J. steroid Biochem. Vol. 35, No. 1, pp. 97–101, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

KINETIC PROPERTIES OF THE 5α-REDUCTASE OF TESTOSTERONE IN THE PURIFIED MYELIN, IN THE SUBCORTICAL WHITE MATTER AND IN THE CEREBRAL CORTEX OF THE MALE RAT BRAIN

A. POLETTI, F. CELOTTI,* R. C. MELCANGI, M. BALLABIO and L. MARTINI
Institute of Endocrinology, Via Balzaretti, 9, 20133 Milano, Italy [*Tel.* 0039-2-29406576; *Fax* 2-29404927]

(*Received* 1 *February* 1989; *received for publication* 30 *August* 1989)

Summary—The 5α-reductase, the enzyme which converts testosterone into dihydrotestosterone (DHT), is present in several CNS structures of the rat. Recent reports from this laboratory indicate that the subcortical white matter and the myelin possess a 5α-reductase activity several times higher than that present in the cerebral cortex. Moreover, previous ontogenetic observations indicate that in all cerebral tissues examined (including the myelin) the 5α-reductase has a higher activity in immature animals.

This study was performed in order to verify whether the differences in the 5α-reductase activity on the various brain components might be due to the presence of different concentrations of the same enzyme or to different isoenzymes. To this purpose, the kinetic properties K_m and V_{max} were measured in the purified myelin as well as in homogenates of the subcortical white matter and of the cerebral cortex, obtained from the brain of adult (60–90-day-old), immature (23-day-old), and aged (>20-month-old) male rats.

The results indicate that the enzymes present in the myelin, in the subcortical white matter and in the cerebral cortex of adult male rats possess a very similar apparent K_m (1.93 ± 0.2, 2.72 ± 0.73 and 3.83 ± 0.49 μM respectively). On the contrary, the V_{max} values obtained in the myelin (34.40 ± 5.54), in the white matter (19.57 ± 2.36) and in the cerebral cortex (6.47 ± 1.03 ng/h/mg protein) of adult animals have been found to be consistently different.

Very similar K_m values were found in the myelin obtained from the brain of immature and very old rats (2.14 ± 0.11 and 3.39 ± 0.75 μM respectively). The V_{max} measured in the myelin purified from the immature rat brain (62.25 ± 4.52) showed a value which was much higher than that found in the myelin of adult animals (34.40 ± 5.54); a V_{max} (34.31 ± 9.41) almost identical to that of adult animals was found in the myelin prepared from the brain of aged rats.

INTRODUCTION

The 5α-reductase, the enzyme which converts testosterone (T) into 5α-androstan-17β-ol-3-one (dihydrotestosterone; DHT), is present in several structures of the central nervous system (CNS) [1]. According to a theory which is now generally accepted, DHT is considered to be the intracellular mediator of many actions exerted by testosterone on its target structures [1].

Recent studies have shown that the 5α-reductase is present both in neurones and in glial cells (astrocytes and oligodendrocytes) [2]. Moreover, other data indicate that this enzymatic activity is particularly elevated in nervous structures mainly composed of white matter [3, 4], as well as in purified myelin membranes [5]. It is important to underline that the enzymatic activity present in isolated myelin membranes appears to be higher (on a mg/protein basis) than that found in any other CNS structure.

The physiological role of the conversion of testosterone into DHT in the myelin membranes and in the white matter structures is not apparent at the moment. To the author's knowledge, the kinetic properties of the 5α-reductase present in the myelin and in the white matter structures (which are mainly composed of myelinated axons) have never been studied. The aim of the present work was to analyze the kinetic properties (apparent Michaelis–Menten constant, K_m and maximal velocity, V_{max}) of the enzyme present in the purified myelin, in homogenates of the subcortical white matter obtained from the brain of adult male rats (60–90-day-old), and to compare them with those of the enzyme present in the cerebral cortex, a gray matter structure. Moreover, since previous observations have indicated that the 5α-reductase present in the myelin sheaths shows a much higher activity in the brain of immature rats than in that of adult animals (with a peak of formation of DHT between 15–25 days) [6], it was felt of interest to evaluate also the kinetic parameters of the 5α-reductase in the myelin purified from the brain of

*Author to whom correspondence should be addressed.

immature (23-day-old) rats. Finally, the kinetic parameters of the 5α-reductase activity were also determined in the myelin obtained from the brain of aged (>20-month-old) male rats.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats (Charles River, Italy) were used throughout these experiments. The animals were maintained in animal quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 6.30 a.m.). Animals were fed a standard pellet diet and water was provided *ad libitum*.

