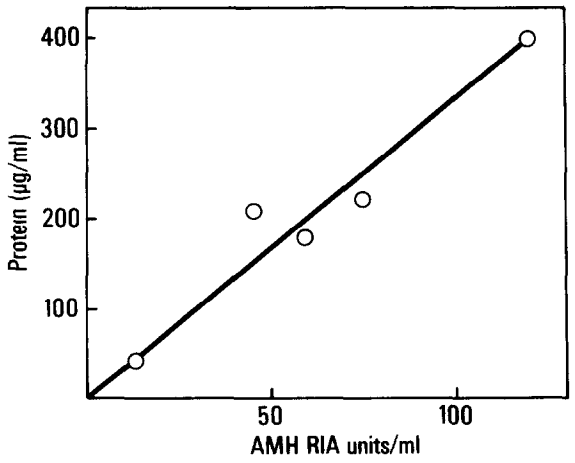


Fig.1. Correlation ($r$ = 0.972) between protein concentration determined by fluorometry, and immunoreactivity determined by a liquid-phase, competition-type radioimmunoassay, of 5 independent solutions of purified AMH in phosphate-buffered saline.

Table 1

Amino acid and sugar composition of bAMH

| | g/100 g | mol/mol |
|---|---|---|
| Aspartic acid | 5.50 | 60.87 |
| Threonine | 4.05 | 50.00 |
| Serine | 5.23 | 73.19 |
| Glutamic acid | 9.80 | 97.82 |
| Proline | 8.70 | 110.87 |
| Glycine | 5.20 | 101.81 |
| Histidine | 2.85 | 21.38 |
| Valine | 2.90 | 36.59 |
| Methionine | 1.63 | 15.94 |
| Isoleucine | 1.48 | 16.66 |
| Leucine | 12.50 | 139.85 |
| Tyrosine | 2.10 | 18.11 |
| Phenylalanine | 3.65 | 35.14 |
| Alanine | 8.95 | 147.82 |
| Lysine | 1.65 | 16.66 |
| Arginine | 10.35 | 87.32 |
| Mannose | 2.05 | 16.85 |
| Galactose | 2.95 | 23.91 |
| *N*-Acetylglucosamine | 3.60 | 23.91 |
| Glucose | 1.04 | 8.33 |
| Sialic acid | 2.09 | 9.96 |
| *N*-Acetylgalactosamine | 1.73 | 11.59 |
| Fucose | + | + |
| Total sugar | 13.46 | |

Amino acid composition was determined in a Jeol autoanalyzer, after a 7 h hydrolysis with 6 N HCl. Sugar composition was analyzed by gas-liquid chromatography as trimethylsilyl derivatives of 1.0 methyl monosaccharides

## 4. DISCUSSION

Although its role in sex differentiation was recognized as early as 1947 by Jost [7], AMH has

eluded biochemical analysis until now. Initially, the presence of the hormone could be detected only through its bioactivity in organ culture [8], and quantitated using a solid-phase, 2-site monoclonal antibody-based radioimmunoassay [9]. The first successful method of purification reported [10] involved the addition of bovine serum albumin (BSA) to the buffer used to elute the hormone from immobilized monoclonal antibody. To obtain AMH free of added protein, several preparations of AMH with BSA were loaded on a column of immobilized monoclonal antibody, and eluted with protein-free buffer [3]. The single purified AMH batch resulting from this procedure was analyzed by radioimmunoassay [9] and fluorometry [5], and 1 AMH RIA unit was reported to correspond to 15 $\mu$g protein. Our present figure, namely a value of $3.06 \pm 0.17$ $\mu$g per RIA unit, is certainly more reliable, since it rests upon analysis of 5 independent batches of purified AMH.

The amino acid sugar compositions shown in table 1 are in agreement with previously published data. The relative equilibrium between acid and basic amino acids is compatible with the isoelectric point of 6.0 which has been determined for the fucose-labelled hormone [11]. The high proportion of hydrophobic amino acids may explain why addition of Nonidet P-40 to partially purified preparations of AMH has been recommended [12]: adsorption of hydrophobic proteins to glass and plastic is a serious problem for those attempting to purify them, and detergents are known to minimize such losses [13].

The glycoprotein nature of AMH had hitherto been demonstrated only by indirect means. Picard et al. [11] incubated fetal bovine testes in the presence of tritiated fucose, submitted the incubation medium to gel filtration and ion-exchange chromatography, and, after various types of biochemical fractionation procedures, showed a correlation between fucose content and biological anti-Müllerian activity of the semi-purified labelled AMH preparation. Anti-Müllerian biological activity is bound by lectins such as wheat-germ agglutinin (WGA) [14,15] and concanavalin A (Con A) [15]. Fucose-labelled AMH binds to WGA, and the binding is abolished by the specific sugar inhibitor of the lectin. Binding to phytohemagglutinin (PHA) is only partial, and reversed by the inhibitors of the E, but not the L subunit of the lectin [14].

