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Kinetic properties of microsomal 3β hydroxysteroid dehydrogenase-isomerase from the testis of *Bufo arenarum* H

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Abstract

3β-hydroxysteroid dehydrogenase 5-ene isomerase (3βHSD/I) activity is necessary for the biosynthesis of hormonally active steroids. A dual distribution of the enzyme was described in toad testes. The present study demonstrates that in testicular tissue of *Bufo arenarum* H., microsomal 3βHSD/I has more affinity for dehydroepiandrosterone (DHEA) than for pregnenolone ($K_m = 0.17 \pm 0.03$ and 1.02 μM, respectively). The Hill coefficient for the conversion of DHEA and pregnenolone were 1.04 and 1.01, respectively. The inclusion of DHEA in the kinetic analysis of pregnenolone conversion affected V_{max} while K_m was not modified, suggesting a non-competitive inhibition of the conversion of pregnenolone. K_i was calculated from replot of Dixon's slope for each substrate concentration. K_i from the intercept and the slope of this replot were similar (0.276 ± 0.01 and 0.263 ± 0.02 μM) and higher than the K_m for DHEA. The K_m and K_i values suggest the presence of two different binding sites. When pregnenolone was present in the assays with DHEA as substrate, no effect was observed on the V_{max} while K_m values slightly increased with pregnenolone concentration. Consequently, pregnenolone inhibited the transformation of DHEA in a competitive fashion. These studies suggest that, in this species, the microsomal biosyntheses of androgens and progesterone are catalysed by different active sites. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: 3HSD/I; Non-competitive inhibition; Toad–testis; Steroid biosynthesis

1. Introduction

The key enzyme in the pathway of steroid biosynthesis is the membrane-bound 3β-hydroxysteroid dehydrogenase-5-ene-isomerase (3βHSD/I). This enzyme catalyses the conversion of 5-ene-3β-hydroxysteroids into the corresponding 4-ene ketosteroids. This reaction is essential for the biosynthesis of steroid hormones, including the adrenal steroid hormones cortisol, corticosterone and aldosterone, as well as the gonadal steroids hormones progesterone, testosterone and estradiol [1–5]. 3βHSD/I is expressed at high levels in classical steroidogenic tissues such as placenta, ovary, adrenal cortex, testis and also in several peripheral tissues including kidney [6] and liver [7].

Molecular cloning studies have revealed that four types of 3HSD/I may be expressed in the rat (types I–IV), two in the human (types I and II), but only one type in the bovine species [8,9].

In human placenta, the enzyme has been found in smooth endoplasmic reticulum (SER) and mitochondria, where it catalyses the production of progesterone and androstenedione [1]. In rat testis it is accepted that 3βHSD/I activity is localized to the SER [10–12], but an additional localisation of the enzyme in mitochondria has been described [13]. Such a localisation has also been described in toad inter-renals (whose function is similar to the mammalian adrenal) and testis [14,15].

Kinetic studies on the placental microsomal 3βHSD/I have shown that K_m for pregnenolone is greater than

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that of dehydroepiandrosterone. These studies also showed that each steroid inhibited the metabolism of the other competitively and K_i obtained were not significantly different from their respective K_m values [16–18].

Testosterone is a 3-oxo-4-ene steroid synthesised from cholesterol in a series of reactions catalysed by specific enzymes located in the mitochondrial and microsomal membranes of the Leydig cell [19]. Among these, 3βHSD/I is a key enzyme in the biosynthetic pathway to testosterone, catalysing the conversions of pregnenolone, 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA) to the biologically active 3-oxo-4-ene steroids progesterone, 17α-hydroxyprogesterone and androstenedione, the immediate precursor of testosterone.

It has been demonstrated that amphibians possess Leydig cells with the same properties as mammals. In effect, these cells exhibit 3βHSD/I activity and synthesise testosterone as in the testis of most mammalian species [15,20,21]. 3βHSD/I activity is markedly increased by the administration of gonadotropin [22–24] and in *Xenopus laevis* and *Rana pipiens* seems to be localised in SER [23], while in *Bufo arenarum* it localises in SER as well as mitochondria [15]. In toad testis, biosynthetic results suggest the predominance of a 5-ene pathway for the biosynthesis of androgens and a 4-ene pathway for 5α-pregnan-3,20-dione biosynthesis [25].

