J. Steroid Biochem. Molec. Biol. Vol. 40, No. 4–6, pp. 545–548, 1991
Printed in Great Britain

0960-0760/91 $3.00 + 0.00
Pergamon Press plc

DIFFERENTIAL INHIBITION OF DEHYDROGENASE AND 5-ene→4-ene ISOMERASE ACTIVITIES OF PURIFIED 3β-HYDROXYSTEROID DEHYDROGENASE. EVIDENCE FOR TWO DISTINCT SITES

VAN LUU-THE,* MASAKAZU TAKAHASHI and FERNAND LABRIE

MRC Group in Molecular Endocrinology, CHUL Research Center and Laval University, 2705 Laurier Boulevard, Quebec, Canada G1V 4G2

Summary—The success in synthesis of [^{3}H]5-androstene-3,17-dione, the intermediate product in the transformation of DHEA to 4-androstenedione by 3β-hydroxysteroid dehydrogenase/5-ene→4-ene isomerase (3β-HSD) offers the opportunity to determine whether or not the two activities reside in one active site or in two closely related active sites. The finding that *N,N*-dimethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (4-MA) inhibits competitively and specifically the dehydrogenase activity whereas a non-competitive inhibition type with a K_i value 1000 fold higher was observed for the isomerase activity, indicated that dehydrogenase and isomerase activities belong to separate sites. Using 5α-dihydro-testosterone and 5α-androstane-3β,17β-diol, exclusive substrates for dehydrogenase activity, it was shown that dehydrogenase is reversible and strongly inhibited by 4-MA and that thus the irreversible step in the transformation of DHEA to 4-androstenedione is due to the isomerase activity.

INTRODUCTION

The enzyme complex 3β-hydroxysteroid dehydrogenase/5-ene→4-ene isomerase, hereafter called 3β-HSD, catalyses the oxidation/isomerization of 3β-hydroxy-5-ene steroids into 3-keto-4-ene configuration [1, 2], and thus plays a major role in the formation of all classes of steroid hormones, namely progesterone, mineralocorticoids, androgens and estrogens. Following the purification of the 42 kDa human placental 3β-HSD, and the subsequent obtention of the antibodies raised in rabbits [3, 4], our group has isolated and characterized human placental [2], bovine ovary [5] and rat ovary [6] cDNA clones. Recently the structure of the human 3β-HSD gene was also determined [7]. It was localized in the p11–p13 band of chromosome 1 [8]. Down-regulation of 3β-HSD expression by 12-*O*-tetradecamoyl phorbol-13-acetate (TPA) was observed in pig granulosa cells [9, 10]. Immunocytochemical localization of 3β-HSD was performed in the gonads and adrenal glands of the guinea pig [11] and rat [12]. In the later species, *in situ* hybridization was also performed [12]. Hormonal regulation of 3β-HSD expression and activity has been investigated in rat ovary [13] and testis [14]. Expression of the human placental 3β-HSD cDNA in mammalian cells [7] has confirmed that the two dehydrogenase and isomerase activities reside within a single 42 kDa protein. However, there is still an uncertainty about whether the two activities are included in a unique or separate sites. Using [^{3}H]5-androstene-3,17-dione, the intermediate product in DHEA transformation, and specific inhibitors such as trilostane and *N,N*-dimethyl-4-methyl-3-oxo-4-aza-5α-andristane-17β-carboxamide (4-MA) [15] we demonstrate that dehydrogenase and isomerase activities are associated with distinct sites within a single protein.

Proceedings of the VIIIth International Congress on Hormonal Steroids, The Hague, The Netherlands, 16–21 September 1990.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

NAD^+, $NAPD^+$, digitonin were purchased from Sigma Chemicals Co (St Louis, MO). Hydroxylapatite Biogel HTP was from Bio-Rad Laboratories (Richmond, CA). [4,7-^{3}H]pregnenolone, [1,2-^{3}H]dehydroepiandrosterone were obtained from NEN Dupont (Missisauga, Ontario, Canada). All non-radioactive steroids were purchased from Steraloids Ltd (Wilton, NH). Trilostane was kindly provided by Sterling-Winthrop Research Institute (Renslaer, New York) and 4-MA was a gift from

Merck Sharp & Dohme Research Laboratories (Rahway, NJ).

