Testosterone Regulates the Synthesis of Major Proteins in Rat Ventral Prostate

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The presence of three major proteins α , β and γ in rat ventral prostate was demonstrated by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Their regulation by androgens was studied by measuring the rates of synthesis of the proteins in minced prostatic tissue by using L-[^{35}S]methionine. The three proteins account for 30–40% of the proteins synthesized in the gland. After castration, their rates of synthesis rapidly decline to about 1% that of normal animals, and this cannot be accounted for by the accompanying decrease in general protein synthesis. Testosterone reverses these changes in castrated animals, so that after 4 days normal synthesis is restored. The regulation is specific for androgens, since cyproterone acetate, an anti-androgen, is inhibitory and oestradiol-17 β and corticosterone are without effect. Preliminary characterization of the proteins indicates that protein α (mol.wt. 22000, pI unknown) is a glycoprotein containing glucose and/or mannose residues and occurs in both the mitochondrial and cytosol fractions. Protein β (mol.wt. 12000, pI 5.4) is also a glycoprotein, but is found exclusively in the cytosol fraction. Protein γ (mol.wt. 8000, pI 5.4) is also found exclusively in the cytosol fraction.

It is well established that the metabolic and secretory activities of male accessory organs are regulated by androgens (Mann, 1964; Brandes, 1974). In rat ventral prostate much is known about the early events in the molecular mechanism of androgen action (King & Mainwaring, 1974). Testosterone is reduced to 5 α -dihydrotestosterone (Anderson & Liao, 1968; Bruchovsky & Wilson, 1968), which can bind with high affinity to a cytoplasmic receptor (Fang *et al.*, 1969; Mainwaring, 1969) capable of interacting with chromatin in the nucleus (Mangan *et al.*, 1968; Fang & Liao, 1971; Mainwaring & Peterken, 1971). Although the activities of numerous enzymes in the prostate (Paris & Brandes, 1974) are stimulated by testosterone, it has not been established whether this is a direct effect on enzyme synthesis by the regulation of mRNA concentration or whether the hormone modulates other factors that affect enzyme activity, such as coenzymes, activators and inhibitors or cell permeability. The androgen-dependent stimulation of aldolase was shown to be due primarily to effects on mRNA concentration, but the increases were modest and precluded further investigation (Mainwaring *et al.*, 1974). Further progress has been hindered by

the failure to detect suitable protein markers induced by the hormone. Clearly studies would be facilitated if a prostatic protein was identified that accounted for a substantial fraction of the total cellular protein synthesis and was induced significantly by androgens.

Recently we have approached the problem by examining the complexity of RNA in prostatic tissue isolated from normal and castrated rats. Analysis of the kinetics of hybridization of poly(A)-containing RNA with its complementary DNA provides information on the numbers of different RNA sequences present in mammalian cells (Bishop *et al.*, 1974; Lewin, 1975). Using this approach we have demonstrated that the concentration of a class of poly(A)-containing RNA molecules is regulated by androgens (Parker & Mainwaring, 1977). The concentrations of approximately two different sequences, accounting for about 50% of the total poly(A)-containing RNA in the ventral prostate, were regulated by testosterone. Since most mRNA molecules described to date contain poly(A) tracts, it is reasonable to predict that these highly abundant poly(A)-containing RNA sequences are mRNA molecules coding for prostatic proteins. Therefore we have studied the complexity of prostatic proteins and investigated the effects of testosterone on their rates of synthesis. In the present paper we describe three major proteins that are useful markers of androgen action in rat ventral prostate.

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Materials and Methods

Animals

Adult male Sprague-Dawley rats (200–250 g) from this Institute were anaesthetized with fluothane and castrated by the scrotal route. Where indicated steroids were injected subcutaneously in arachis oil in the flank region. Doses were (per animal) corticosterone and testosterone, 1.25 mg, oestradiol-17 β , 10 μ g, and cyproterone acetate, 25 mg.