In this paper, we describe the kinetic properties of microsomal 3βHSD/I activity in the testis of *B. arenarum* and demonstrate that DHEA inhibits in a non-competitive fashion the conversion of pregnenolone to progesterone.

2. Materials and methods

2.1. Materials

Pregnenolone, progesterone, dehydroepiandrosterone (DHEA) and androstenedione were from Sigma (St. Louis, MO) and [7(*n*)-^{3}H]pregnenolone (19 Ci/mmol), [4-^{14}C]progesterone (48.9 mCi/mmol), [1,2-^{3}H]DHEA (54 Ci/mmol) and [4-^{14}C]androstenedione (53.9 mCi/mmol) were from NEN (Boston, MA). Silica gel plates 60GF 254 on aluminium were purchased from Merck (Darmstadt, Germany). All other chemicals were of reagent grade.

2.2. Tissue preparations

Testicular tissue was obtained from male *B. arenarum* collected during the breeding season (130–140 g). Toads were maintained at 25°C for 1 week prior to use. Toads were killed with ether in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction. Testes were quickly removed and then carefully separated from Bidder's organ, excised and homogenised in 10 mM Tris–HCl buffer, pH 7.4, containing 20% glycerol, 0.4 mM 2-mercaptoethanol and 0.1 mM EDTA (T buffer) (250 mg of tissue/ml). Microsomal fraction was separated from the homogenate by differential centrifugation [14]. Briefly, after sedimentation of the nuclear fraction at 800 g for 10 min, mitochondria were sedimented from the supernatant by centrifugation at 15 000 × *g* for 20 min. For the separation of the microsomal fraction, the 15 000 × *g* supernatants were centrifuged at 105 000 × *g* for 90 min. All steps were carried out at 4°C. All fractions were used immediately for enzyme assays. Protein was estimated by the method of Lowry et al. using bovine serum albumin as standard [26].

2.3. Enzyme assay

3βHSD/I activity was assayed in microsomal preparations containing 50 μg protein in T buffer (pH 7.4, at 28°C), with 0.5 mM NAD^+ as a cofactor. The final volume was 1 ml. The reaction was started by the addition of 25 μM [^{3}H]pregnenolone or 10 μM [^{3}H]DHEA and samples were incubated for 20 min [15]. These conditions were established in preliminary experiments as the linear zones of time and enzyme curves. The reaction was stopped with 3 ml of cold dichloromethane and media were extracted twice with the same solvent.

2.4. Chromatographic systems

Substrates and products were separated by TLC. The solvent systems used were: benzene:ethanol (95:5, v/v) for pregnenolone and progesterone; chloroform:acetone (95:5, v/v) for DHEA and androstenedione. The amounts of [^{3}H]progesterone and [^{3}H]androstenedione produced were quantified after the last chromatographic step. The losses were estimated by recovering of ^{14}C tracer [27,28]. After the appropriate correction for recovery, the mass of the products was calculated from the known specific activities of the substrate. The specific activities of the enzyme were expressed as nmole of product per minute per miligram of protein. Positions of radioactive steroids on TLC were determined by fluorography with EN^{3}HANCE spray (NEN, Boston, MA) as a fluorographic intensifier and Kodak films X-OMAT LS (Sigma). No other products different from progesterone or androstenedione were detected after the exposure of TLC to autoradiography. Standard 3-oxo-4-ene steroids as progesterone, androstenedione and testosterone were detected by UV absorption. Spraying with primuline [29] revealed non-

absorbing standard steroids as pregnenolone, DHEA and 5α-pregnan-3,20-one.

2.5. Kinetic studies

The 3βHSD/I activity was determined by measuring the microsomal conversion of [^{3}H]pregnenolone to [^{3}H]progesterone and [^{3}H]DHEA to [^{3}H]androstenedione. Incubations were carried out for 10 min at 28°C. Protein concentration was adjusted so that the rate of progesterone and androstenedione formation was linear for up to 10 min. Kinetic parameters — K_m and V_{max} — were measured with various concentrations of both substrates, 0.05–3 μM for DHEA and 0.2–3 μM for pregnenolone. At the end of the incubations, steroids were extracted, separated and quantified as described above. K_m and V_{max} were estimated from Lineweaver–Burk and Wilkinson linearisations. Hill-plots were performed using V_{max} and initial velocities values. Hill coefficients were calculated from the slope of Hill-plots.