Purification of 3β-HSD

3β-HSD was purified to homogeneity as judged by SDS–PAGE from human placentas by a phenyl-Sepharose 4B-CL column followed by double hydroxyapatite column as described previously [4, 15].

Enzymatic assay

Purified enzyme were incubated at 37°C in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA and 1 mM NAD^+ for 10 min under constant agitation. Immediately after incubation, steroids were extracted twice with 2 ml of methylene dichloride. The extract was then dried with anhydrous sodium sulfate and evaporated under a stream of nitrogen. The metabolites were isolated and separated by TLC on silica gel in the benzene-acetone (4:1) separating system. Radioactivity was measured by a liquid scintillation spectrometer.

Synthesis of [1,2-^{3}H]5-androstene-3,17-dione

The reaction was carried out in a closed system kept under slightly positive air atmosphere. 5 mg of DHEA in 500 μl of ice-cold stabilized acetone was mixed with 1 mCi [^{3}H]DHEA (50 Ci/mmol). 5 ml of Jone's reagent was carefully added under inert atmosphere. After 15 min, the reaction was terminated by adding 3 ml of ice-cold water. After standing in the cold room overnight, the precipitate was filtered, washed, dried *in vacuo* and purified through an LH-20 column. The purified product showed no adsorption at 240 nm, thus indicating the absence of contamination from 4-androstenedione. The melting point (125–129°C) was identical with authentic 5-androstene-3,17-dione. The yield was 3.5 mg (70%).

RESULTS AND DISCUSSION

The two step transformation of DHEA to 4-androstenedione via 3β-HSD requires the intervention of 3β-hydroxysteroid dehydrogenase and 5-ene→4-ene isomerase activities and the production of the intermediate product, 5-androstene-3,17-dione:

$$\text{DHEA} \xrightarrow[\text{dehydrogenase}]{} \text{5-androstene-3,17-dione} \xrightarrow[\text{isomerase}]{} \text{4-androstenedione}$$

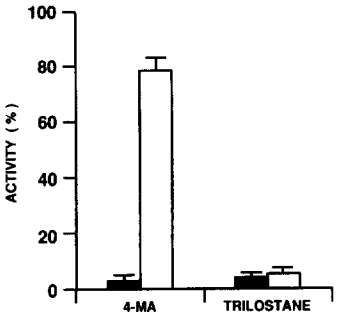

Fig. 1. Effect of 4-MA on the activity of purified human placental microsomal 3β-HSD. Incubation was performed as described in Materials and Methods in the presence of 20 μM of DHEA (■) or 5-androstene-3,17-dione (□) and the indicated inhibitors at the concentration of 10 μM. The 100% activity corresponds to incubation in the presence of substrate alone. Each value was obtained from duplicate experiments.

Success in obtaining tritiated intermediate allowed us to investigate differential inhibition of dehydrogenase and isomerase activities. As illustrated in Fig. 1, trilostane inhibits strongly both DHEA and 5-androstene-3,17-dione transformation, whereas 4-MA affects preferentially DHEA and does not affect significantly 5-androstene-3,17-dione. Dixon plot analysis shows that trilostane is a competitive inhibitor at both dehydrogenase and isomerase sites with K_i values of 36 and 160 nM, respectively (Fig. 2). On the other hand, 4-MA is competitive when DHEA is used as a substrate with a K_i value of 56 nM but non-competitive when 5-androstene-3,17-dione is used (K_i value = 55 μM, 1000 fold higher (Fig. 3).