Sample preparation

Cerebral cortex and white matter homogenates. After decapitation, the brain was rapidly removed and the cerebral cortex (CC) and the subcortical white matter (SWM) were dissected. Both cerebral tissues were homogenized in Krebs–Ringer (5% w/v for the CC; 3% w/v for SWM) and were frozen in liquid nitrogen until further processing.

Purification of myelin. The purification has been performed by the method of Keenan[7], with slight modifications. In brief, a 5% (w/v) homogenate of rat brain was prepared in 0.32 M of sucrose at 0–4°C, the suspension (3.5 ml/each tube) was centrifuged at 75,000*g* (29,000 rpm) for 10 min in a Beckman SW 50.1 rotor. The pellet formed was resuspended in 0.85 M of sucrose (4.5 ml/each tube) and centrifuged again in the same conditions. The floating crude myelin layer was collected, diluted with water to 4.5 ml/each tube and centrifuged at 42,000*g* (21,000 rpm) for 5 min. The resulting pellet was resuspended in water and centrifuged as before twice more. The final pellet was resuspended in 0.85 M of sucrose and centrifuged at 75,000*g* (29,000 rpm) for 10 min; the floating purified myelin layer was collected, washed twice with Krebs–Ringer buffer at 1000*g* for 10 min and the pellets obtained were frozen in nitrogen until incubation.

The purity of myelin was evaluated by electron microscopy as previously described [6].

Incubation

The incubations were performed in Krebs–Ringer buffer solution (250 μl, pH 7.1) in the presence of a NADPH generating system (NADP, disodium salt, Boehringer Mannheim 4.66×10^{-3} M; glucose 6-phosphate, disodium salt, Boehringer Mannheim 11.76×10^{-2} M and glucose 6-phosphate dehydrogenase from yeast grade 1, Boehringer Mannheim 7×10^{-2} IU) and [^{14}C]testosterone (SA ~ 56.9 mCi/mmol, Amersham England). The incubation was carried out at 37°C in a Dubnoff metabolic shaker under a stream of O_2–CO_2 (98:2).

The following incubation conditions were tested:

(a) Effect of different amounts of tissue. In these studies the time of incubation was kept constant at 2 h and [^{14}C]testosterone at 3.2×10^{-6} M. The amounts of tissue studied were as follows: purified myelin from 0.04 to 0.7 mg of protein, white matter homogenate from 0.04 to 0.3 mg of protein and cerebral cortex homogenate from 0.1 to 0.8 mg of protein. In the case of the white matter, the maximum amount of tissue was limited to 0.3 mg of protein/sample due to the presence of an irregular chromatographic behaviour of testosterone metabolites when higher amounts of white matter are incubated; this is probably due to the interference of the high amounts of lipids presents in this particular sample.

(b) Effect of different times of incubation. In these studies the amount of tissue was kept constant and the times of incubation were of 10, 30, 60, 90, 120 and 180 min; testosterone was added at the same concentration as before.

On the basis of the results obtained in (a) and (b), the apparent Michaelis–Menten constant (K_m) and the V_{max} were calculated for the purified myelin and for the homogenates of subcortical white matter and of cerebral cortex, utilizing the following conditions of incubation. Time of incubation: 1 h at 37°C. Substrate ([^{14}C]testosterone): from 0.5 to 3 μM; each experiment was performed utilizing five different concentrations of the substrate and triplicate determinations. Amounts of protein incubated: ~0.3 mg for the cerebral cortex, ~0.2 mg for the white matter and ~0.1 mg for the purified myelin. Vials without tissue provided the blanks for each concentration of substrate.

Protein content was evaluated, according to the method of Bradford[8].

Detection of metabolites

At the end of the incubation the reaction was stopped by freezing the samples to −20°C. Tritium labelled DHT (about 5000 dpm each) were added to each sample in order to evaluate the recoveries. The metabolites formed were extracted twice with diethylether, non-radioactive DHT (40 μg/100 ml) was added to each sample in order to aid visualization of the steroid on the TLC plates. Extracted samples were separated on a thin layer silice gel plate (Merck 60 F_{254}, DC) eluting three times with a mixture of dichloromethane–diethylether (11:1 v/v). The DHT spots were identified with iodine vapours, scraped off and the radioactivity counted in a Packard 300 C liquid scintillation spectrometer. Quench corrected dpm of the isotope were obtained by a standard calibration curve. The identification of the metabolite was performed by recrystallization to constant ^{3}H/^{14}C ratio as previously described [5].

