PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 71 (1999) 239–246

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Pregnenolone metabolized to 17α-hydroxyprogesterone in yeast: biochemical analysis of a metabolic pathway

E. Degryse[a,*], G. Cauet[a], R. Spagnoli[b], T. Achstetter[a]

[a] *Yeast Department, Transgène SA, Strasbourg, France*
[b] *Department for Biotechnological Research, Hoechst Marion Roussel, Romainville, France*

Received 22 January 1999; accepted 23 August 1999

Abstract

The cDNA coding for the human 3β-hydroxy-5-ene steroid dehydrogenase/5-ene-4-ene steroid isomerase (3β-HSD) has been expressed in yeast. When expressed from identical vectors except for the coding sequence, the specific activity of the type I is lower than that of the type II enzyme. A mutant of the human 3β-HSD type II lacking the putative membrane spanning domain 1 was generated by site directed mutagenesis: its apparent K_m for pregnenolone (PREG) is significantly increased and its V reduced to the level of the type I enzyme. The influence of the kinetic properties of 3β-HSD in the accumulation of 17α-hydroxyprogesterone was probed by co-expression of the bovine 17α-hydroxylase cytochrome P450 (P45017α) cDNA. The metabolism of PREG was followed with time using the membrane fraction. Kinetic properties of the 3β-HSD were modulated such that its activity was in excess, limiting or balanced with respect to the activity of the P45017α and the accumulation of intermediates and products recorded. Conditions for the generation of the by-products resulting from the 17,20-Lyase activity of the P45017α were found. The potential applications of the system are discussed.

1. Introduction

In the steroid hormone biosynthesis [1], one enzyme localized to the adrenal cortex, plays a central role in the generation of a wide variety of products. The bovine 17α-hydroxylase cytochrome P450 (P45017α, EC 1.14.99.9) converts pregnenolone (PREG) and progesterone (PROG) into the respective 17α-hydroxy forms (Fig. 1). In addition, bovine P45017α can also have a 17,20-Lyase activity on 17α-hydroxypregnenolone (17-OH PREG) only, producing dehydroepiandrosterone (DHEA) [2]. Interestingly, the panoply of products generated from PREG is enriched with 17-OH PROG from 17-OH PREG and androstenedione (A-dione) from DHEA when the P45017α is combined with 3β-hydroxy-5-ene steroid dehydrogenase (3β-HSD, EC 1.1.1.145)/5-ene-4-ene steroid isomerase (EC 5.33.1). Indeed, 3β-HSD catalyses the reaction transforming PREG, 17-OH PREG and DHEA into respectively PROG, 17-OH PROG and androstenedione (Fig. 1 and [3]). The distributive function of the initial part of the corticoid pathway is the starting point of the biosynthesis of important hormonal products: the glucocorticoids, the mineralocorticoids and the sex hormones. The medical importance of the glucocorticoids has raised interest in the transposition of this pathway in a microbial host. Yeast has been chosen as a host because it is an industrially well-known and robust species. Furthermore, the cDNA coding for the bovine P45017α was expressed in yeast [4,5]. The

Abbreviations: A-dione, androstenedione; DHEA, dehydroepiandrosterone; 3β-HSD, 3β-hydroxy-5-ene steroid dehydrogenase/5-ene-4-ene steroid isomerase; P45017α, 17α-hydroxylase cytochrome P450; P45011β, 11β-hydroxylase cytochrome P450; 17-OH PREG, 17α-hydroxypregnenolone; 17-OH PROG, 17α-hydroxyprogesterone; MSD, membrane-spanning domain; P450red, NADPH-cytochrome P450 reductase; PREG, pregnenolone; PROG, progesterone.

* Corresponding author. Tel.: +33-(0)14981-5171; fax: +33-(0)14981-5240.

E-mail address: degryse@compuserve (E. Degryse).

0960-0760/00/$ - see front matter
PII: S0960-0760(99)00137-5

cDNAs and/or genes coding for 3β-HSD [3] have been cloned from a wide variety of species including man and rat. In human, two types have been described: type I 3β-HSD is expressed in placenta and in skin; type II 3β-HSD is present in adrenals and gonads. The cDNA was expressed in Hela or insect cells [6,7], whereas its expression in yeast has not yet been reported.

The present goal is to co-express in a single cell the cDNAs coding for 3β-HSD plus P45017α into an active form. Next, the pathway should be engineered in order to accumulate the preferred endproduct (17-OH PROG) avoiding the generation of by-products (DHEA, A-dione). Finally, since the pathway is not linear (Fig. 1), it should be emphasised that it does not suffice to overexpress the second enzyme in order to increase the product yield. To our best knowledge, it is the first time that this type of pathway has been analysed, providing information how to direct the flow of intermediates towards production of glucocorticoids.