Differential inhibitory effects of 4-MA and trilostane thus clearly indicate that dehydrogenase and isomerase active sites are distinct within a single protein. Localization of dehydrogenase and isomerase within a unique 42 kDa protein has been confirmed by expression of 3β-HSD cDNA inserts in mammalian cells [7]. Using DHT and 5α-androstane-3β,17β-diol, which are exclusive substrates for dehydrogenase activity, Dixon plot analysis (Fig. 4) shows that 4-MA inhibits transformation of both substrates competitively with K_i values of 16 and 3.7 μM for DHT and 5α-androstane-3β,17β-diol, respectively. Since DHT and 5α-androstane-3β,17β-diol are interconverted by 3β-HSD, the results indicate that the dehydrogenase step is reversible while the isomerase activity is irreversible.

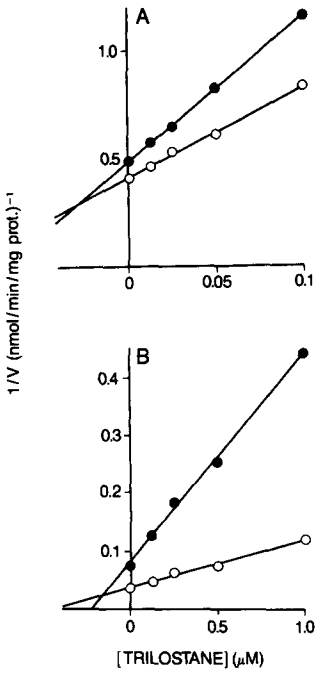

Fig. 2. Dixon plot analysis of the effect of trilostane on the transformation of DHEA (A) and 5-androstene-3,17-dione (B) by purified human placental microsomal 3β-HSD. Concentrations of [^{3}H]DHEA were 100 μM (○—○) and 20 μM (●—●), and those of [^{3}H]5-androstene-3,17-dione were 10 μM (○—○) and 5 μM (●—●).

CONCLUSION

While both dehydrogenase and isomerase activities reside within a single protein in mammalian tissues as evidenced by enzyme purification from bovine ovaries [17], ovine adrenal [18], rat testis [19] and adrenal [20] and human placenta [3, 4] as well as by expression of human placental 3β-HSD cDNA in mammalian cells [7], they were isolated on separate proteins from bacterial sources [21]. Comparison between amino acid and nucleotide sequences of bacterial isomerase [22] with human placental 3β-HSD [2] did not reveal significant homology (unpublished data). However, a common ancestor between human placental 3β-HSD cDNA sequence and flavonoids sequence have been evidenced [23].

Our finding that 4-MA differentially inhibits dehydrogenase and 5-ene→4-ene isomerase activities clearly indicates that although the two activities reside within a single protein in mammalian tissues, they have separated sites. Selectivity of 4-MA inhibition offered the opportunity to study the regulation of these two activities as well as their interaction. Since 3β-HSD did not transform 3α-steroids, we can speculate that the binding for the dehydrogenase site is near the position number 3 on the steroid, whereas the site for the isomerase activity binds to the flattened B or A ring. Androstane compound which did not have a

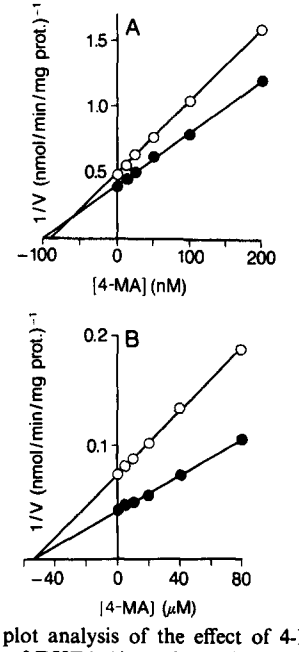

Fig. 3. Dixon plot analysis of the effect of 4-MA on the transformation of DHEA (A) and 5-androstene-3,17-dione (B) by purified human placental microsomal 3β-HSD. [^{3}H]DHEA was used as substrate at the concentrations of 100 μM (●—●) and 20 μM (○—○) and those of [^{3}H]5-androstene-3,17-dione were 20 μM (●—●) and 5 μM (○—○).