Materials

Chemicals were of the highest available purity and supplied by either Sigma (London) Chemical Co., London S.W.6, U.K., or BDH, Poole, Dorset, U.K. Other chemicals used were as follows: testosterone propionate (Androject; Intervet Laboratories, Morden, Surrey, U.K.); electrophoretically pure proteins for use as molecular-weight markers on polyacrylamide gels (Miles-Seravac, Maidenhead, Berks., U.K.); sodium dodecyl sulphate (Serva, Heidelberg, W. Germany); highly purified sodium deoxycholate (Fisons, Loughborough, Leics., U.K.); *p*-rosaniline reagent (Searle, High Wycombe, Bucks., U.K.); L-[³⁵S]methionine (200–400 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). CNBr-derived fragments of myoglobin were a gift from Mr. J. W. McCauley of this Institute.

Isolation of subcellular fractions

(a) *Nuclear and postnuclear supernatant fractions.* These fractions were prepared essentially as described by Chung & Coffey (1971). Ventral prostate was homogenized in 10 vol. (or 100–200 vol. for radioactive tissue) of 0.25 M-sucrose containing 3 mM-MgCl₂ and 0.1 % Triton X-100, with a motor-driven homogenizer (Silverson Machines, Chesham, Bucks., U.K.) at 500 rev./min for 30 s. The homogenate was then centrifuged at 1000g for 10 min to obtain the post-nuclear supernatant fraction. The sediment was resuspended in 2.1 M-sucrose containing 1 mM-MgCl₂ and 0.01 M-Tris/HCl, pH 7.5, and centrifuged at 50000g for 1 h at 4°C. The sediment constituted the nuclear fraction.

(b) *Mitochondrial, microsomal and cytoplasmic fractions.* Differential centrifugation of the 1000g supernatant fraction was used to prepare these fractions; the sediments recovered after centrifugation at 10000g for 10 min and 100000g for 100 min provided the mitochondrial and microsomal fractions respectively. The final non-particulate fraction was retained as the cytosol fraction.

Incorporation of radioactive amino acids

Protein synthesis was measured as described by Higgins *et al.* (1976a). Freshly minced tissue (100–200 mg) was incubated for 1 h at 37°C in 10 ml of Dulbecco's modified Eagle's medium (without

methionine) (Dulbecco & Freeman, 1959) with 50–100 μ Ci of L-[³⁵S]methionine (200–400 Ci/mmol) under an atmosphere of O₂/CO₂ (19:1). Incorporation was linear with time during this period, provided that no more than about 300 mg of tissue was used. An equal volume of 0.5 M-sucrose containing 3 mM-MgCl₂ and 0.1 % Triton X-100 was then added and the tissue homogenized to prepare a postnuclear supernatant fraction as described above.

Analytical electrophoresis

(a) *Detection of protein.* Electrophoresis was carried out in slab gels consisting of 17 % (w/v) polyacrylamide and 0.1 % (w/v) *NN'*-methylenebisacrylamide (10 cm $\times$ 16 cm $\times$ 0.2 cm) containing 0.4 % sodium dodecyl sulphate with a 5 % stacking gel (1 cm $\times$ 16 cm $\times$ 0.2 cm) (Studier, 1970) and the buffer system described by Laemmli (1970). The gels were stained with 1 % (w/v) Naphthalene Black 10B in 40 % (v/v) methanol/7 % (v/v) acetic acid for 1 h at room temperature (20–25°C) and destained overnight by using the same solvent without the dye.

(b) *Detection of radioactivity.* Radioactive samples were first concentrated 10-fold by precipitation with 5 % (w/v) trichloroacetic acid and redissolving them in an appropriate volume of 1 % sodium dodecyl sulphate. The samples were heated at 100°C, and after dialysis against 1 % sodium dodecyl sulphate, 0.1 % β -mercaptoethanol and 62.5 mM-Tris/HCl, pH 7.0, were electrophoresed as described above. Gels were stained for proteins and then prepared for radioautography by impregnating them with 2,5-diphenyloxazole (Bonner & Laskey, 1974). After drying, the gels were placed in contact with Kodak X-omat H film at –70°C for up to 3 weeks. The appropriate regions of the gel, located by using the radioautographs, were then cut out and digested with H₂O₂ (0.4 ml) at 50–60°C for 12–18 h, and radioactivity was determined.