2. Materials and methods

2.1. Construction of transfer vectors

2.1.1. Human 3β-HSD type I cDNA

'Transfer vectors' contain an expression cassette with a yeast promoter, the cDNA of interest, the *PGK* terminator and an *E. coli* replicon. In the case of the cDNA coding for 3β-HSD type I, the plasmid (kindly provided by Labrie) — published previously [9] — was modified to contain a *Mlu*I site at its 3′ and a *Sal*I site at its 5′, to allow direct cloning in our expression vectors. pTG10064 is a transfer vector which contains the *CYC1L* promoter controlling the coding sequence for 3β-HSD type I bordered by *Sal*I and *Mlu*I restriction sites. The sequence preceding the initiating ATG is the following: 5′-**GTCGAC**GGGGCCATG ACG GGC (The *Sal*I site is in bold and the initiating ATG is underlined.).

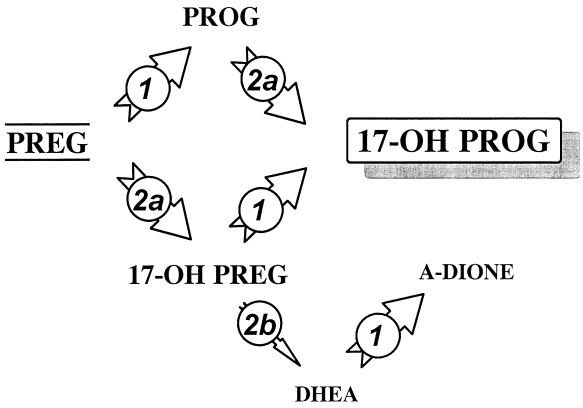

Fig. 1. Metabolism of PREG based on the catalytic properties of the human 3β-HSD and the bovine P45017α. Ascending arrows labelled '1' symbolize the human 3β-HSD activities on different substrates. Descending arrows symbolise the P45017α activities on different substrates. The hydroxylase activity is indicated by label '2a'. The width of the arrows suggests similar activities, installing a balanced metabolism. The smaller '2b' arrow indicates the 17,20-Lyase activity of the bovine P45017α, which could be reduced by for example a limiting P450red activity.

2.1.2. Human 3β-HSD type II cDNA

The cDNA for type II 3β-HSD was described previously [6]. A transfer vector (pTG10095) was generated which contains the coding sequence of the human 3β-HSD type II cDNA flanked by *Sal*I and *Mlu*I sites downstream the *GAL10/CYC1* promoter. The sequence preceding the initiating ATG is the following: 5′-**GTCGAC**CACGATG GGC.

2.1.3. Reshaping of the human 3β-HSD type I cDNA

The 5′ of the human 3β-HSD type I cDNA was reshaped such that the sequence of the expression block (*CYC1s/PGK*) would be identical to that of the type II gene (except for differences within the coding region): pTG10488. The sequence at the 5′ reads: CATAAATTA**GTCGAC**CACGATG GGCTGGAGC.

2.1.4. Construction of a transfer vector for bovine P45017α

The cDNA coding for the P45017α [10] was cloned downstream of the *CYC1L* promoter in a transfer vector generating pTG10058.

2.2. Generation of a 3β-HSD without predicted MSD1

Simard et al. [11] showed the relationship between the presence of the predicted MSD1 and a decreased K_m for the substrates. The elimination of the MSD1 predicted for both human type I and II 3β-HSD might increase the K_m of the 3β-HSD enzyme above that of the P45017α. The MSD1 was first eliminated 'virtually' by re-iteratively introducing small changes in the primary sequence of the human 3β-HSD and diagnosis through published algorithms [12,13]. A M13 template containing the human 3β-HSD type I form was mutagenised in order to remove the MSD1. A *Stu*I fragment was cloned into a human 3β-HSD type II transfer vector yielding pTG10468. A comparison of the MSD1 amino acid sequences is shown below. Differences with 3β-HSD are indicated in lower case, predicted MSD1 is underlined.

β-HSD type I:
PFLKR ACQDVSVIIHTACIIDVFGVT HRESIMN
3β-HSD type II:
PFLKR ACQDVSVvIHTACIIDVFGVT HRESIMN
3β-HSD type II MSD1^{-}:

PFLKR ACQDVSVIIHTAt IIDVtGVT HRESIMN

2.3. Expression plasmids

Recombination vectors were generated containing the yeast 2 μm, a replicon, a $CYC1_{prom}$ - PGK_{term} expression cassette and different selection markers: *URA3-d* (pTG10257) or *LEU2* (pTG10260). These recombination vectors are identical to those previously described (pTG10042 (*URA3-d*) and pTG10158 (*LEU2-based*)) except that the *Xba*I site contained in the 2 μm region was replaced by a *Xba*I° marker, obtained through filling in of the natural *Xba*I site by the Klenow polymerase and religation. 'Expression plasmids' are generated by incorporating the cDNA of interest into the expression cassette of a recombination vector by recombination between homologous flanking sequences during transformation in yeast or *E. coli* [4,8].