In the incubation conditions described, usually the formation of 5α-androstane-3α,17β-diol (3α-diol) represents 10–15% of all the 5α-reduced metabolites formed. Moreover, the purified myelin does not form this metabolite. Finally, 5α-androstane-3β,17β-diol

(3β-diol) is formed in unmeasurable amounts by the brain in the conditions described. Because of this, it was felt appropriate to use only the DHT data in order to perform the kinetic analysis here to be described.

Statistical analysis

The experimental data concerning the different incubation conditions (tissue, time) were analyzed by means of the linear regression analysis; the data obtained in the kinetic study were analyzed utilizing the Lineweaver–Burk plot with appropriate computer programs.

RESULTS AND DISCUSSION

Figure 1a shows the data on the linear regression analysis of the formation of DHT as a function of the amount of protein incubated for the myelin, the white matter, and the cerebral cortex. The regression lines obtained for the 3 tissues considered show correlation coefficients of 0.957, 0.984, and 0.894, indicating that the amount of DHT formed is linear as a function of the amount of tissue incubated in the range of the concentrations utilized. In the case of the white matter, the maximum amount of tissue incubated was limited to 0.3 mg of protein/sample, due to the technical difficulties reported in the Experimental Section. The amount of steroid formed is also linear as a function of time of incubation: correlation coefficients of 0.966, 0.974 and 0.963 have been found respectively for the purified myelin, the white matter homogenate and the cerebral cortex homogenate (Fig. 1b).

The apparent Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) for the three tissues examined are shown in Fig. 2. These parameters were calculated using the Lineweaver–Burk plot. In the experimental conditions adopted, in the presence of an excess of cofactors, the apparent K_m was very

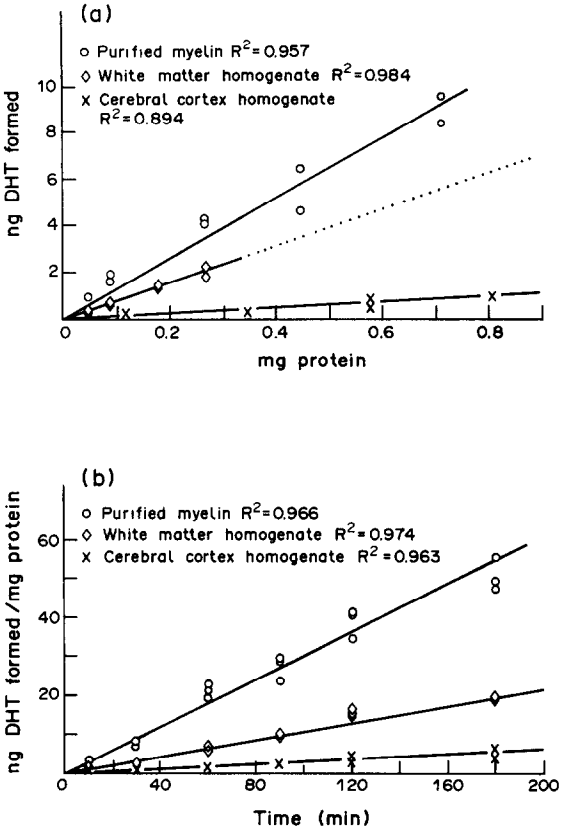

Fig. 1. (a) Effect of different amount of tissue on DHT formation. Incubation time: 2 h (37°C). [^{14}C]testosterone ~3 μM, in presence of a NADPH generating system. (b) Effect of different incubation times on DHT formation. [^{14}C]testosterone ~ 3 μM, in presence of a NADPH generating system.