2.6. Inhibition studies

Inhibition kinetics of the enzyme was determined using the 3βHSD/I assay with five non-saturating concentrations of pregnenolone or DHEA and five non-saturating concentrations of the substrates. Dixon analysis allowed the determination of both the type of inhibition and the inhibition constant [30]. The effects of the inhibitor on the kinetics parameters were confirmed using Direct Linear Plot [31] and a non-linear least squares curve-fitting program [32].

2.7. Radioactivity

Scintillation counting was carried out with a Mark III, Model 6882, equipment (Tracor Analytic, Chicago, IL) in which quenching is corrected individually for each sample through automated optimal energy-window opening. The equipment automatically rejected samples with chemiluminiscence. The scintillation cocktail contained 4 g 2,5-diphenyloxazole (PPO) and 0.25 g dimethyl-1,4-bis [2-(5-phenyloxazole)] benzene (POPOP) per litre toluene. The chemiluminescent effect was eliminated keeping the samples in the dark at 4°C during 24 h.

2.8. Statistical analysis

Experiments were expressed as means ± S.E.M. Comparison of means were performed using Student's *t*-test. The kinetic parameters from Direct Linear Plot were compared using the Kruskal–Wallis non-parametric ANOVA test.

3. Results

3.1. Kinetic studies

Apparent K_m and V_{max} values of the microsomal enzyme were compared using pregnenolone and DHEA as substrates and calculated using Lineweaver–Burk and Wilkinson linearisations. As shown in Table 1, DHEA was the preferred substrate for microsomal 3βHSD/I. Fig. 1A and Fig. 2A show the kinetics of saturation for both substrates. The Hill coefficient (determined from the slopes of Hill-plots) for the conversion of DHEA and pregnenolone were 1.04 ($r^2 = 0.918$) and 1.01 ($r^2 = 0.944$), respectively, indicating the possibility of two enzymes catalysing each substrate conversion or one enzyme with two non-interacting sites (Fig. 1B and Fig. 2B).

3.2. Effects of 5-ene-3β-hydroxysteroids on the conversion of DHEA to androstenedione and pregnenolone to progesterone

Increasing concentrations of pregnenolone did not decrease the rate of conversion of DHEA (10 μM) into androstenedione, whereas the conversion of pregnenolone (25 μM) to progesterone was reduced to 50% of the control value when 1 μM of DHEA was present (Fig. 3). Under these conditions, the total androstenedione produced from DHEA was 0.064 μM (data not shown). Indeed, when the effect of increasing amounts of progesterone and androstenedione on the transformation of pregnenolone to progesterone was analysed, it could be seen that 0.1 μM androstenedione produced no significant inhibition of progesterone formation (data not shown). As a consequence, it is possible to conclude that the inhibitory effect of DHEA did not depend on androstenedione biosynthesis. These studies

Table 1
K_m and V_{max} of microsomal 3βHSD/I activity for pregnenolone and DHEA[a]

Substrate	Linearisation	K_m (μM)	V_{max} (nmole/min/mg)
Pregnenolone	Lineweaver–Burk	1.00 ± 0.20**	0.39 ± 0.04
	Wilkinson	1.02 ± 0.02	0.40 ± 0.02
DHEA	Lineweaver–Burk	0.17 ± 0.03**	0.40 ± 0.09
	Wilkinson	0.22 ± 0.06	0.37 ± 0.05

[a] K_m and V_{max} were measured with various concentrations of either pregnenolone (0.2–3 μM) or DHEA (0.05–3 μM) with microsomes in the presence of 0.5 mM NAD^+ in T buffer. K_m and V_{max} were calculated by Lineweaver–Burk and Wilkinson linearisations. Values represent means of eight experiments ± S.D.