(c) *Detection of carbohydrate.* Electrophoresis was carried out in cylindrical 17 % (w/v) polyacrylamide gels (7 cm $\times$ 0.5 cm) with a stacking gel (1 cm $\times$ 0.5 cm) containing 0.4 % sodium dodecyl sulphate (Laemmli, 1970) at 0.67 mA per gel for 16 h.

Carbohydrate residues were detected with Schiff reagent by using the method of Glossmann & Neville (1971) as modified by Segrest & Jackson (1972).

Isoelectric focusing

Isoelectric focusing was carried out in polyacrylamide gels containing 7 % (w/v) acrylamide, 0.2 % (w/v) *NN'*-methylenebisacrylamide and 3.5 M-urea, as described in detail by Wrigley (1968). Two pH gradients containing mixtures of Ampholines have been used: a narrow-range pH gradient containing 1 % (v/v) pH range 4–6 plus 1 % (v/v) pH range 5–7; a broad-range pH gradient containing

0.2% (v/v) pH2.5–4, 1.6% pH3–10 and 0.2% pH9–11.

Affinity chromatography on lectin columns

Two sugar-specific lectins from *Lens culinaris* (LCH) with specificity for glucose and mannose residues (Hayman & Crumpton, 1972) and *Ricinus communis* (RCA₁) with specificity for terminal α -galactose residues (Nicholson *et al.*, 1974) were used to prepare affinity-chromatography columns to fractionate carbohydrate-containing material (Hayman & Crumpton, 1972). Postnuclear supernatant fractions, dialysed against 1% (w/v) sodium deoxycholate, were applied to the affinity columns (5.0cm $\times$ 2.5cm). Columns of both LCH and RCA₁ lectins were first washed with 1% sodium deoxycholate to remove unbound protein, and then LCH-lectin columns were eluted with 0.3M- α -methyl-D-mannose in 1% sodium deoxycholate and RCA₁-lectin columns with 0.3M-D-galactose in 1% sodium deoxycholate. Eluted protein was then dialysed against 1% deoxycholate, freeze-dried and precipitated with ethanol at -20°C overnight. After centrifugation the precipitates were dried under N₂ and treated with sodium dodecyl sulphate before polyacrylamide-gel electrophoresis.

Other determinations

Radioactivity was measured with a Nuclear-

Chicago II liquid-scintillation counter by using scintillation fluid consisting of 6g of butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (Ciba ARL, Duxford, Cambridge, U.K.) in 250ml of Triton X-100, 45ml of water and 705ml of toluene. Efficiency for ^{35}S was 48–50%. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Identification of androgen-dependent proteins

Prostatic proteins regulated by testosterone have been identified in postnuclear supernatant fractions by polyacrylamide-gel electrophoresis (Plate 1). Among the components that change significantly after manipulation of the androgenic status of the animals are three major proteins with R_F values of approx. 0.58 (α), 0.80 (β) and 0.90 (γ). Proteins α and γ were not detectable at 16 days after castration, whereas protein β was still detectable but greatly decreased. These effects could be reversed by androgen therapy of the castrated animals. Daily administration of testosterone *in vivo* for 3–5 days resulted in the reappearance of the proteins α , β and γ , as judged by Naphthalene Black staining.

Fig. 1. Effect of castration on prostatic protein synthesis

Approx. 150mg portions of prostatic tissue isolated from groups of up to six rats at different periods after castration were each incubated with 100 μCi of [^{35}S]methionine. (a) Total protein synthesis/gland was determined by precipitation with trichloroacetic acid. (b) [^{35}S]Methionine incorporation into proteins α (■), β (●) and γ (▲). Post-nuclear supernatant fractions were analysed by electrophoresis on polyacrylamide gels and, after radioautography, the appropriate regions of the gels were counted for radioactivity.