The expression blocks (from pTG10064, pTG10095, pTG10468, pTG10488 and pTG10058) were introduced in the newly generated *URA3-d, URA3* or *LEU2* recombination vectors yielding the expression plasmids shown in Table 1. To be noticed that the *CYC1* promoters used (*CYC1s* and *CYC1L*) differ in the length of the sequence preceding the initiating ATG [5,8].

2.4. Classical molecular biology techniques and recombination in vivo in E. coli

Molecular biology methods are according to Sambrook et al. [14]; strains and methods for in vivo recombination have been described previously [4].

2.5. Transformation of Saccharomyces cerevisiae

Strain W303-1B (*MATα*, ρ^+, *ura3-1, leu2-3, -112, his3-11, -15, trp1-1, ade2-1*, can^R, cyr^+(?), [15]) was used. A satisfactory protocol for the simultaneous transformation of two plasmids is the following: cells are made competent [16] and transformed with 1 μg of pTG-*URA3-d* and 10 μg pTG-*LEU2* (no carrier DNA was utilised). After transformation and plating out on agar plates (containing YNBG + casamino acids 0.01% + tryptophan, adenine, histidine and leucine, each at 100 μg/ml), candidate colonies were confirmed on selective medium (YNBG + casamino acids 0.01% + tryptophan, adenine and histidine at 100 μg/ml). A high number of colonies were double transformants (> 30%). PCR was used to confirm the simultaneous presence of the selective marker and the cDNA associated with it for each plasmid.

2.6. Total membrane preparation

After harvest, the cells were broken by vortexing for five, 1 min cycles with glass beads (v/v). The extraction buffer is composed of 50 mM potassium phosphate buffer pH 7.4 containing the 'protease inhibitor cocktail' (Complete™ supplied by Boehringer Mannheim). Cells and debris were removed by centrifugation at 3000 *g* for 5 min. The supernatant is then centrifuged at 105000 *g* for 90 min. The pellet is resuspended in an appropriate volume of 50 mM potassium phosphate buffer pH 7.4 and stored at −80°C until use.

2.7. Specific activity determination

2.7.1. P45017α

P45017α hydroxylase activity was measured on yeast total membranes using 20 μM [^{3}H]-PREG (at 200 Ci/mol) as substrate in 250 μl containing 50 mM potassium phosphate buffer pH 7.4 containing 1 mM KCN, NADPH (1 mM) glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (2 IU/ml). Incubations were carried out at 30°C for 10 min. The steroids were analysed as described below.

2.7.2. 3β-HSD

3β-HSD activity was measured on yeast total membranes using 20 μM [^{3}H]-PREG (at 200 Ci/mol) as substrate in 250 μl containing 50 mM potassium phosphate buffer pH 7.4, NAD+ (1 mM), sodium pyruvate (5 mM) and lactate dehydrogenase (2 IU/ml). Incubations were carried out at 30°C for 10 or 20 min

Table 1
Summary of the representative vectors constructed for the expression of 3β-HSD and P45017α cDNAs. Details on constructions are given in Materials and methods. The roman number I and II associated with 3β-HSD represent the human 3β-HSD type I and II respectively

Expression plasmid	Selection marker	cDNA	Promoter	Corresponding transfer vector
pTG10261	*URA3-d*	3β-HSD **I**	*CYC1L*	pTG10064
pTG10264	*URA3*	3β-HSD **I**	*CYC1L*	pTG10064
pTG10602	*URA3-d*	3β-HSD **I**	*CYC1 s*	pTG10488
pTG10262	*URA3-d*	3β-HSD **II**	*CYC1 s*	pTG10095
pTG10485	*URA3-d*	3β-HSD **II**, $MSD1^-$	*CYC1 s*	pTG10468
pTG10269	*LEU2*	P45017α	*CYC1L*	pTG10058

depending on the expression level of the different strains. The steroids were analysed as described below.

2.8. Bioconversion in vitro

Metabolism of PREG in total membrane fractions: The reaction mixture contained total membranes from strains expressing 3β-HSD and P45017α cDNAs, [^{3}H]-PREG (5 μM unless indicated otherwise). NADPH (0.5 mM), NAD+ (0.5 mM), glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (2 IU/mln), pyruvate (5 mM) and lactate dehydrogenase (2 IU/ml), in 50 mM potassium phosphate buffer pH 7.4. After different time intervals at 30°C, 250 μl aliquots were extracted and the steroids analyzed. PREG disappearance is not linear with protein amount at high concentration.

2.9. Steroid analysis

After extraction of the reaction mixtures with dichloromethane, the radiolabelled steroids are separated by reverse-phase HPLC on Ultrasphere ODS (Beckman) and quantitated using a FLO-One radiodetector (Packard) coupled to the HPLC system. Details on the separation systems used will be provided upon request.

