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PARTIAL CHARACTERIZATION OF PLACENTAL 3β-HYDROXYSTEROID DEHYDROGENASE (EC 1.1.1.145), Δ^{4-5}ISOMERASE (EC 5.3.3.1) IN HUMAN TERM PLACENTAL MITOCHONDRIA

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SUMMARY

A partial characterization of human term placental 3β-HSDH in mitochondria is reported. Apparent K_M of pregnenolone: 70 nM. A dose-dependent stimulation of 3β-HSDH by NAD^+ or $NADP^+$ was observed in the range from 10^{-6} to 10^{-3} M (K_M value of NAD^+: 20 μM). At equimolar concentrations NAD^+ is more than 10-fold as effective a cofactor of the 3β-HSDH than $NADP^+$. pH optimum: 9.5 (glycine–NaOH buffer). Temperature optimum 40–45°C. A rapid loss of 3β-HSDH activity was found after preincubation of the enzyme at 37°C after 30 min; less than 50% of initial enzyme activity is present. No inhibition was obtained by Mg^{2+}, Ca^{2+} Sr^{2+} and Ba^{2+} (1–100 mM). A strong inhibition was achieved with 1 mM Zn^{2+}, Cd^{2+}, Cu^{2+} and 10 mM and 100 mM Fe^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}.

INTRODUCTION

Cholesterol taken up from the maternal circulation is the precursor for human placental progesterone synthesis [1]. Two enzyme systems are involved in the enzymatic conversion of cholesterol to progesterone. The cholesterol side chain cleavage enzyme is necessary for splitting of a C_6 fragment from the side chain of the cholesterol molecule and thus also for formation of pregnenolone. This enzymatic reaction is thought to be the slowest and therefore rate-limiting step by which the steroidogenesis of pregnenolone and furthermore progesterone can be controlled. The enzyme system which converts pregnenolone to progesterone itself consists of two different enzymes: 3β-hydroxysteroid: NAD (P) oxidoreductase (EC 1.1.1.145) and the 3-ketosteroid Δ^{4-5}-isomerase (EC 5.3.3.1.). The conventional abbreviation for the latter enzyme system is 3β-HSDH, which comprises not only the dehydrogenase but also the isomerase step. Dehydrogenation is the initial and rate-limiting step of this reaction [2, 3]. In the bacterium *Pseudomonas testosteroni* both enzymes can be isolated separately [4–6]. In tissue of mammals, this enzyme is associated with subcellular fractions [7–9]; it is assumed that the enzyme is localized in different parts of the same protein.

In the human placenta, the 3β-HSDH is present both in the mitochondria and the microsomes [10]. A partial characterization of the microsomal 3β-HSDH was given by Ferre *et al.*[10], Gibb[11], and of the mitochondrial 3β-HSDH by Wiener and Allen[12], Ferre *et al.*[10], and Edwards *et al.*[13]. Different results were obtained concerning the K_M value of the substrates pregnenolone and dehydroepiandrosterone and other enzyme characteristics. In the present study, various enzyme characteristics were reinvestigated and several new features of human term placental 3β-HSDH in mitochondria are reported. Preliminary results have already been published by our group [14–15].

MATERIALS AND METHODS

Chemicals

[4-^{14}C]-Pregnenolone (55.4 mCi/mmol) was purchased from the Radiochemical Center, Amersham, Buckinghamshire (England) and rechromatographed prior to use by thin layer chromatography (t.l.c.) (acetone-ethylacetate, 8:2, v/v) and on a Sephadex LH 20 column (*n*-heptane–benzene–methanol, 85:10:5, v/v). NAD^+ and $NADP^+$ were obtained from Boehringer Mannheim (West Germany). All other reagents were of highest purity available. Solvents were freshly redistilled prior to use.

Mitochondria isolation

Human placental mitochondria were isolated by means of a common ultracentrifugation technique as described by Hochberg *et al.*[16]. The purity of the isolated mitochondria fraction was checked by electron microscopy. The protein concentration of the mitochondrial stock solution was 34 mg/ml [17]. The

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stock solution was stored at −20°C and the enzyme was stable for up to at least 6 months.