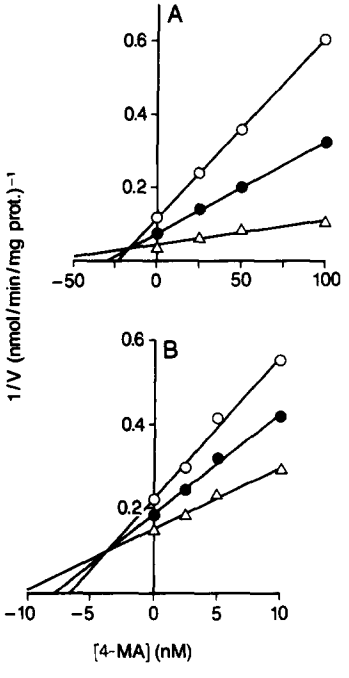

Fig. 4. Dixon plot analysis of the effect of 4-MA on the transformation of DHT (A) and 5α-androstane-3β, 17β-diol (B) by purified human placental microsomal 3β-HSD. Incubation was performed as described in Materials and Methods in the presence of 10 μM (△—△), 5 μM (●—●) and 2.5 μM (○—○) of substrate and the indicated quantity of 4-MA.

flattened A or B ring did not bind to the isomerase site and have higher turnover but lower affinity (unpublished results).

Acknowledgements—The authors wish to thank Dr J. Simard and J. Côté for helpful discussions and Mrs Elaine Leclerc and Josée Poulin for assistance in preparation of the manuscript.