2.10. K_{mapp} determination

The K_{mapp} was determined using single transformants expressing one of the different human 3β-HSD variants. The reaction mixture contained the components described above except that instead of total membranes, microsomal fractions were used (see below). K_{mapp} was calculated from the data by non-linear regression [17] but represented as Lineweaver–Burk plots with the V normalised to 1 (Fig. 2).

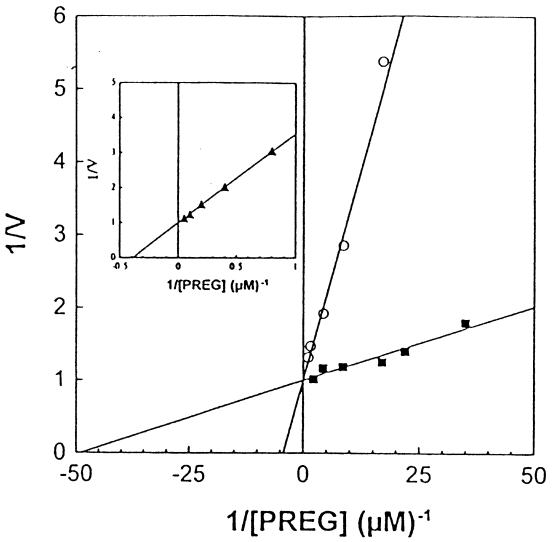

Fig. 2. Lineweaver–Burk plots of the activities on varying PREG concentrations of the 3β-HSD variants expressed in yeast. The 3β-HSD type I cDNA was expressed from pTG10602 (■) and the 3β-HSD type II cDNA from pTG10262 (○). The inset shows the 3β-HSD type II, MSD1$^-$ cDNA expressed from pTG10485 (▲). The V was normalized to 1, in order to allow a comparison between the widely varying K_{mapp} values.

3. Results

3.1. Expression of the P45017α cDNA

A prerequisite for the present study is the production of active forms of the P45017α and 3β-HSD. For the P45017α cDNA this was shown in yeast [10,18]. When the bovine P45017α cDNA was expressed under control of the *CYC1L* promoter from a 2 μ-based shuttle vector with the *URA3-d* selection marker in yeast [10] its specific activity was 13 nmol/min/nmol. In the presence of an excess of purified rat liver NADPH-cytochrome P450 reductase (P450red, EC 1.6.2.4) a K_m value of 0.2 μM for PREG and 1.4 μM for PROG were found [11]. A low conversion of 17-HO PREG into DHEA could be detected in vitro. No evidence for an in vivo 17,20-Lyase activity could be obtained on PROG.

3.2. Co-expression of the P45017α and 3β-HSD: constant P45017α activity

It seemed appropriate to maintain a constant P45017α cDNA enzyme level, its activity being dependent on limiting endogenous electron donors (P450red and cytochrome *b5*). For this purpose, the P45017α cDNA was associated with *LEU2* as a selection marker on a medium copy number vector (pTG10269). Since it is known that the endogenous 2 μ (and a fortiori any 2 μ based artefactual vector) can be out-competed by a 2 μ vector containing a defective selection marker allele [19], it was essential to demonstrate that the expression level of the P45017α cDNA remains more or less constant. In first instance the activity was determined with PREG as a substrate in the membrane fraction. With one exception (indicated * in Table 2), the P45017α activities determined vary by less than 20% around the mean (Table 2). The specific activity of the P45017α in the W303/pTG10264+pTG10269 association were higher because the cells were harvested after a longer induction time on glycerol. The specific activities indicate that the association of a medium (*LEU2*) with a high copy (*URA3-d*) number plasmid does not induce a dramatic co-plasmid instability, within the time course of the experiments. At a practically constant P45017α,

it is to be analysed whether the 3β-HSD cDNA can be expressed in yeast to varying levels.

3.3. Specific activities of recombinant human 3β-HSD type I and type II

The expression of the human 3β-HSD cDNA in yeast was not yet described. The specific activity of the yeast-made 3β-HSD was measured in cell-free extracts with PREG as substrate (Table 2). The specific activity of the 3β-HSD type I (using pTG10261) is much lower than that of 3β-HSD type II (using pTG10262). Since apart from the coding sequence, differences exist between the expression blocks due to subcloning manoeuvres, an expression block identical to that of the type II, except for the coding sequence, was created for the type I cDNA (pTG10602). When both the promoter and the sequence preceding the initiating ATG of the 3β-HSD type I cDNA are made identical to that of the type II expression block, the type II cDNA product is more active than the type I. Furthermore, the activity of the putative MSDI$^-$ mutant form of the type II (pTG10485), is close to that of the natural 3β-HSD type I (pTG10602). It is to be concluded that in yeast the 3β-HSD type I and MSD1$^-$ type II have both (nearly) the same activity but are (nearly 6-fold) less active than the wild type 3β-HSD type II. In conclusion, the human 3β-HSD cDNA expressed in yeast is active, with the type II form being more active than the type I. A mutant form has been generated which retains the catalytic potential of the type I form.