Protein synthesis has been measured in minced tissue by using L-[^{35}S]methionine incorporation. After castration, total incorporation of L-[^{35}S]methionine into protein was decreased by half after 2–3 days and ultimately to about 5% that of normal animals after 15 days (Fig. 1a). The incorporation of L-[^{35}S]methionine into proteins α , β and γ was detected by radioautography after electrophoresis on polyacrylamide gels, revealing several labelled proteins, including four major ones (Plate 2a). Three of these correspond closely to the three proteins α , β and γ , although one of them corresponds to only the trailing edge of protein α revealed by Naphthalene Black stain. The fourth major radioactive component on the radioautographs also corresponds to a protein revealed by staining, but varied in amount from preparation to preparation and was not studied in detail. Assuming then that these radioactive components correspond to the stainable proteins α , β and γ , it is clear that incorporation of [^{35}S]methionine into these bands is greatly decreased after castration and that testosterone administered *in vivo* restores their rates of synthesis. The proportion of total protein synthesis in normal animals ranged from 5 to 8% for

α , 15 to 20% for β and 6 to 9% for γ . After castration, synthesis of each protein had decreased by half after about 1 day and ultimately to about 5% for α and 1% for β and γ proteins after 7 days (Fig. 1b). Androgen treatment reversed these changes, as shown in Figs. 2(a) and 2(b). A single injection of testosterone into animals castrated for 14 days stimulated total protein synthesis within 1 day. A value characteristic of normal animals was reached after about 3–4 days, after which total protein synthesis continued to rise, often reaching a rate exceeding that of normal animals. Similarly, the synthesis of the proteins α and β increased significantly after 1 day, and all three proteins achieved normal synthesis rates after 3–4 days. A similar response time was obtained when animals 7 days after castration were treated with testosterone (G. Scrace & M. Parker, unpublished work). That testosterone affects both general and specific protein synthesis is confirmed by comparing the radioactivity incorporated into each of the proteins when fixed amounts of radioactivity are applied to the gels. Thus the proportion of [^{35}S]methionine incorporated into the proteins β and γ declines after castration and is increased by testosterone.

Fig. 2. Effect of testosterone on prostatic protein synthesis

Approx. 150mg portions of prostatic tissue were isolated from groups of up to six rats treated with testosterone for various periods after 14 days after castration. The tissue was incubated with 100 μCi of [^{35}S]methionine. (a) Total protein synthesis/gland was determined by precipitation with trichloroacetic acid. (b) [^{35}S]Methionine incorporation into proteins α (■), β (●) and γ (▲). Postnuclear supernatant fractions were analysed by electrophoresis on polyacrylamide gels and, after radioautography, the appropriate regions of the gels were counted for radioactivity. The points represent the means of duplicate determinations.

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of prostatic proteins

Postnuclear (1000g) supernatant fractions (100 μ g) of prostates from rats of different hormonal status were analysed by electrophoresis on polyacrylamide gels containing 0.4% sodium dodecyl sulphate (see the Materials and Methods section). Electrophoresis was from top to bottom and the proteins were stained with Naphthalene Black. The material loaded on gels was from: (1) normal rats; (2) rats 1 day after castration; (3) rats 5 days after castration; (4) rats 16 days after castration; (5) rats 16 days after castration+1 day of testosterone treatment; (6) rats 16 days after castration+3 days of testosterone; (7) rats 16 days after castration+5 days of testosterone; (8) normal rats.