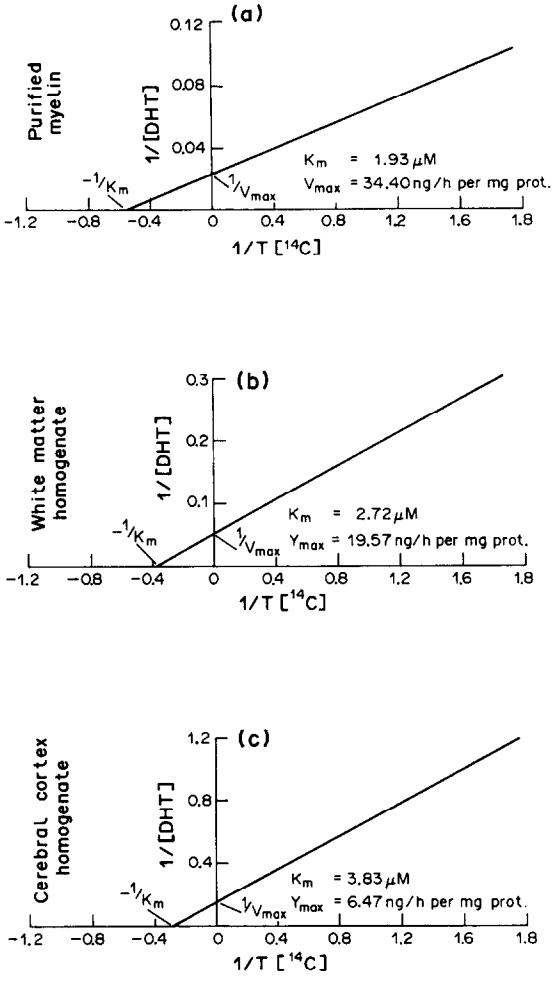

Fig. 2. (a) Average of 8 experiments each performed with 5 different substrate concentrations in triplicate. The incubation where performed with ~100 μg of protein for 1 h at 37°C in presence of a NADPH generating system. (b) Average of 3 experiments each performed with 5 different substrate concentrations in triplicate. The incubation were performed with ~200 μg of protein for 1 h at 37°C in presence of a NADPH generating system. (c) Average of 3 experiments each performed with 5 different substrate concentrations in triplicate. The incubation where performed with ~300 μg of protein for 1 h at 37°C in presence of a NADPH generating system.

Table 1. Kinetic properties of testosterone 5α-reductase in the purified myelin of the male rat brain at different ages

Age	(23 days)	(60–90 days)	(>20 months)
Cases	5	8	3
$K_m \pm$ SE	2.14 ± 0.11	1.93 ± 0.2	3.39 ± 0.75
$V_{max} \pm$ SE	62.25 ± 4.52	34.40 ± 5.54	34.31 ± 9.41

$K_m = \mu M$; V_{max} = ng/h/mg of protein. Incubation time = 60 min. Range of testosterone [T^{14}C] from 0.5 to 3 μM. Protein incubates ~0.1 mg for the purified myelin, in presence of a NADPH generating system.

similar in the three structures examined, i.e. 1.93 ± 0.2 μM in the purified myelin, 2.72 ± 0.73 μM in the white matter, and 3.83 ± 0.49 μM in the cerebral cortex homogenates. On the contrary, the V_{max} values obtained for the various tissues have been found to be consistently different, being 34.4 ± 5.54, 19.57 ± 2.36 and 6.47 ± 1.03 ng/h/mg of protein $\pm$ SE respectively. This indicates that the myelin possesses a concentration of 5α-reductase which is much higher than that present in the white matter; moreover, both the myelin and the white matter have a concentration of the enzyme considerably higher than the cerebral cortex, a gray matter structure. Table 1 shows the data of the K_m and V_{max} in the myelin isolated from the brain of prepuberal (23-day-old), adult (60–90-day-old) and very old (>20-month-old) animals. Very small variations in the K_m values have been found at the different ages considered. On the contrary, the V_{max} values have been found to be almost twice as high in the myelin obtained from immature animals than in the myelin obtained from adult or old animals. The V_{max} values are practically identical in 2–3-month-old and in >20-month-old animals.

The data here presented indicate that, in the brain of normal adult male rats, the white matter contains a concentration of 5α-reductase which is much higher than that present in the cerebral cortex; moreover, the concentration of the enzyme is considerably more elevated in the myelin than in either the white or the gray matter. The high V_{max} values obtained in these kinetic studies for the myelin and the subcortical white matter support the results of previous experiments in which the ability to transform testosterone into DHT was studied using single point assays in different white matter structures microdissected using the Palkovits technique [5, 9], as well as in isolated myelin membranes [5].

The K_m values obtained for the enzyme in the myelin, the white matter and the cerebral cortex, are very similar, all in the μM range, indicating that the enzyme present in the different nervous structures has the same affinity for testosterone, the substrate used in the present experiments. This suggests that the enzyme may be present, in the three types of samples examined, in the same form. The K_m values obtained for the enzyme in the present series of experiments are in good agreement with those described in the literature for the rat brain, even if a direct comparison with previous data cannot be made because of the variety of tissue preparations used by the different authors. Denef *et al.*[10] and Krieger *et al.*[11] have used slices of rat mesencephalic tissue (2.15 and 4.1 μM), Noma *et al.*[12] hypothalamic homogenates (0.74 μM) and Kohsaka *et al.*[13] diencephalic homogenates (2.2 μM).