3.4. Kinetic parameters of the yeast-borne 3β-HSD

The K_{mapp} (PREG as substrate) values were determined to be 20 ± 3 nM and 230 ± 30 nM for the 3β-HSD type I and II variants respectively (Fig. 2). The K_m values observed agree with those obtained on microsomes prepared from human placenta [20]. The discrepancy with the values published by Lachance et al. [6] could be the use of a microsomal preparation in the former cases and of a cell lysate, possibly containing parasite reactions which reduce the actual substrate concentration, in the latter case. The mutant (pTG10485) lacking the putative MSD1 has a K_{mapp} value of 2.6 ± 0.05 μM for PREG (Fig. 2) which is significantly increased when compared to both natural forms (type I and II). This confirms the role of this peptide segment (aa71–91) in the kinetic parameters of the 3β-HSD. The creation of a mutant enzyme with a K_m value higher than the one of the P45017α is interesting in view of their competition for the same substrate, PREG. At this point the variants of the 3β-HSD with significant differences in K_m values and specific activities can be used to probe the appearance of 17-OH PROG during the metabolism of PREG (Fig. 1).

3.5. Time course of the PREG metabolism

In order to analyse the time course of the metabolism of PREG in vitro, total membranes were prepared from the yeast cells co-expressing the bovine P45017α together with the human 3β-HSD variants (pTG10264, pTG10261, pTG10602 and pTG10485). The transformed clones cover the range of different 3β-HSD activities and K_{mapp} at constant P45017α. Time courses and appearance of the different metabolic products are shown in Fig. 3. Three phases can be distinguished.

During the initial phase, the decrease in PREG is accompanied by an accumulation of the intermediates 17-OH PREG and PROG. Kinetic differences have a clear impact on the increase in the intermediates in the first phase. Indeed, during metabolism of PREG in membranes prepared from W303/(pTG10264 + pTG10269; see Fig. 3*A*) mostly 17-OH PREG accumulates and only very little PROG,

Table 2
Specific activities scored in the total membrane fractions of recombinant yeast differentially co-expressing the 3β-HSD and P45017α cDNAs. The plasmid used for the co-expression of the P45017α is the medium copy number pTG10269. The copy number status of each plasmid used for 3β-HSD cDNA co-expression is indicated. Details on plasmids used for 3β-HSD cDNA expression are given in Materials and methods and in Table 1. The substrate used was PREG at a saturating concentration. The specific activities indicate the product measured for each enzyme in the total membrane fraction

	3β-HSD, nmoles PROG/min/mg	P45017α, nmoles 17-OH PREG/min/mg	Relative specific activities, 3β-HSD/P45017α
pTG10261 (Hcn[a])	0.34	0.33	1.03
pTG10264 (mcn[b])	0.32[c]	0.74[c]	0.43
pTG10602 (Hcn[a])	3.5	0.38	9.2
pTG10262 (Hcn[a])	18.0	0.50	36.0
pTG10485 (Hcn[a])	1.8	0.40	4.5

[a] Refers to the high copy number (Hcn) of the yeast expression plasmid.
[b] Refers to the medium copy number (mcn) of the yeast expression plasmid used for the 3β-HSD cDNA expression.
[c] Cultures were induced for a longer time, hence the recorded specific activities are higher.