EXPLANATION OF PLATE 2

(a) Radioautograph of [^{35}S]methionine-labelled prostatic proteins and (b) polyacrylamide-gel electrophoresis of prostatic proteins after fractionation on LCH-lectin affinity columns

(a) Minced prostatic tissue was incubated with [^{35}S]methionine. The 1000g postnuclear supernatant fraction was then analysed on polyacrylamide gels containing sodium dodecyl sulphate. Radioactive proteins were detected by radioautography (see the Materials and Methods section). The material loaded on the gels was from: (1) rats 14 days after castration; (2) rats 14 days after castration+1 day of testosterone treatment; (3) rats 14 days after castration +2 days of testosterone; (4) rats 14 days after castration+3 days of testosterone; (5) normal rats. Protein bands were stained with Naphthalene Black. (b) A 1000g supernatant fraction of normal rat prostate containing 30 mg of protein was fractionated on an LCH-lectin affinity column. Portions (100 μg) of (1) the starting material, (2) the proteins that failed to bind and (3) those that bound to the LCH-lectin column were analysed by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels.

Fig. 3. Effect of castration and androgen treatment on the relative synthesis of proteins α , β and γ . The percentage of [35 S]methionine incorporated into proteins α (■), β (●) and γ (▲) was measured. Postnuclear supernatant fraction of prostatic proteins was isolated (a) from rats at different periods after castration and (b) from animals treated with testosterone for different periods 14 days after castration. In each case 30000 c.p.m. was applied to the gels. The points represent the means of duplicate determinations.

Alterations in synthesis of α protein are much less marked (Figs. 3a and 3b).

Restoration of specific and general protein synthesis in castrated animals is androgen-dependent. Oestradiol-17 β and corticosterone were ineffective, but the anti-androgen cyproterone acetate almost completely inhibited the stimulatory effects of testosterone (Table 1).

Characterization of the proteins

Since the major proteins the synthesis of which is regulated by testosterone in rat seminal vesicle are glycoproteins (Higgins *et al.*, 1976a), the possibility that proteins α , β and γ are also glycoproteins has been examined in two ways. First, on polyacrylamide gels protein β , and to a lesser extent protein α , gave a positive reaction to Schiff reagent. Secondly, approx. 5% of the total prostatic protein could be bound to the LCH-lectin column. When this fraction was analysed by polyacrylamide-gel electrophoresis (Plate 2b), it had a mobility corresponding to that of protein α . Less than 1% of the prostatic proteins was bound by the RCA₁-lectin column and none of these corresponded to proteins α , β or γ . These results suggest that protein α contains glucose and/or mannose, but not terminal galactose residues. Proteins β and γ do

Table 1. Steroid specificity of specific prostatic protein synthesis

Groups of three rats 7 days after castration were injected daily with the steroid indicated. After 4 days, prostates were removed and incubated with 50 μ Ci of [35 S]methionine, and the incorporation in total and specific proteins was measured as described in the Materials and Methods section.

Steroids	[35 S]Methionine incorporation (c.p.m./h per gland)			
	Total proteins	α	β	γ
None	177 000	2710	2010	826
Testosterone	1 696 000	158 000	274 000	59 800
Oestradiol-17 β	267 000	5160	3930	1070
Corticosterone	176 000	2690	1990	820
Cyproterone	193 000	3840	2200	900
Testosterone + cyproterone	309 000	8250	11 900	4000

not appear to contain any of these residues, although the former stains positively with Schiff's reagent.

On polyacrylamide gels containing sodium dodecyl sulphate, proteins α , β and γ migrate as proteins of

Fig. 4. Molecular-weight determinations of prostatic proteins α , β and γ

Mobilities of proteins α , β and γ were determined in polyacrylamide gels containing sodium dodecyl sulphate and compared with those of purified commercial proteins: β -galactosidase (1), lactate peroxidase (2), catalase (3), alcohol dehydrogenase (4), carbonic anhydrase (5), myoglobin (6), globin (7), ribonuclease (8), cytochrome *c* (9) and CNBr fragments of myoglobin (10, 11 and 12). The arrows indicate the mobilities of proteins α , β and γ , relative to Bromophenol Blue as a tracking dye.

mol. wts. approx. 22000, 12000 and 8000 respectively (Fig. 4). These values may be overestimates, since proteins containing appreciable amounts of carbohydrate are known to behave anomalously on sodium dodecyl sulphate-containing gels (Segrest & Jackson, 1972).

