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GLYCOSYLATED NATURE OF TESTOSTERONE 5α-REDUCTASE 2 PURIFIED FROM HUMAN PROSTATE

Eric QUEMENER[1*], Yolande AMET[1], Georges FOURNIER[2], Sylvie DI STEFANO[1], Jean-Hervé ABALAIN[1], and Hervé-Henri FLOCH[1]

[1] *Département de Biochimie et Biologie Moléculaire, Faculté de Médecine, 22, avenue Camille Desmoulins, BP 815, 29279 BREST Cédex, FRANCE*

[2] *Service d'Urologie, CHU Augustin Morvan, 5, avenue Foch, BP 824, 29285 BREST Cédex, FRANCE*

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Summary : 5α-reductase 2 from human prostate solubilized into an active and stable form using a non-ionic detergent octyl glucoside was successfully purified using a four-step chromatographic procedure. The enzyme was obtained as an apparently homogeneous protein exhibiting an apparent molecular weight of 42 kDa upon SDS-PAGE. Con A, DBA, UEA-I, and RCA60 lectins recognized this protein. After treatment with O-glycosidase and neuraminidase, a protein of an apparent molecular weight about 30 kDa appeared. On the other hand, N-glycosidase treatment of this enzyme had no effect. These results indicate that the human prostate testosterone 5α-reductase 2 is an O-glycosylated sialoglycoprotein with a peptide moiety of about 30 kDa; the oligosaccharide side chains contain mannose, N-acetyl galactosamine, fucose, galactose and sialic acids.

The enzyme 5α-reductase (3-oxo-5α-steroid : NADPH+ 4-ene-oxydoreductase; EC 1.3.1.22) catalyzes the conversion of testosterone (17ß-hydroxy-4-androsten-3-one) to 5α-dihydrotestosterone (17ß-hydroxy-5α-androstan-3-one). This is a crucial step in the mediation of androgenic activity in prostatic tissue (**1,2**). In this tissue, 5α-reductase activity has been implicated in the etiology of benign hypertrophy and cancer (**3,4,5,6**). It has now been proved that there are two isoenzymes chronologically identified as type 1 and type 2. The 5α-reductase 2 is expressed in high levels in the prostate with optimum activity in acidic pH (**7,8,9**).

In the view of the importance of this enzyme, numerous attempts to solubilize and purify 5α-reductase were performed (**10,11,12,13**). This has been a particularly difficult problem to resolve because of its extreme susceptibility to detergents and its strong membrane association (**14,15**).

Using a non ionic detergent, n-octyl ß-D-glucopyranoside, we were able to solubilize 5α-reductase 2 from nuclear membranes into an active form. Then we have purified the solubilized enzyme using a four-step chromatographic procedure. Loss of activity during the various

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purification processes was overcome by the addition of bovine serum albumin and n-octyl glucoside in the incubation reaction mixture. Purified 5α-reductase 2 from human prostate has an apparent molecular weight of 42 kDa upon SDS-PAGE (**16**). However, 5α-reductase from human and rat prostates have been predicted from cDNA sequence analysis to have a molecular weight of 29 kDa (**17,7,8**). The purpose of the present paper is to show that this difference is due to the glycosylated nature of the purified enzyme.

MATERIAL AND METHODS

Tissues

Human prostatic tissues were obtained from patients undergoing prostatectomy for benign hyperplasia. They were minced and kept in homogeneous aliquot parts in liquid nitrogen until use.

Chemicals

[4-^{14}C]testosterone (50 mCi/mmole) was obtained from Amersham Int. plc (Bucks., England). NADPH, n-octyl ß-D-glucopyranoside, bovine serum albumin, biotinylated lectins and avidin-alkaline phosphatase were obtained from Sigma (St Louis, Mo, USA). Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden) and DEAE-Trisacryl from IBF (Paris, France). Hydroxylapatite (Bio-gel HTP) and all products for SDS-PAGE were purchased from Bio-Rad Labs (Richmond, CA, USA). Immobilon-P transfer membranes were from Millipore Corporation (Bedford MA 01730, USA). N-glycosidase, O-glycosidase and neuraminidase were purchased from Boehringer Mannheim GmbH Biochemica (Germany). Testosterone-Sepharose was prepared in our laboratory by coupling testosterone hemisuccinate to AH-Sepharose 4B (Pharmacia). Other chemicals were of analytical grade and were bought from Merck. All solvents were HPLC grade from SDS (Peypin, 13124 France).