** Differences were significant between substrates with $P < 0.01$.

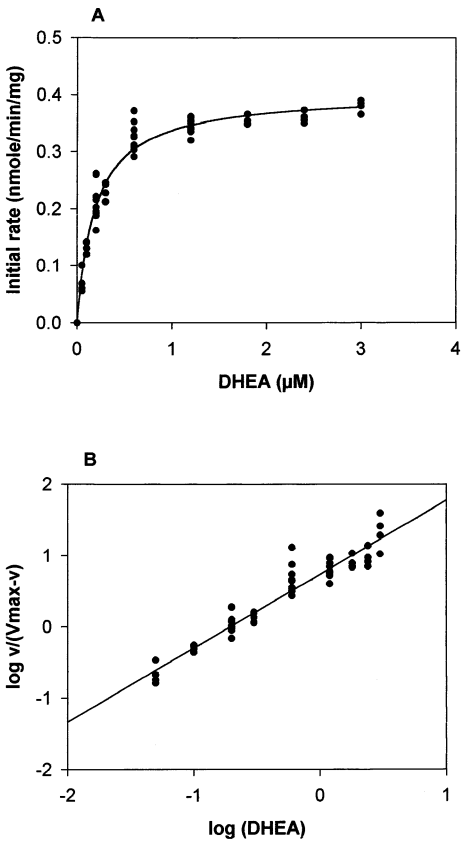

Fig. 1. (A) V versus S curve for DHEA transformation. Experiments were carried out with nine nonsaturating concentrations of the substrate (0.05–3 μM). Incubations were performed using 20 μg proteins in T buffer (pH 7.4, at 28°C) with 0.5 mM NAD^+, in a final volume of 1 ml during 10 min. $V_{max} = 0.4$ nmole/min/mg; $K_m = 0.19$ μM. (B) Hill plot, n Hill = 1.04.

and the values of Hill coefficient apparently showed that the two substrates were metabolised by two enzymes and that the substrate of one enzyme may influence the activity of the other enzyme. This hypothesis led us to determine the relationship between these 3βHSD/I activities and hence, to study the effects of the substrate of one enzyme on the utilisation of the other steroid substrate in non-saturating conditions.

3.3. Effect of pregnenolone on microsomal conversion of DHEA to androstenedione

When pregnenolone (0.1–3 μM) was present in the assays of 3βHSD/I with DHEA as substrate (0.05–3.0 μM), no effect was observed on the V_{max} while K_m values slightly increased with pregnenolone concentration (Fig. 4). Consequently, pregnenolone inhibited the conversion of DHEA to androstenedione in a competitive fashion. Under these conditions, K_i and K_m for pregnenolone transformation were different (4.27 and 1.0 μM, respectively). The inhibition kinetic experiments were conducted in triplicate.

3.4. Effect of DHEA on microsomal conversion of pregnenolone to progesterone

The inclusion of 0.1, 0.5, 1 or 3 μM DHEA in the kinetic analysis of pregnenolone conversion greatly affected progesterone production; the biggest effect could be seen on V_{max}, while K_m was not affected (Table 2). Under these conditions, the higher concentration of DHEA reduced the V_{max} to 10% of control values (Table 2). Thus, DHEA would cause an inhibition of

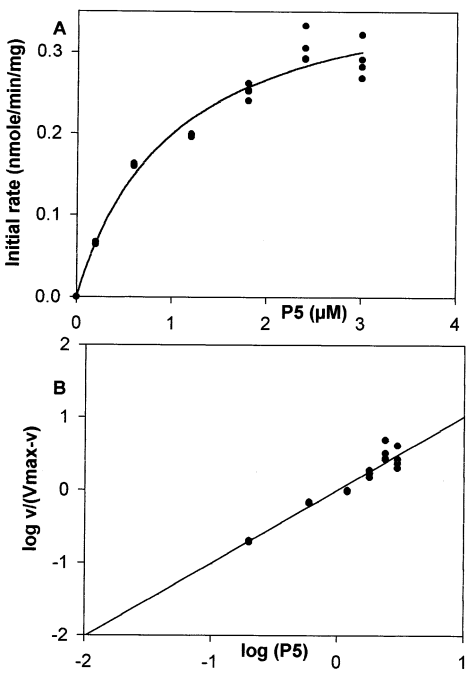

Fig. 2. (A) V versus S curve for pregnenenolone transformation. Experiments were carried out with six nonsaturating concentrations of the substrate (0.2–3 μM). Incubations were done as in Fig. 1. $V_{max} = 0.4$ nmole/min/mg; $K_m = 1.03$ μM. (B) Hill plot, n Hill = 1.01.