Isoelectric focusing on polyacrylamide gels indicated that both proteins β and γ had isoelectric points of about 5.4. Surprisingly, protein α was not detected on focusing gels containing pH gradients ranging from 3.0 to 9.7. Unfortunately, stable pH gradients were not obtained below 3.0 or above 9.7.

Tissue-fractionation studies indicate that proteins β and γ are restricted to the 100000g supernatant or cytosol fraction, whereas protein α may also be found in the 10000g sediment from prostate homogenates. Electron-microscopic analysis showed that this latter fraction is extremely heterogeneous, containing mitochondria, lysosomes, secretion granules and other microbodies (L. Peachey, G. T. Scrace & M. G. Parker, unpublished work). Other procedures are needed to identify the intracellular location of protein α ,

Discussion

Most progress in the elucidation of the molecular mechanism by which steroid hormones control protein synthesis has been made by using systems in which a major protein is regulated by the steroid. Although much of what is known about the action of androgens has been obtained by using the rat ventral prostate, suitable marker proteins of androgen action that account for a substantial fraction of the protein synthesis of the tissue have not been reported. Here we have demonstrated the existence of three major proteins representing 30–40% of the total protein and whose synthesis is testosterone-dependent. The three proteins α , β and γ should be useful indicators of androgen action in molecular terms. Preliminary experiments reported here on steroid specificity suggest that the regulation appears to be modulated by a specific androgen receptor, since cyproterone acetate, which competes with 5α -dihydrotestosterone for binding to androgen-specific receptor (Fang & Liao, 1969; Stern & Eisenfeld, 1969), inhibits the action of testosterone. In addition, oestradiol and corticosterone, neither of which bind to testosterone receptor, have no effect on the synthesis of the proteins.

Since 50% of the poly(A)-containing RNA present in the ventral prostate consists of about two sequences whose concentrations are acutely dependent on testosterone (Parker & Mainwaring, 1977), it is reasonable to predict that the synthesis of a similar number of major proteins should also be regulated by testosterone. This assumes, of course, that the poly(A)-containing RNA species function as mRNA. Evidence to date suggests that poly(A) tracts are a feature of most mRNA molecules (Darnell *et al.*, 1973). It is likely that the mRNA species for the proteins α , β and γ are coded for by the abundant poly(A)-containing RNA sequences.

The rapid decline in both specific and general protein synthesis after castration and the restoration of synthesis by testosterone could result from changes in the total number of synthetic cells rather than alterations in protein-synthetic capacity of individual cells. Although not discounted directly, the kinetics of proliferative changes in the prostate probably do not account for the effects reported here. In particular, cell division does not occur to any significant extent in the first 36–48 h after the administration of testosterone to castrated rats (Coffey *et al.*, 1968; Rennie *et al.*, 1975), whereas increases in general and specific protein synthesis occur much more rapidly.

The synthesis of a rat liver protein $\alpha 2u$ is also under androgenic control (Roy & Neuhaus, 1967; Roy *et al.*, 1977; Kurtz *et al.*, 1976a,b), but the regulation of this protein appears to be complex and certainly involves other hormonal factors. The androgenic regulation of protein synthesis in rat seminal vesicle

has received much attention (Higgins *et al.*, 1976a,b), but their results are quite different from those reported here for the prostate. In the rat seminal vesicle changes in the synthesis of specific proteins follow general protein synthesis, there being no evidence that androgens extend differential effects on individual proteins (S. J. Higgins, J. M. Burchell & W. I. P. Mainwaring, unpublished work).

So far the identity and function of proteins α , β and γ are quite unknown. It is not even clear whether they contribute to prostatic secretions and thus constitute part of the seminal plasma. Nonetheless it is expected that studies of their hormonal regulation will allow us to investigate the molecular mechanism of androgenic hormones.

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