The carbohydrate composition of AMH, namely its relatively high galactose content, and the coexistence of mannose, galactosamine and glucosamine suggest that both *N*-oligosaccharide linkages, characteristic of proteins secreted into the plasma, and *O*-glycosidic linkages, typical of epithelial cell secretions are present [16]. At this point, it is impossible to determine whether the latter are of the high mannose or of the complex type. The fucose and sialic acid could belong to either the *N*-linked complex chains or the *O*-linked oligosaccharides [17], although the relatively low amounts of these sugars make their distribution into both types of chains unlikely. The 3 lectins shown to interact with AMH, namely WGA, Con A and PHA [14,15], bind to both types of *N*-linked chains [18,19], and evidence for the interaction of WGA with *O*-glycosidically linked chains has also been presented [20]. The determination of the exact composition of the *N*-glycosidically linked chains of AMH must await the undertaking of a detailed structural analysis of the hormone.

The contribution of carbohydrate to the biological activity of AMH may be even more difficult to investigate. Addition of tunicamycin (1–2 $\mu$g/ml) to culture medium does not affect the biological anti-Müllerian activity of either explanted fetal testicular tissue, or its incubation medium [22], showing that *N*-glycosidically linked oligosaccharide chains, whose synthesis is specifically inhibited by tunicamycin [23], are not crucial for hormone bioactivity. The role of *O*-linked chains is not known, because the conditions required for selective alkaline $\beta$-elimination of *O*-linked carbohydrate [24] or for total chemical deglycosylation [25] are incompatible with the preservation of AMH bioactivity. It would also be interesting to determine whether the carbohydrate moiety of AMH is implicated in the relatively long period required for its secretion by bovine Sertoli cells in tissue culture [4] and whether differences in carbohydrate structure may explain why AMH is found in the serum of some species but apparently not in others [4].

## REFERENCES


[1] Blanchard, M.G. and Josso, N. (1974) Pediatr. Res. 8, 968–971.

[2] Vigier, B., Picard, J.Y., Tran, D., Legeai, L. and Josso, N. (1984) Endocrinology 114, 1315–1320.

[3] Picard, J.Y. and Josso, N. (1984) Mol. Cell. Endocrinol. 34, 23–29.

[4] Vigier, B., Picard, J.Y., Campargue, J., Forest, M.G., Heyman, Y. and Josso, N. (1985) Mol. Cell. Endocrinol., in press.

[5] Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220.

[6] Chambers, R.E. and Clamp, J.R. (1971) Biochem. J. 125, 1009–1018.

[7] Jost, A. (1947) Arch. Anat. Microsc. Morphol. Exp. 36, 271–315.

[8] Picon, R. (1969) Arch. Anat. Microsc. Morphol. Exp. 58, 1–19.

[9] Vigier, B., Legeai, L., Picard, J.Y. and Josso, N. (1982) Endocrinology 111, 1409–1411.

[10] Josso, N., Picard, J.Y. and Vigier, B. (1981) CR Acad. Sci. Ser. D 293, 447–450.

[11] Picard, J.Y., Tran, D. and Josso, N. (1978) Mol. Cell. Endocrinol. 12, 17–30.

[12] Budzik, G.P., Powell, S.M., Kamagata, S. and Donahoe, P.K. (1983) Cell 34, 307–314.

[13] Suelter, C.H. and De Luca, M. (1983) Anal. Biochem. 115, 112–119.

[14] Picard, J.Y. and Josso, N. (1980) Ann. Endocrinol. 41, 538–544.

[15] Budzik, G.P., Swann, D.A., Hayashi, A. and Donahoe, P.K. (1980) Cell 21, 909–915.

[16] Zinn, A.B., Plantner, J.J. and Carlson, D.M. (1977) in: The Glycoconjugates (Horowitz, M.I. and Pigman, W. eds) vol.1, pp.69–86, Academic Press, New York.

[17] Kornfeld, R. and Kornfeld, S. (1980) in: The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J. ed.) pp.1–34, Plenum, New York.

[18] Cummings, R.D. and Kornfeld, S. (1982) J. Biol. Chem. 257, 11230–11234.

[19] Hammarström, S., Hammarström, M.L., Sundblad, G., Arnarp, J. and Lönngren, J. (1982) Proc. Natl. Acad. Sci. USA 79, 1611–1615.

[20] Goldstein, I.J., Hammarström, S. and Sundblad, G. (1980) Biochim. Biophys. Acta 405, 53–61.

[21] Peters, B.P., Ebisu, S., Goldstein, I.J. and Flashner, M. (1979) Biochemistry 18, 5505–5511.

[22] Josso, N., Picard, J.Y. and Tran, D. (1980) in: Testicular Development Structure, and Function (Steinberger, A. and Steinberger, E. eds) pp.21–32, Raven, New York.

[23] Duskin, D. and Bornstein, P. (1977) Proc. Natl. Acad. Sci. USA 74, 3433–3437.

[24] Pigman, W. and Downs, F. (1977) in: The Glycoconjugates (Horowitz, M.I. and Pigman, W. eds) vol.1, pp.80–83, Academic Press, New York.

[25] Sairam, M.R. (1976) Arch. Biochem. Biophys. 176, 197–205.