Incubation procedure

Incubations were carried out at 37°C under air using 10 ml Tris-HCl buffer, 30 mM, pH 7.4, 250 mM sucrose, 6.25 mM $MgCl_2$, 1 mM $CaCl_2$. For routine tests, 1 mM NAD^+ was used as cofactor. As precursor, 0.53 μM [4-^{14}C]-pregnenolone (approx. 6×10^5 d.p.m.) was added to each test. The mitochondrial stock solution was diluted 1:60 and 50–100 μg mitochondrial protein was added to each incubation. Incubations were performed at 37°C, open in a shaking water bath (Köttermann). When not separately indicated, aliquots of the incubation medium were withdrawn after 8–10 min of incubation for steroid separation.

Steroid separation

Radioactive pregnenolone and progesterone were extracted with diethylether. 5 μg unlabeled carrier of each steroid was added and the mixture was applied on silica gel t.l.c. plates (20 × 20 cm, thickness 0.25 mm) (Merck, Darmstadt, West Germany). t.l.c. plates were developed using a solvent system of acetone and ethylacetate (8:2, v/v) (developing time: 60 min). After development, steroids were visualized by ultraviolet fluorescence (256 nm) or by iodine staining. Localized steroids were scraped off the thin layer plates and transferred into scintillation vials. The recovery rate of radioactive steroids was about 90–95%.

Scintillation measurements

For scintillation counting, 10 ml of the scintillation solution consisting of 5 g 2,5-diphenyloxazole (PPO), 80 g naphthalene, 330 ml xylene and 666 ml dioxane per liter were added to each vial [18]. Scintillation measurements were performed in a scintillation counter from Nuclear Chicago, Mark II (Chicago, Frankfurt, West Germany) operating at 84% efficiency for carbon-14 and 60% efficiency for tritium.

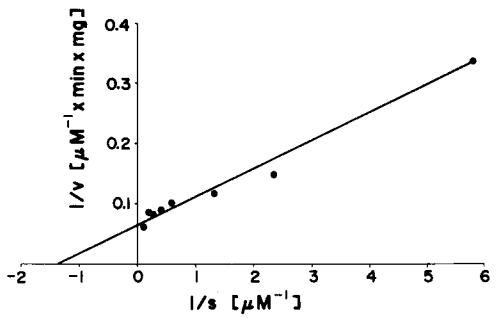

Fig. 2. Lineweaver–Burk plot for the determination of the K_M value for pregnenolone (apparent K_M: 70×10^{-9} M) in tests with mitochondrial 3β-HSDH isolated from human term placenta.

Scintillation counting was performed using a preset time of 10 min and a preset count of 100,000 c.p.m.

RESULTS

Enzyme kinetics

Enzyme kinetics were studied in relation to both time and protein concentration (Fig. 1). A first-order enzyme kinetics (initial velocity) can be achieved for an incubation time up to 10 min using a protein concentration of the enzyme solution less than 5 μg/ml. A linear correlation of 3β-HSDH activity and protein concentration of the enzyme solution could be demonstrated up to a protein concentration of 10 μg/ml (Fig. 1).

All enzymatic tests described below were performed using 50–100 μg of 3β-HSDH protein per test and an incubation period of 10 min.

Substrates

The K_M value for pregnenolone was determined by using substrate concentrations from 0.05 to 1 μM. By means of Lineweaver–Burk plot for pregnenolone ranging from $10^{-1} - 10 \times K_M$, the apparent K_M of pregnenolone was calculated to be 7.0×10^{-8} M (Fig. 2).

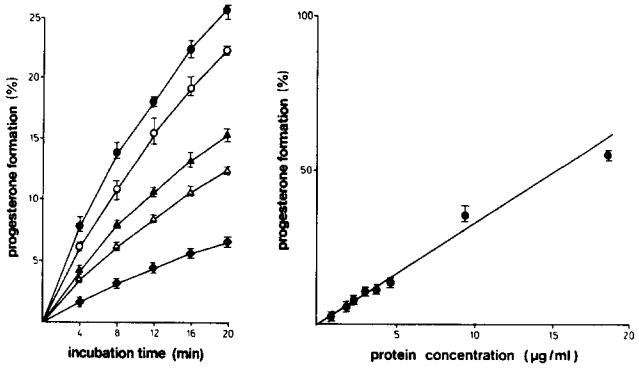

Fig. 1. Left panel: Time-related linearity of the enzyme kinetic of the human placental 3β-HSDH in mitochondria using different enzyme protein concentrations. 0.9 μg/ml (◆), 1.8 μg/ml (△), 2.3 μg/ml (▲), 3.1 μg/ml (○) and 4.7 μg/ml (●). $x \pm$ range ($n = 3$). Right panel: 3β-HSDH activity in relation to enzyme protein concentration. (incubation time of 10 min) $\bar{x} \pm$ range ($n = 3$).