It also appears from the data that the concentrations of the enzyme (as evaluated by the V_{max}) in myelin membranes shows a significant difference between the immature and the adult brain. The higher V_{max} of the enzyme in the myelin prepared from the brain of young animals is in agreement with previous data obtained in this [6] and in other laboratories [14], which have shown that in different CNS structures (hypothalamus, white matter and cerebral cortex) the 5α-reductase is more active before puberty. On the contrary, the 5α-reductase of the myelin obtained from the CNS does not appear to show any quantitative variation during the process of aging, since the results obtained for the myelin of 60–90-day-old rats are identical to those found in >20-month-old rats. This is in agreement with the data of this laboratory obtained using single point determinations in the hypothalamus [15], the cerebral cortex and in the subcortical white matter of old male rats [Melcangi *et al.*, unpublished observations].

Acknowledgements—The experiments described here were supported by grants of the Consiglio Nazionale delle Ricerche, Roma, Italy (through the project 'Preventive and Rehabilitative Medicine' and 'Oncology' contract Nos 87.00373.56, 87.01343.44) and of the Ministero della Pubblica Istruzione, Roma, Italy. Such support is gratefully acknowledged.

REFERENCES

1. Martini L.: The 5α-reduction of testosterone in the neuroendocrine structures. Biochemical and physiological implication. *Endocr. Rev.* **3** (1982) 1–25.
2. Melcangi R. C., Celotti F., Ballabio M., Castano P., Massarelli R., Poletti A. and Martini L.: DHT formation in isolated and cultured rat brain cells. *Neurosci. Lett.* **33** Suppl. (1988) Abstr. S127.
3. Melcangi R. C., Celotti F., Poletti A., Negri-Cesi P. and Martini L.: The 5α-reductase activity of the subcortical white matter, the cerebral cortex, and the hypothalamus of the rat and the mouse: possible sex differences and effect of castration. *Steroids* **49** (1987) 259–270.
4. Celotti F., Melcangi R. C., Negri-Cesi P., Ballabio M. and Martini L.: Differential distribution of the 5α-reductase in the central nervous system of the rat and the mouse: are the white matter structures of the brain target tissue for testosterone action? *J. Steroid Biochem.* **26** (1987) 125–129.
5. Melcangi R. C., Celotti F., Ballabio M., Poletti A., Castano P. and Martini M.: Testosterone 5α-reductase activity in the rat brain is highly concentrated in white matter structures and in purified myelin sheaths of axon. *J. Steroid Biochem.* **31** (1988) 173–179.
6. Melcangi R. C., Celotti F., Ballabio M., Castano P., Poletti A., Milani S. and Martini L.: Ontogenetic development of the 5α-reductase in the rat brain: cerebral cortex, hypothalamus, purified myelin and isolated oligodendrocytes. *Dev. Brain Res.* **44** (1988) 181–188.
7. Keenan R. W. and Jones M.: A rapid and simple

microprocedure for myelin isolation. *J. Neurochem.* **34** (1980) 231–235.
8. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal-dye binding. *Analyt. Biochem.* **72** (1976) 248–254.
9. Celotti F., Maggi R., Melcangi R. C., Negri-Cesi P. and Martini L.: Metabolism of androgen in the rat and hamster neuroendocrine structures. In *Endocrinology* (Edited by F. Labrie and L. Proulx). Excerpta medica, Amsterdam (1984) pp. 305–309.
10. Denef C., Magnus C. and McEwen B. S.: Sex differences and hormonal control of testosterone metabolism in rat pituitary and brain. *J. Endocr.* **59** (1973) 605–621.
11. Krieger N. R., Scott R. G. and Jurman M.: Testosterone 5α-reductase in rat brain. *J. Neurochem.* **40** (1983) 1460–1464.
12. Noma K., Sato B., Yano S. and Yamamura Y.: Metabolism of testosterone in hypothalamus of male rat. *J. Steroid Biochem.* **6** (1975) 1261–1266.
13. Kohsaka M., Kaneyuki T., Shohmori T.: Influences of central acting drugs on Δ^4-3-ketosteroid 5α-oxidoreductase activity in rat diencephalon. *Folia Psych. Neurol. Jap.* **31** (1977) 661–669.
14. Massa R., Justo S. and Martini L.: Conversion of testosterone into 5α-reduced metabolites in the anterior pituitary and in the brain of maturing rats. *J. Steroid Biochem.* **6** (1975) 567–571.
15. Piva F., Zanisi M., Messi E., Maggi R., Celotti F., Negri-Cesi P., Motta M. and Martini L.: Aging and the Hypothalamic-Pituitary Complex of male Rat. In *Neuroendocrine System and Aging* (Edited by P. Vezzadini, A. Facchini and G. Labo). EURAGE, Bologna (1986) pp. 27–35.