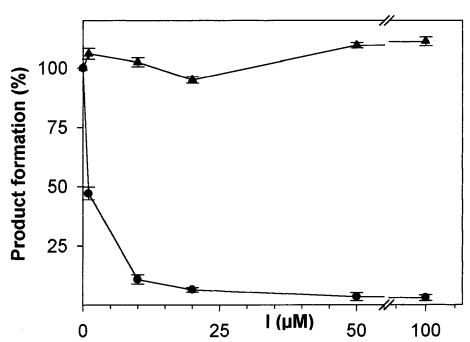

Fig. 3. Effect of increasing amount of pregnenolone on the conversion of [^{3}H] DHEA to androstenedione (A4) (▲) and effect of increasing amount of DHEA on the conversion of [^{3}H] pregnenolone to progesterone (P4) (●). Substrate concentrations were saturating, 10 μM for DHEA and 25 μM for pregnenolone. Incubations were performed using 50 μg proteins in T buffer (pH 7.4, at 28°C) with 0.5 mM NAD^+, in a final volume of 1 ml. I: inhibitor concentration, DHEA.

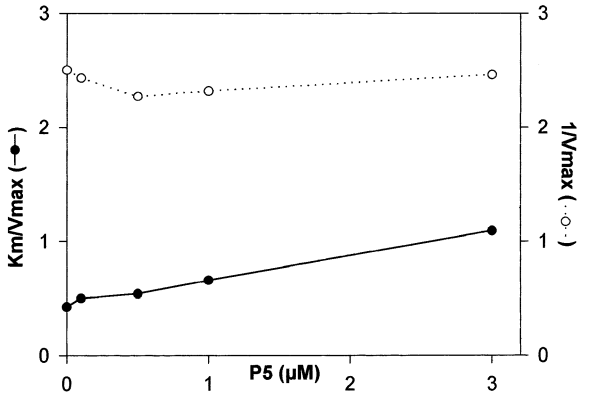

Fig. 4. K_m/V_{max} and $1/V_{max}$ ratios for each pregnenolone (P5) concentrations (0.1, 0.5, 1 and 3 μM). Substrate (DHEA) concentrations used were between 0.05 and 3 μM. Different K_m and V_{max} values were calculated for each P5 concentrations.

the conversion of pregnenolone to progesterone, but this inhibition would not be due to a competition for the binding to the substrate site since K_m was not affected (Table 2). Experiments were carried out in triplicate.

3.5. Dixon analysis

To determine whether DHEA and pregnenolone bind to the same site or a different active site, K_i values were determined by Dixon analyses. Fig. 5 shows linear Dixon plots for six substrate concentrations. Therefore, the slope and intercept replots for each inhibitor concentration were performed in order to validate the two-site model. Fig. 6A, which also exhibits linear behaviour, shows that V_{max} from Dixon intercept was similar to V_{max} from Lineweaver–Burk plot (0.38 and 0.40, respectively). K_i was calculated from replot of Dixon's slope for each substrate concentration (Fig. 6B). K_i from the intercept and the slope of this replot were similar (0.276 ± 0.01 and 0.263 ± 0.02 μM) and higher than the K_m for DHEA (0.17 ± 0.03 μM). The K_m and K_i values suggest the presence of two different binding sites.

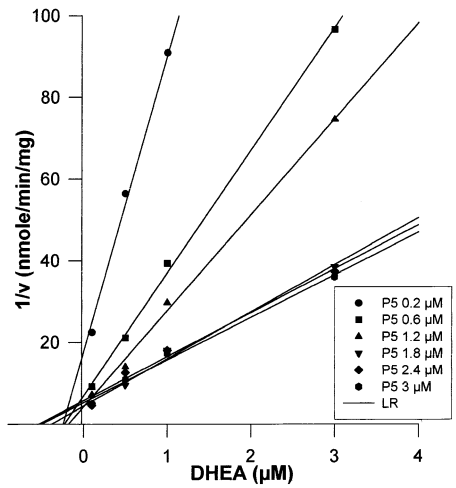

Fig. 5. Dixon plot of inhibition by DHEA for different substrate (pregnenolone, P5) concentration. Velocities (V) were calculated as nmole progesterone produced per minute and per miligram microsomal protein. Correlation coefficients for each substrate concentration were: 0.2 μM ($r^2 = 0.986$); 0.6 μM ($r^2 = 0.999$); 1.2 μM ($r^2 = 0.997$); 1.8 μM ($r^2 = 0.991$); 2.4 μM ($r^2 = 0.998$); 3 μM ($r^2 = 0.996$).