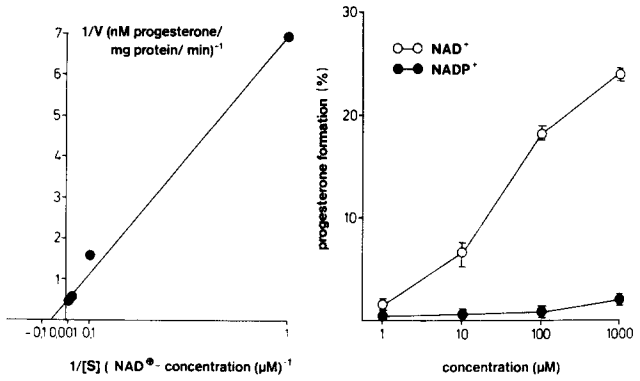

Fig. 3. Left panel: Lineweaver–Burk plot for the determination of the K_M value of NAD^+ (apparent K_M: 20×10^{-6} M). Right panel: Activity of the placental mitochondrial 3β-HSDH in relation to the concentration of the cofactors NAD^+ and $NADP^+$. $\bar{x} \pm$ range ($n = 3$).

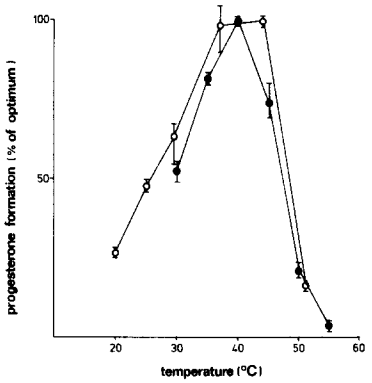

Fig. 4. Temperature optimum for the human placental 3β-HSDH. Results of two separate test series are plotted together. $\bar{x} \pm$ range ($n = 3$).

Cofactors

NAD^+ and $NADP^+$ are necessary as cofactors for the 3β-HSDH enzyme. A dose-dependent increase of the enzyme activity was found using the respective cofactor in a concentration ranging from 10^{-6} to 10^{-3} M. At equimolar concentrations, NAD^+ was more than 10-fold as effective as $NADP^+$. At high concentrations of NAD^+ (up to 10^{-2} M), no further increase of 3β-HSDH activity could be observed. The apparent K_M value for NAD^+ was determined to be 20×10^{-6} M.

Temperature optimum

The influence of the temperature on the activity of the 3β-HSDH was investigated in two separate test series at different temperatures between 20 and 55°C (Fig. 4). There is a maximum 3β-HSDH activity between 40 and 45°C which will be referred to as temperature optimum (100% enzyme activity) as shown in Fig. 5. A gradual enhancement of the 3β-HSDH activity (25–100%) was found when the temperature was increased from 20–40°C. When incubations were carried out a higher than optimal temperatures (40–55°C), the activity of the 3β-HSDH rapidly decreased until no enzymatic conversion could be measured.

pH optimum

The pH dependence of the 3β-HSDH was analyzed using two different buffer systems (Tris–HCl buffer: pH 6.5–9.0 and glycine–NaOH buffer: pH 9.0–11.0) in a 0.1 M concentration (Fig. 5). The pH optimum of the 3β-HSDH was found to be at pH 9.5.