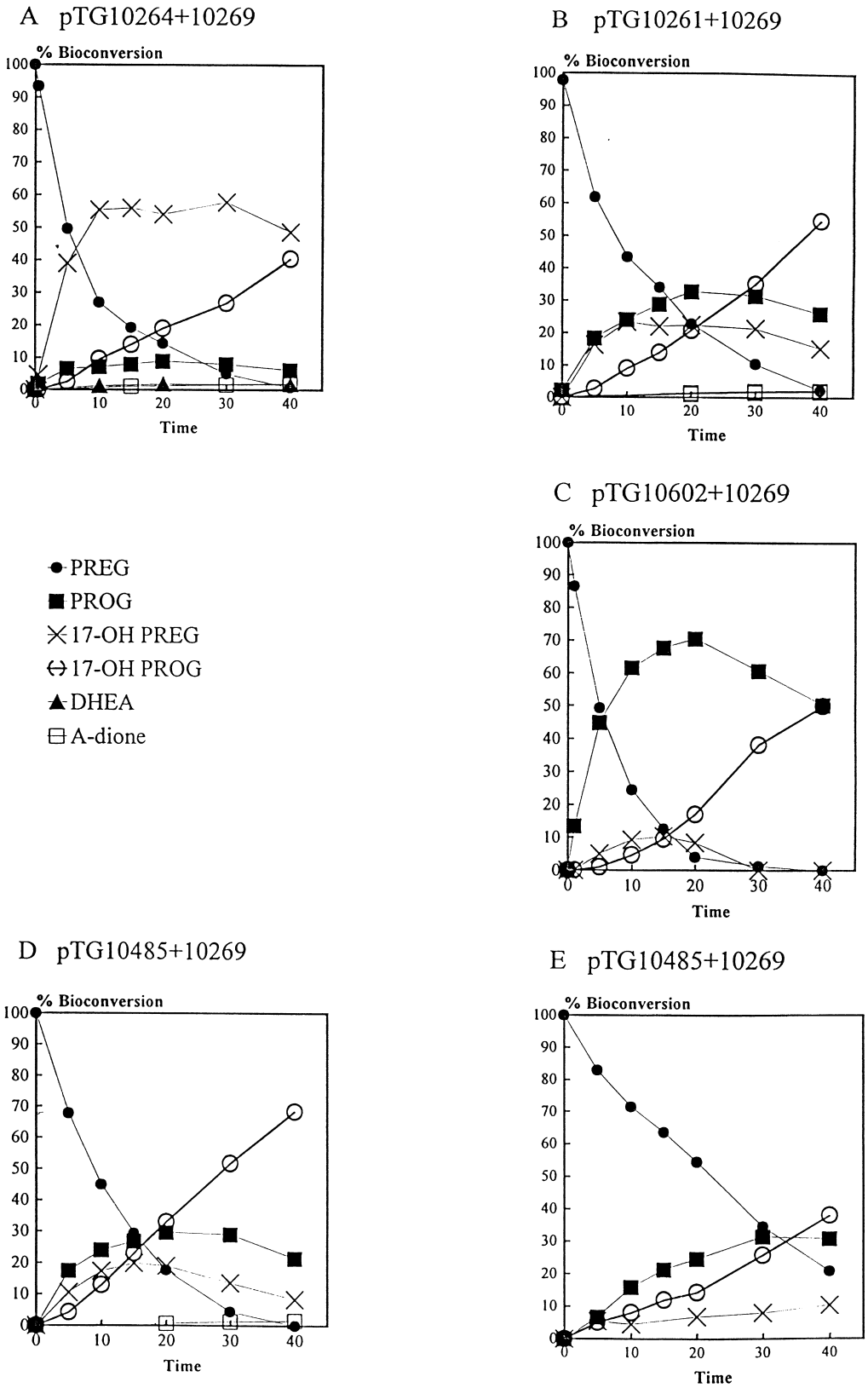

Fig. 3. Progress curves showing the metabolism of PREG in vitro of recombinant yeast total membrane fractions. Incubations were carried out as described under Materials and methods using per time point 140 μg total membrane protein for A; 185 μg for B; 88 μg for C; 80 μg for D and 320 μg for E. Using the protein amounts and the sum of 3β-HSD and P45017α specific activities presented in Table 2, the following units (nmol PREG disappearing/min/250 μl) can be calculated: 0.124 for A; 0.148 for B; 0.34 for C; 0.176 for D and 0.7 for E. PREG was used at a concentration of 5 μM in all cases except E where its concentration was 20 μM. Symbols as indicated in the figure. Pertinent information concerning plasmids used in A to E (as indicated in this figure) is contained in Table 1. The abcissa represents the 'Time' scale, in minutes.

whereas the membranes prepared from W303/(pTG10602 + pTG10269; see Fig. 3*C*) accumulate more PROG than 17-OH PREG. The metabolism on (5 μM) PREG is balanced for membranes prepared from W303/(pTG10261 + pTG10269; see Fig. 3*B*) and from W303/(pTG10485 + pTG10269; see Fig. 3*D*) since a nearly identical increase in both intermediates is found. In the latter case equilibration is achieved because of the increased K_m for PREG of mutant 3β-HSD MSD1$^-$. When tested on saturating amounts of PREG, these membranes accumulate as expected an excess of PROG (Fig. 3*E*). During the second phase, the pools of the intermediates attain a relatively constant level. This is particularly true when the P45017α is in excess (Fig. 3*A*). The time span of the nearly constant intermediate pool level is shorter when the metabolism of PREG was balanced in first instance (Fig. 3*B* and 3*D*) and is not reached when the 3β-HSD is in excess (Fig. 3*C*). The intermediates remain nearly constant when PREG has reached a concentration where its decrease is paralleled by an increase in 17-OH PROG (Fig. 3*A*, 3*B* and 3*D*). The third phase mirrors the first phase except that the PREG level has dropped completely and that the accumulation of the product, 17-OH PROG, is set by the limiting enzyme. For example, when an excess of PROG has accumulated its disappearance depends on the activity of the P45017α (Fig. 3*C*); when 17-OH PREG has accumulated, it will be converted into 17-OH PROG according to the limiting activity of the 3β-HSD (Fig. 3*A*).