3.6. Direct linear plot and non-linear least squares fitting

Although in the toad testis DHEA showed a non-competitive inhibition on the conversion of pregnenolone to progesterone, this is not the case in other species. As a consequence, it became necessary to analyse the data by a mathematical approach other than Dixon analysis. We chose direct linear plot and non-linear fitting to reduce data transformation.

Direct linear plotting evidenced an extremely significant effect on V_{max} ($P \leq 0.0001$) while the effect on K_m was considered not significant ($P = 0.389$). Table 2 shows the K_m and V_{max} median values for each inhibitor concentration. In order to test for significant differences, the individual intersection values for each treatment were considered and compared using the Kruskal–Wallis test. A non-linear curve-fitting program (Enzyme, NIH, Lutz et al., 1986) corroborated

Table 2
K_m and V_{max} for microsomal conversion of pregnenolone to progesterone in the presence of increasing amounts of DHEA[a]

	Lineweaver–Burk		Wilkinson		Direct linear plot*	
DHEA (μM)	K_m (μM)	V_{max} (nmole/min/mg)	K_m (μM)	V_{max} (nmole/min/mg)	K_m (μM)	V_{max} (nmole/min/mg)
0	1.00 ± 0.02	0.39 ± 0.04	1.02 ± 0.02	0.40 ± 0.02	0.99	0.39
0.1	1.14 ± 0.09	0.31 ± 0.01	0.98 ± 0.13	0.29 ± 0.02	1.03	0.27
0.5	1.24 ± 0.48	0.14 ± 0.03	0.96 ± 0.08	0.12 ± 0.02	1.22	0.13
1	1.28 ± 0.12	0.09 ± 0.01	1.18 ± 0.27	0.08 ± 0.01	1.31	0.08
3	1.18 ± 0.16	0.03 ± 0.01	1.03 ± 0.09	0.04 ± 0.02	1.94	0.05

[a] K_m and V_{max} for pregnenolone were calculated in the presence of increasing amounts of DHEA. Substrate concentrations were between 0.2 and 3.0 μM. K_m and V_{max} were calculated by Lineweaver–Burk and Wilkinson linearisations. Values represent means of three experiments ± S.D.
* Median values from Direct Linear Plot.

the same inhibitory effect showing a significant effect on V_{max} only and, thus, consistent with a classic pure non-competitive model ($K_i = 0.245 \pm 0.015$ μM). This K_i value is similar to that calculated by Dixon plot.

4. Discussion

Earlier studies [14,15], provided evidence for a dual localisation of 3βHSD/I in the interrenal and testes of *B. arenarum*. The purpose of this work was to examine the kinetic properties of the microsomal 3βHSD/I in testis of *B. arenarum* H. As in mammals the microsomal enzyme utilises pregnene and androstene steroids as alternative substrates. However, the enzyme has a greater affinity for DHEA than for pregnenolone. These results suggest a limited formation of progesterone in favour of androstenedione production for androgen biosynthesis. This could be due to the inhibition exerted on the conversion of pregnenolone to progesterone by DHEA. A 1 μM concentration of DHEA resulted in a 50% inhibition of progesterone biosynthesis. The inhibition was not due to the formation of androstenedione during the experiments. This was shown by the fact that 1 μM androstenedione produced no inhibition of progesterone biosynthesis.