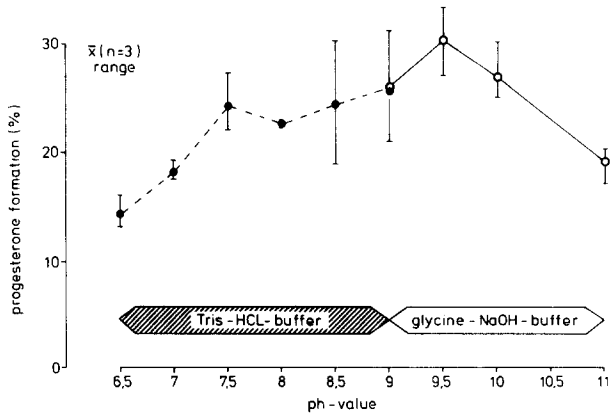

Fig. 5. Influence of pH value and buffer system (0.1 M) on the activity of the human placental 3β-HSDH. $\bar{x} \pm$ range ($n = 3$).

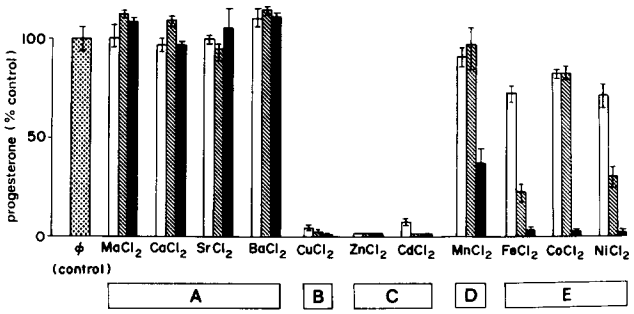

Fig. 6. Influence of metal ions (□ 1 mM, ▨ 10 mM, ■ 100 mM) on the activity of the human placental 3β-HSDH. Metal ions belong to the following chemical groups. A: 2nd main group, B: 1st subgroup, C: 2nd subgroup, D: 7th subgroup, E: 8th subgroup. $\bar{x} \pm$ range ($n = 3$).

Metal ions

The effect of various bivalent metal ions on the activity of the 3β-HSDH was tested in 1, 10 and 100 mM concentrations using a Tris-HCl buffer without $MgCl_2$ and $CaCl_2$ (Fig. 6). The pH value of each metal salt solution was readjusted to pH 7.4 prior to incubation studies. No metal ion tested stimulated the human placental 3β-HSDH enzyme. Slight changes in 3β-HSDH activity (80–120% compared to controls) seem to depend on methodological errors. No effect could be detected by 1 mM, 10 mM or 100 mM Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}. A weak inhibition (50–80% 3β-HSDH activity) was found with 1 mM Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+}. At higher concentrations (10 mM), Mn^{2+} and Co^{2+} showed still a weak inhibition whereas Fe^{2+} and Ni^{2+} inhibited the enzyme moderately (10–50%). At a 100 mM concentration, a strong inhibition (residual enzyme activity below 10%) was obtained with Fe^{2+}, Co^{2+} and Ni^{2+}, whereas Mn^{2+} showed only a moderate inhibition.

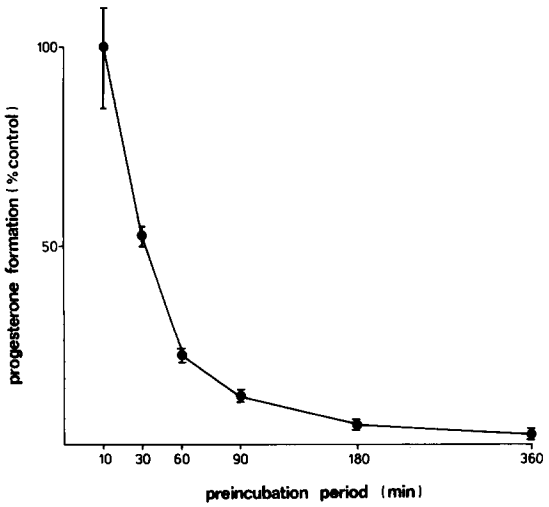

Fig. 7. Activity of the human placental 3β-HSDH in dependence of the preincubation period of the enzyme at 37°C. The progesterone formation after different preincubation times of the 3β-HSDH was related to the initial value taken as 100%. $\bar{x} \pm$ range ($n = 3$).

Even at a 1 mM concentration Cu^{2+}, Zn^{2+} and Cd^{2+} showed a strong and at higher concentrations an almost complete inhibition of the 3β-HSDH. These metal ions were found to possess the highest inhibitory potency in tests with 3β-HSDH.