The appearance of the by-products is clearly correlated with the accumulation of 17-OH PREG. Whenever the activity of the 3β-HSD is in excess over that of the P45017α (Fig. 3*B* and 3*D*) only A-dione accumulates. Both byproducts, DHEA and A-dione accumulate when the 3β-HSD is limiting (Fig. 3*A*). It is concluded that in yeast the distributive function of the initial part of the steroid hormone pathway is controlled by the level of 17-OH PREG. This level is dependent on the relative expression levels of the 3β-HSDH/P45017α and of their kinetic properties.

4. Discussion

The metabolic pathway starting at PREG forms a branch point catalysed by 3β-HSD and P45017α which in the next step, involving the same enzymes, converges towards one product: 17-OH PROG (Fig. 1). Since both enzymes intervene twice in the bioconversion of PREG into 17-OH PROG, a slight imbalance in one activity over the other will become amplified. In an attempt at balancing the flux, two parameters were varied: the relative expression levels of the cDNAs and the relative K_ms of the enzymes for their substrates.

The specific activity obtained on membrane preparations of yeast for the type I enzyme was 3 nmol/min/mg prot, comparable to the activity found in crude cell extracts after expression in insect cells [7]. The specific activity of the pure enzyme was 270 nmol/min/mg [7]. The specific activities determined in membrane fractions shows that the activity of the yeast-made 3β-HSD type I is (6-fold) lower than that of the type II. Two putative MSD are predicted by all three different algorithms for mammalian 3β-HSD, but one for bovine and rat type II [3]. The generation of a human 3β-HSD type II which lacks the predicted MSD1 Whenever the MSD1 is no longer predicted (MSD1$^-$ mutant, pTG10485) the apparent K_m for PREG is increased ([11], this paper) and the V is reduced for both rat [11] and human 3β-HSD (this paper). It is tempting to speculate that the putative MSD1 (aa 71–91), which on the primary sequence resides between the putative cofactor (aa 6–36) and substrate binding sites (aa 176–186 and aa 251–274; [3]), serves as a membrane anchor facilitating substrate capture. It appears as if the type II enzyme has optimal kinetic properties and either reducing (type I) or increasing the apparent K_m for PREG by affecting the MSD1 (pTG10485), lowers its catalytic activity.

In order to account for the total 3β-HSD and P45017α activities present in the cell, the analysis of PREG metabolism in vitro was carried out using the membrane fraction of double transformants. It is shown that using a reiterative adjustment of the expression level of 3β-HSD compared to P45017α it is possible to equilibrate the metabolism on PREG via a heterologous system. Equilibration is achieved when PROG and 17-OH PREG accumulate to nearly the same level, as shown for membranes prepared from W303/(pTG10261 + pTG10269; Fig. 3*B*). This occurs when the 3β-HSD/P45017α activity ratio equals 1 (Table 2). The influence of the expression level was analysed, either by reducing the copy number (Fig. 3*A*) or by increasing the expression level (Fig. 3*C*). A 3β-HSD/P45017α activity ratio of 0.4, obtained by a reduced copy number (pTG10264) results in the accumulation of 17-OH PREG (see Fig. 3*A*). When the activity of the 3β-HSD type I exceeds that of the P45017α, PROG accumulates (in W303/(pTG10602 + pTG10269); Fig. 3*D*). The study of the influence of the K_m for PREG of the 3β-HSD becomes practically possible by using the mutant form (pTG10485) whose apparent K_m for PREG is increased by about a 100-fold to reach the μmolar level and which is expressed to nearly the same specific activity as the type I enzyme (Table 2). The system is balanced (Fig. 3*D*) at a subsaturating initial PREG concentration (5 μM). At saturating PREG, PROG accumulates (Fig. 3*E*) as in the case of an excess activity of 3β-HSD over the P45017α (compare Fig. 3*C* and *E*).

It was suggested recently [21] that the in vivo activity of recombinant yeast cells expressing particular enzymes could be an alternative to the large scale-production and in vitro reconstitution of a biosynthetic segment. Applied to the glucocorticoid hormones, this aspect becomes possible. However, the presence of parasitic reactions reduce the product yield in vivo [10,22]. Their identification will be reported in extenso (Cauet et al., submitted).

Acknowledgements

This research was supported by Hoechst Marion Roussel. The expert technical assistance of Mireille Dietrich, Catherine Ledoux and Patricia Kleinpeter is gratefully acknowledged. We are indebted to F. Labrie and V. Luu-The for their generous gift of cDNAs coding for the human 3β-HSD type I and II and B. Dumas for his interest in this work.