REFERENCES

1. Samuels L. T., Helmreich M. L., Lasuter M. B. and Reinch H.: An enzyme in endocrine tissues which oxidizes Δ^{5-3} hydroxyde steroids to α, β unsaturated ketones. *Science* **113** (1951) 490–491.
2. Luu-The V., Lachance Y., Labrie C., Leblanc G., Thomas J. L., Strickler R. C. and Labrie F.: Full length cDNA structure and deduced amino acid sequence of human 3β-hydroxy-5-ene steroid dehydrogenase. *Molec. Endocr.* **3** (1989) 1310–1312.
3. Luu-The V., Côté J. and Labrie F.: Purification and characterization of human placental 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase. *Clin. Invest. Med.* **11** (1988) C-200.
4. Luu-The V., Takahashi M. and Labrie F.: Purification of microsomal and mitochondrial 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase from human placenta. *Ann. N.Y. Acad. Sci.* **595** (1990) 386–388.
5. Zhao H. F., Simard J., Labrie C., Breton N., Rhéaume E., Luu-The V. and Labrie F.: Molecular cloning, cDNA structure and predicted amino acid sequence on bovine 3β-hydroxy-5-ene steroid dehydrogenase/Δ^5-Δ^4 isomerase. *FEBS Lett.* **259** (1990) 153–157.
6. Zhao H. F., Labrie C., Simard J., de Launoit lY., Trudel C., Martel C., Rhéaume E., Dupont E., Luu-The V., Pelletier G. and Labrie F.: Characterization of 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4 isomerase cDNA and differential tissue specific expression of the corresponding mRNAs in steroidogenic and peripheral tissues. *J. Biol. Chem.* **266** (1991) 583–593.
7. Lachance Y., Luu-The V., Labrie C., Simard J., Dumont M., de Launoit Y., Guérin S., Leblanc G. and Labrie F: Characterization of human 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase gene and its expression in mammalian cells. *J. Biol. Chem.* **265** (1990) 20469–20475.
8. Bérubé D., Luu-The V., Lachance Y., Gagné R. and Labrie F.: Assignment of the human 3β-hydroxysteroid dehydrogenase gene (HSB3) to the p13 band of chromosome 1. *Cytogenet. Cell Genet.* **52** (1989) 199–200.
9. Chedrese P. J., Luu-The V., Labrie F., Juorio A. V. and Murphy B. D.: Evidence for the regulation of 3β-hydroxysteroid dehydrogenase messenger RNA by human chorionic gonadotrophin in luteinized porcine granulosa cells. *Endocrinology* **126** (1990) 2228–2230.
10. Chedrese P. J., Zhang D., Luu-The V., Labrie F., Juorio A. V. and Murphy B. D.: Regulation of the mRNA expression of 3β-hydroxy-5-ene steroid dehydrogenase in porcine granulosa cells in culture: a role for the protein kinase C pathway. *Molec. Endocr.* **4** (1990) 1532–1538.
11. Dupont E., Luu-The V., Labrie F. and Pelletier G.: Light microscopic immunocytochemical localization of 3β-hydroxy-5-ene-steroid dehydrogenase/Δ^5-Δ^4 isomerase in the gonads and adrenal glands of the guinea pig. *Endocrinology* **126** (1990) 2906–2909.
12. Dupont E., Zhao H. F., Rhéaume E., Simard J., Luu-The V., Labrie F. and Pelletier G.: Localization of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase in the rat gonads and adrenals by immunocytochemistry and *in situ* hybridization. *Endocrinology* **127** (1990) 1394–1403.
13. Martel C., Labrie C., Dupont E., Couet J., Trudel C., Rhéaume E., Simard J., Luu-The V., Pelletier G. and Labrie F.: Regulation of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase expression and activity in the hypophysectomized rat ovary: interactions between the stimulatory effect of human chorionic gonadotropin and the luteolytic effect of prolactin. *Endocrinology* **127** (1990) 2726–2737.
14. Labrie C., Martel C., Trudel C., Couet J., Dupont E., Takahashi M., Luu-The V., Pelletier G. and Labrie F.: Hormonal regulation of testicular 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3-HSD) expression and activity in intact and hypophysectomized rats. Submitted.
15. Takahashi M., Luu-The V. and Labrie F.: Inhibitory effect of synthetic progestins, 4-MA and cyanoketone on human placental 3β-hydroxysteroid dehydrogenase/Δ^5-ene-4-ene isomerase activity. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 231–236.
16. Takahashi M., Luu-The V. and Labrie F.: Purified microsomal and mitochondrial 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase from human placenta. Characterization of substrate and inhibitor specificity. *Ann. N.Y. Acad. Sci.* **595** (1990) 383–385.
17. Cheatum S. G. and Warren J. C.: Purification and properties of 3β-hydroxysteroid dehydrogenase and Δ^5-3-ketosteroid isomerase from bovine corpora lutea. *Biochim. Biophys. Acta* **122** (1966) 1–13.
18. Ford H. C. and Engel L. L.: Purification and properties of the Δ^5-3β-hydroxysteroid dehydrogenase isomerase system of sheep adrenal cortical microsomes. *J. Biol. Chem.* **249** (1974) 1363–1368.
19. Ishii-Ohba H., Inano H. and Tamaoki B. I.: Purification and properties of testicular 3β-hydroxy-5-ene-steroid dehydrogenase and 5-ene-4-ene isomerase. *J. Steroid Biochem.* **25** (1986) 555–560.
20. Ishii-Ohba H., Saiki N., Inano H. and Tamaoki B. I.: Purification of rat adrenal 3β-hydroxysteroid dehydrogenase with steroid 5-ene-4-ene isomerase. *J. Steroid Bichem.* **24** (1987) 753–760.
21. Talalay P. and Wang V. S.: Enzymic isomerization of Δ^5-3-ketosteroids. *Biochem. Biophys. Acta* **18** (1955) 300–301.
22. Kuliopulos A., Shortle D. and Talalay P.: Isolation and sequencing of the gene encoding 5′-3-ketosteroid isomerase of pseudonomas testosteroni, overexpression of the protein. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 8893–8897.
23. Baker M. E., Luu-The V., Simard J. and Labrie F.: A common ancestor for mammalian 3β-hydroxysteroid dehydrogenase and plant dihydroflavonol reductase. *Biochem. J.* **269** (1990) 558–559.