Solubilization and purification of 5α-reductase

5α-reductase was solubilized and purified from prostatic tissue, as previously reported (**16**), using 0.5 % n-octyl ß-D-glucopyranoside and using DEAE-Trisacryl, Hydroxylapatite, and testosterone-Sepharose. The last gel filtration chromatography on Sepharose 4B initially used was omitted because it resulted in association of the enzyme to very bulky aggregates.

Protein electrophoresis and Western blot

Protein samples were applied to SDS-polyacrylamide gels (SDS-PAGE) : 6 cm long x 10 cm wide x 0.5 mm thick; 4 % polyacrylamide stacking gel / 12 % polyacrylamide resolving gel. Gel electrophoresis was performed as described by Laemmli (**18**).

Proteins separated on SDS-PAGE were electrotransferred onto 0.45 µm Millipore immobilon PVDF transfer membrane according to the method of Towbin (**19**).

Lectin-binding tests

Immobilon membranes were saturated for 2 hours at room temperature with bovine serum albumin (BSA) by incubating them in 3 % BSA in Tris buffered saline (TBS, 10 mM Tris, 0.9 % NaCl, pH 7.4). BSA was previously treated with 10 mM periodic acid as described by Glass et al (**20**) to remove lectin-binding properties.

The protein saturated immobilon sheets were overlaid with diluted biotinylated lectins (Con A from *Concanavalin ensiformis*, UEA-I from *Ulex europeaus*, PHA-E from *Phaseolus*, RCA_{60} from *Ricinus communis*, DBA from *Dolichos biflorus*, and WGA from *Titricum vulgaris*) 5 µg/ml in TBS pH 7.4, 0.2 % Tween 20, 0.6 % BSA, for 2 hours at room temperature. For localization of Con-A binding activities, 1 mM $CaCl_2$ and $MgCl_2$ were included in the buffers. Controls contained 0.25 M inhibitory sugar. Immobilon sheets were washed six times with avidin-alkaline phosphatase conjugate for 2 hours at room temperature. The glycoprotein-lectin complexes were localized by staining for phosphatase activity in the presence of NBT.

Enzymatic deglycosylations

Enzymatic deglycosylations of the purified 5α-reductase were carried out following the conditions recommended by the suppliers. Testosterone-Sepharose eluate aliquotes containing

10 µg protein were incubated at 37°C fot 24 hours with 50 mU neuraminidase, 2.5 mU O-glycosidase, or 1 U N-glycosidase. At the end of incubations, proteins were precipitated by cold acetone. Samples before and after deglycosylation were analysed by SDS-PAGE.

RESULTS

Lectin-binding activities of purified 5α-reductase 2 are shown in **Table 1**. Con A, UEA-I, DBA, and RCA60 recognized specifically 5α-reductase 2; on the other hand WGA did not recognize the protein, indicating that 5α-reductase 2 is a glycoprotein whose glycan moiety is composed of mannose, fucose and N-acetylgalactosamine, but devoid of N-acetylglucosamine.

To further identify the nature of glycosylation, purified 5α-reductase 2 was submitted to enzymatic deglycosylations. When eluates from testosterone-Sepharose were incubated in the presence of neuraminidase or O-glycosidase, a protein with a molecular weight of about 30 kDa appeared on SDS-PAGE; however N-glycosidase had no effect on purified 5α-reductase 2

Table 1 : **Lectin-Binding Tests**

Lectin	Sugar	Binding Activity
Con A (from *Concanavalin ensiformis*)	D-Mannose	+ +
UEA-I (from *Ulex europeaus*)	L-Fucose	+ +
PHA-E (from *Phaseolus*)	Oligosaccharides	+
DBA (from *Dolichos biflorus*)	N-Acetyl galactosamine	+
RCA60 (from *Ricinus communis*)	N-Acetyl galactosamine, D-Galactose	+ -
WGA (from *Triticum vulgaris*)	N-Acetyl glucosamine	-

Eluates from testosterone-Sepharose 4B chromatography which contained purified 5α-reductase were subjected to analytical SDS-PAGE and electrotransferred onto Immobilon membranes. Membranes were incubated with biotinylated lectins diluted in TBS, washed with TBS and incubated with avidin-alkaline phosphatase conjugates. Glycoprotein-lectin complexes were localized by staining for phosphatase activity.
(++) : strong staining; (+) : staining; (+ -) : weak staining; (-) : no staining.