3β-HSDH stability

The stability of enzyme activity was tested after preincubation of the enzyme at 37°C without cofactors and radioactive pregnenolone for different time intervals. After addition of [4-^{14}C]-pregnenolone and cofactors, the enzyme activity was determined. The 3β-HSDH activity in dependence of the preincubation interval is shown in Fig. 7. A rapid loss of enzyme activity was noted within the first 90 min of preincubation; a 50% loss of 3β-HSDH activity was already reached after 30 min. The loss of enzyme activity during preincubation is not due to an inhibition of the 3β-HSDH by pregnenolone, progesterone or progesterone metabolites formed from endogenous cholesterol, as could be shown by the following test. The 4-fold amount of 3β-HSDH enzyme protein as used for 3β-HSDH tests was preincubated in the same manner as in tests presented in Fig. 7 but without radioactive pregnenolone. After corresponding time intervals, the preincubation was stopped by extraction with diethylether. The diethylether extracts containing the endogenous steroids present in the enzyme preparation were added separately to 3β-HSDH tests. Up to 90 min of preincubation, no change in 3β-HSDH activity could be observed.

DISCUSSION

In the human placenta, the 3β-HSDH is located both in the mitochondria and in the microsomes. A partial characterization of this enzyme has been reported by various investigators [10–13, 19–20]. The enzymatic activity is equally distributed between mitochondrial and microsomal fractions [10].

In this paper, several physicochemical characteristics of the human placental 3β-HSDH (e.g. substrate saturation, cofactors, temperature stability of the

enzyme, influence of metal ions), have been reinvestigated as a precondition, for studies concerning the regulation of human placental progesterone synthesis.

Linear enzyme kinetics (initial velocity) were obtained up to 10 min using an enzyme protein concentration of 5–10 μg/ml. A linear correlation between 3β-HSDH activity and enzyme protein concentration could be demonstrated up to 10 μg enzyme protein per ml. Incubation conditions were selected to obtain conversion rates of less than 20% of total [4-^{14}C]pregnenolone in view of a strong feedback inhibition of the 3β-HSDH by its own product, progesterone [12, 14–15, 21].

Different K_M values have been reported in the literature for pregnenolone: $3 \pm 0.8 \times 10^{-6}$ M (mitochondria, microsomes) [10], 170×10^{-9} M (homogenates) [22], 35×10^{-8} M (microsomes) [23], $35–42 \times 10^{-9}$ M (microsomes) [11]. The K_M value (70×10^{-9} M) for pregnenolone reported in this paper for the human placental mitochondrial 3β-HSDH is in good accordance with the data of Blomquist *et al.*[23] and Gibb *et al.*[11] obtained in tests using placental microsomes. The high K_M value of Ferre *et al.*[10] may be due to the photometric determination of the enzyme activity or be due to the limitations of studies with membrane-bound enzymes [24].

Like other oxidoreductase systems, the 3β-HSDH requires nicotinamide purine nucleotides for hydrogen transfer [25]. The enzyme activity was closely related to the presence of NAD^+ as could be shown by no 3β-HSDH activity in tests without NAD^+. At equimolar concentrations, NAD^+ was found to be more effective than $NADP^+$ which is also reported in the literature [8]. The K_M value of NAD^+ was reported to be 1.9×10^{-6} M (microsomes) [23], 5×10^{-6} M (microsomes) [11], 20×10^{-6} M (homogenates) [22], and 50×10^{-6} M (microsomes, mitochondria) [10]. The K_M value (20×10^{-6} M) for NAD^+ reported in this paper is in good accordance with the literature [10, 20].

The influence of pH on enzyme activity depends on changes of the ionizable groups of the enzyme. As a function of the pH value, the dissociation constants of the various enzyme, cofactors, substrate and inhibitor complexes changes. The pH optimum (pH 9.5) (glycine–NaOH buffer) of this enzyme is slightly higher than data reported by Ferre *et al.*[10]. When pregnenolone was used as substrate, the optimal activity of mitochondrial and microsomal 3β-HSDH occurs between pH 7.4–8.0 with Tris–HCl and pH 8–8.5 with glycylglycine buffer [10]. During investigations with human placental homogenates *in vitro*, a pH optimum was found between pH 10 and 12 [22]. The pH optimum of the microsomal and mitochondrial 3β-HSDH of human placentas was found to be pH 10–10.5 assaying the dehydrogenation step [8]. The isomerization activity was assayed at pH 7.0 at which no dehydrogenating activity could be detected. The microsomal 3β-HSDH in ovine corpora lutea displayed a broad pH optimum between pH 6.6 and 8.0 [26].