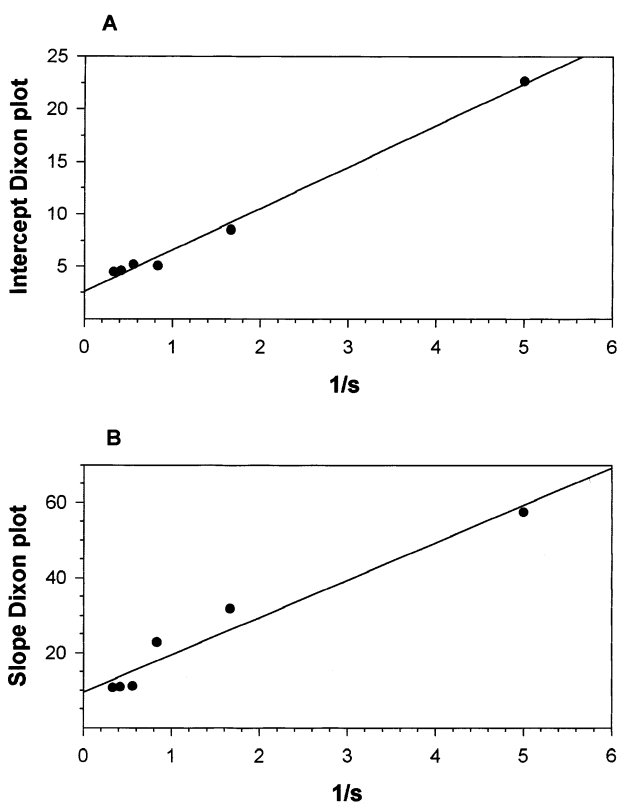

Fig. 6. (A) Dixon intercept for each inhibitor concentration. Intercept corresponds to V_{max}. Correlation coefficient: $r^2 = 0.993$. (B) Dixon slope for each inhibitor concentration. Correlation coefficient: $r^2 = 0.949$.

Our present findings demonstrated that DHEA was a non-competitive inhibitor of pregnenolone transformation ($K_i = 0.245 \pm 0.015$ μM). However, pregnenolone is a competitive inhibitor, albeit poor, for the biosynthesis of androstenedione ($K_i = 4.27 \pm 0.09$ μM). Furthermore, microsomal 3βHSD/I of *B. arenarum* has a higher affinity for DHEA than for pregnenolone and DHEA inhibits pregnenolone transformation with a K_i slightly higher than the K_m for DHEA ($K_i = 0.245 \pm 0.015$ μM; $K_m = 0.17 \pm 0.03$ μM). On the contrary pregnenolone inhibits androstenedione biosynthesis with a higher K_i than the K_m for pregnenolone as well as the K_m for DHEA ($K_i = 4.27 \pm 0.09$ μM; $K_m = 1 \pm 0.02$ μM). Furthermore, Hill coefficient analysis suggested that the two proposed sites are located in two different enzymes or that they are non-interacting. The results presented in this work are different from those obtained in microsomal placental 3βHSD/I. In those studies, DHEA inhibited progesterone biosynthesis in a competitive manner. However, in another steroidogenic enzyme such as 7α/β-hydroxylase, DHEA was a non-competitive inhibitor of 7α/β-hydroxylation of pregnenolone. The authors suggested more than one enzyme [33].

In conclusion, these studies show that the 3βHSD/I reactions in the pathway to androgens and progesterone-derived C_{21} steroids are catalysed by different active sites: one for pregnenolone and the other for DHEA.

This conclusion admits several possibilities: it is indeed uncertain whether these differences are due to two isoenzymes corresponding to two genes, two enzyme sites on the same protein, the same gene with different post-translational events or the same enzyme in different lipid environments. In pig testis, the latter possibility for the 3βHSD/I was suggested [34,35]. This author found that phosphatidylserine inhibits the conversion of 5,16-androstandien-3β-ol to 4,16-androstandien-3-one but not the conversion of DHEA to androstenedione. This suggested separate 3βHSD/I for DHEA and andien-β. This possibility was also postulated for another steroidogenic enzyme, 17α-hydroxylase-C17,20-lyase [36]. This enzyme catalyses the 17α-hydroxylation of pregnenolone and progesterone. The same enzyme expresses lyase activity for the C17–20 bond at the 21-carbon steroids yielding 19-carbon steroids. A single or dual activity expression depends, in this case, on the lipid composition of the environment [36].

It is possible to speculate that the microsomal localisation permits androgen biosynthesis to occur through a pathway preferred to the formation of progesterone. This is due to a higher affinity of 3βHSD/I for DHEA than for pregnenolone and to the inhibition exerted by DHEA on progesterone biosynthesis. This inhibition is stronger than the inhibition exerted by pregnenolone on the biosynthesis of androstenedione (K_i for preg-

nenolone is higher than K_m for DHEA). For progesterone biosynthesis in the microsomal fraction, it could be necessary to inhibit DHEA formation. The 4-ene pathway that could be localised in mitochondria would be important for the biosynthesis of progesterone metabolites like 5α-pregnane-3,20-dione and 17α,20α-dihydroxy-4-pregnen-3-one [15,25].

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