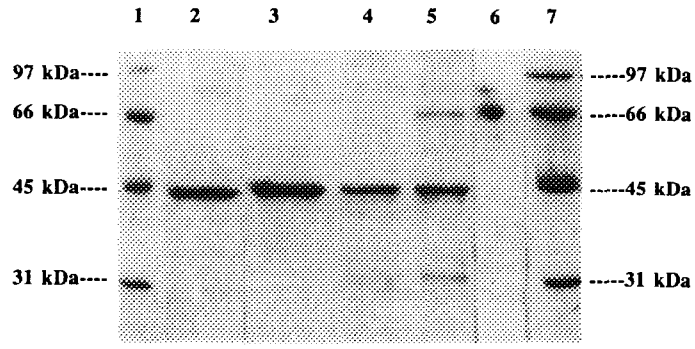

Figure 1 : **Enzymatic deglycosylations of purified 5α-reductase.** Aliquots of eluates from testosterone-Sepharose chromatography were incubated for 24 hours at 37°C in the presence of neuraminidase, O-glycosidase, or N-glycosidase. At the end of the incubation, proteins were precipitated in the presence of cold acetone and subjected to analytical SDS electrophoresis, using 12 % (w/v) polyacrylamide gel. Proteins were stained with Coomassie blue.
lanes 1,7 : molecular weight markers; **lane 2** : testosterone-Sepharose eluate;
lane 3 : eluate + N-glycosidase; **lane 4** : eluate + O-glycosidase;
lane 5 : eluate + neuraminidase; **lane 6** : neuraminidase control.

(**Figure 1**). During O-glycosidase and neuraminidase treatments, the 42 kDa protein does not completely disappeared because of partial hydrolysis of the glycoprotein. Additional bands on lane 5 were due to the presence of the neuraminidase protein as shown in lane 6.

DISCUSSION

SDS-PAGE of the 5α-reductase 2 purified from human prostate revealed an apparent molecular weight of 42 kDa (**16**). However, Andersson et al (**17,7,8**) have calculated the molecular weight of the dog and human prostate 5α-reductase, from deduced cDNA sequences, as 29 kDa. This difference could be due to glycosylation of the native enzyme. Working from prostatic tissue we purified the native glycosylated form with a high molecular weight, while Andersson and Russell, working from cDNA, could only identify the peptidic moiety with a lower molecular weight. A priori argument for this hypothesis was the presence, in the sequence deduced from the cDNA clone, of 14 serines and 10 threonines that represent potential O-glycosylation sites. However there was no consensus sequence for N-glycosylation (Asn-$X_{amino\ acid}$-Thr/Ser).

The results presented here show evidence that the purified 5α-reductase 2 is a O-glycosylated sialoglycoprotein. Initially, lectin-binding tests revealed the presence of oligosaccharide side chains associated with the enzyme, composed of mannose, fucose and N-acetylgalactosamine, but devoid of N-acetylglucosamine. N-acetylgalactosamine and N-acetylglucosamine are usually implicated in O-glycosylation and in N-glycosylation respectively. Consequently enzymatic deglycosylations confirmed rigorously the above. A protein of about 30 kDa appeared on SDS-PAGE after O-glycosidase treatment, while N-glycosidase had no effect.

On the other hand, neuraminidase treatment of purified 5α-reductase 2 had the same effect as O-glycosidase treatment indicating the presence of sialic acids associated with the enzyme. The negative charges of sialic acids residues could explain the binding of the 5α-reductase 2 on DEAE-Trisacryl during the purification procedure (**16**).

The glycosylated nature of the 5α-reductase 2 appears to be contradictory with the hydrophobicity which was observed during the purification procedure. We assume that hydrophilic saccharidic residues were more or less entrapped when the enzyme was solubilized from its membranous environment leading to the release of the hydrophobic regions when excess detergent was removed, leading to aggregation of the 5α-reductase.

Glycosylation of 5α-reductase 2 can explain the difference observed between the molecular weight of the native enzyme purified from prostatic tissue and the molecular weight of the peptide deduced from cDNA; but it does not explain the difference with the molecular weight of 23 kDa observed more recently by Thigpen et al (**21**). These authors carried out immunoblots of tissue homogenates using antibodies raised against synthetic peptide representing amino acids 227 to 251 of the sequence deduced from the 5α-reductase type 2 gene by Andersson et al (**8**). We assume that there are two forms of the enzyme : a glycosylated form with an apparent molecular weight of about 42 kDa upon SDS-PAGE, and a non glycosylated form with an apparent SDS-PAGE molecular weight of 23 kDa. We have only purified the glycosylated form, while the antibodies used by Thigpen et al should have more affinity for the non glycosylated form.

Glycosylation may change markedly the quaternary structure of a protein and thus modify the biological activity of enzymes, moreover many pathological states, especially in cancer, are characterized by changes in the carbohydrate structure of cellular glycoproteins (**22**). Thus, the data presented in this paper should lead to further investigations about the possible regulation of the testosterone 5α-reductase activity in the prostate by modification of its glycosylation, especially in pathological situations such as benign hypertrophy and cancer.

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