References

[1] W.L. Miller, Molecular biology of steroid hormone synthesis, Endocrine Revs 9 (1988) 295–318.
[2] H.J. Barnes, M.P. Arlotto, M.R. Waterman, Expression and enzymatic activity of recombinant cytochrome P450 17α-hydroxylase in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 88 (1991) 5597–5601.
[3] J. Simard, F. Durocher, F. Mébarki, C. Turgeon, R. Sanchez, Y. Labrie, J. Couet, C. Trudel, E. Rhéaume, Y. Morel, V. Luu-The, F. Labrie, Molecular biology and genetics of the 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase gene family, J. Endocrinol. 150 (1996) 5189–5207.
[4] E. Degryse, In vivo intermolecular recombination in *Escherichia coli*: application to plasmid constructions, Gene 170 (1996) 45–50.
[5] V. Nacken, T. Achstetter, E. Degryse, Probing the limits of expression levels by varying promoter strength and plasmid copy number in *Saccharomyces cerevisiae*, Gene 175 (1996) 253–260.
[6] Y. Lachance, V. Luu-The, C. Labrie, J. Simard, M. Dumont, Y. de Launoit, S. Guérin, G. Leblanc, F. Labrie, Characterisation of human 3β-hydroxysteroid dehydrogenase/delta5–delta4 isomerase gene and its expression in mammalian cells, J. Biol. Chem. 265 (1990) 20469–20475.
[7] M. Zhou, R. Breton, H.-X. Lin, Rapid purification of overexpressed 3β-hydroxysteroid dehydrogenase, Prot. Sci 2 (Suppl 1) (1993) 626–M.
[8] E. Degryse, B. Dumas, M. Dietrich, L. Laruelle, T. Achstetter, In vivo cloning by homologous recombination in yeast using a two-plasmid-based system, Yeast 11 (1995) 629–640.
[9] V. Luu-The, Y. Lachance, C. Labrie, G. Leblanc, J.L. Thomas, R.C. Strickler, F. Labrie, Full length cDNA structure and deduced amino acid sequence of human 3β-hydroxy-5-ene steroid dehydrogenase, Mol. Endocrinol. 3 (1989) 1310–1312.
[10] B. Dumas, G. Cauet, E. Degryse, R. Spagnoli, T. Achstetter, in: M.C. Lechner (Ed.), Cytochrome P450. 8th International Conference, John Libbey Eurotext, Paris, 1994, pp. 527–530.
[11] J. Simard, Y. de Launoit, F. Labrie, Characterisation of the structure-activity relationships of rat types I and II 3β-hydroxysteroid dehydrogenase/delta5-delta4 isomerase by site-directed mutagenesis and expression in HeLa cells, J. Biol. Chem. 266 (1991) 14842–14845.
[12] D. Eisenberg, E. Scharz, M. Komaromy, R. Wall, Analysis of membrane and surface protein sequences with the hydrophobic moment plot, J. Mol. Biol. 179 (1984) 125–142.
[13] P. Klein, M. Kanehisa, C. de Lisi, The detection and classification of membrane-spanning proteins, Biochim. Biophys. Acta 815 (1985) 468–476.
[14] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor University Press, Cold Spring Harbor, 1989.
[15] M.D. Crivellone, M. Wu, S. Takemori, Assembly of the mitochondrial membrane system, J. Biol. Chem. 263 (1988) 14323–14333.
[16] V. Lauermann, Ethanol improves the transformation efficiency of intact yeast cells, Curr. Gen. 20 (1991) 1–3.
[17] R.G. Duggleby, A nonlinear regression program for small computers, Anal. Biochem. 110 (1981) 9–18.
[18] T. Sakaki, M. Shibata, Y. Yabusaki, H. Murakami, H. Okhawa, Expression of bovine cytochrome P450c17 cDNA in *Saccharomyces cerevisiae*, DNA 8 (1989) 409–418.
[19] E. Erhart, C.P. Hollenberg, The presence of a defective *LEU2* gene on 2 μ DNA recombinant plasmids of *Saccharomyces cerevisiae* is responsible for curing and high copy number, J. Bact. 156 (1983) 625–635.
[20] W. Gibb, Substrate and nucleotide specificity of placental microsomal 3 beta-hydroxysteroid dehydrogenase, Steroids 37 (1981) 23–31.
[21] A. Wiseman, Genetically-engineered mammalian cytochromes P-450 from yeast — potential applications, Tibtech 11 (1993) 131–136.
[22] T. Sakaki, M. Akiyoshi-Shibata, Y. Yabusaki, K. Manabe, H. Murakami, H. Okhawa, Progesterone metabolism in recombinant yeast simultaneously expressing bovine cytochromes P450c17 (CYP17A1) and P450c21 (CYP21B1) and yeast NADPH-P450 oxidoreductase, Pharmacogen 1 (1991) 86–93.