The optimal temperature reported by Ferre *et al.*[10] of 37°C is lower than the value found by our study group (40–45°C). At higher temperatures, a rapid decrease of enzyme activity and at 55°C no enzymatic activity could be measured. This effect may be due to a thermal inactivation or enhanced destruction of the enzyme by proteolytic digestion.

In the literature, there is little information on the regulatory effect of metal ions on 3β-HSDH activity. In our studies, bivalent metal ions such as Zn^{2+}, Cd^{2+} and Cu^{2+} were found to be potent enzyme inhibitors showing a nearly complete enzyme inhibition even in 1 mM concentrations, whereas Fe^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} showed only a weak inhibition. No effect was observed by 1–100 mM Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}. Different effects of metal ions were observed on steroidogenic enzymes. In tests with bovine placental 17β-estradiol dehydrogenase $ZnCl_2$ at a concentration of 5×10^{-6} M was found to enhance the enzymatic activity by about 38%. Higher concentrations lead to about 20% activation. In contrast, $MgCl_2$ and $CaCl_2$ at concentrations of 5×10^{-6} M and 2×10^{-5} M lead to about 25% and 30% inactivation respectively [27]. In this enzyme, zinc might influence the active site of the enzyme. Such a role has been established for some other dehydrogenases such as horse liver dehydrogenase [28], whereas a zinc atom is coordinated to two cysteinyl residues, one histidyl residue and a water molecule at the active site of the enzyme.

Manganese is known to be important in the reproductive system of mammalians. Animals kept on a diet lacking manganese lose their libido and fecundity due to an impaired function of ovary and testis [29–30]. The manganese levels in the endometrium and the plasma of normal women undergo cyclic changes during the menstrual cycle with a rise of manganese concentration during the proliferative phase [31]. The stimulatory effect of malate on the progesterone synthesis in human placental mitochondria was increased by the presence of Mn^{2+} due to the activatory role of Mn^{2+} for mitochondrial $NADP^+$-linked malic enzyme [32].

In tests with adrenal mitochondria from the rat, Mason *et al.*[33] observed with different metal ions (Ca^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}) (0.25–2.5 mM) that only calcium ions produced any marked stimulation of the cholesterol side chain cleavage, suggesting a specific calcium ion effect. A maximal stimulation was observed using 1 mM calcium ions (enzyme activity 230% of controls taken as 100%), while at a Ca^{2+} concentration of 2.5 mM, the enzyme activity was only enhanced to 140% of controls.

During *in vitro* incubation of the mitochondrial 3β-HSDH at 37°C, a rapid loss of enzyme activity was found within the first 90 min. After 30 min of incubation, less than 50% of initial enzyme activity

could be found. The inhibition of the 3β-HSDH during the preincubation period by steroids (e.g. pregnenolone, progesterone or progesterone metabolites) formed from endogenous cholesterol present in the mitochondrial preparation could be excluded by addition of diethylether extracts of the preincubated enzyme to 3β-HSDH tests performed sparately. The loss of enzymatic activity may be due to the instability of the enzyme after isolation or due to a proteolytic digestion of the enzyme by proteases still present after mitochondrial isolation. Similar results have been reported for other steroidogenic enzymes. The instability of the cholesterol side chain cleavage enzyme in bovine adrenal mitochondria during different preincubation periods at 30°C was observed by Hochberg *et al.*[16], who found 75% of initial enzyme activity after 30 min and 55% after 90 min. The instability of an enzyme may be an important consideration when assaying relative inactive preparations of the enzyme which require longer incubation periods.

The characterization of the 3β-HSDH is a precondition for the investigation of regulatory mechanism controlling the activity of this enzyme [14–15]. The inhibition of the 3β-HSDH may be important for the termination of human pregnancy via progesterone withdrawal and induction of labor. Especially various steroidogenic inhibitors of human placental 3β-HSDH have been analyzed [34–35] and our own results have been partially presented [14–15].

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