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Characterization of equine GST A3-3 as a steroid isomerase

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ABSTRACT

Glutathione transferases (GSTs) comprise a superfamily of enzymes prominently involved in detoxication by making toxic electrophiles more polar and therefore more easily excretable. However some GSTs have developed alternative functions. Thus, a member of the Alpha class GSTs in pig and human tissues is involved in steroid hormone biosynthesis, catalyzing the obligatory double-bond isomerization of Δ^5-androstene-3,17-dione to Δ^4-androstene-3,17-dione and of Δ^5-pregnene-3,20-dione to Δ^4-pregnene-3,20-dione on the biosynthetic pathways to testosterone and progesterone. The human GST A3-3 is the most efficient steroid double-bond isomerase known so far in mammals. The current work extends discoveries of GST enzymes that act in the steroidogenic pathways in large mammals. The mRNA encoding the steroid isomerase GST A3-3 was cloned from testis of the horse (*Equus ferus caballus)*. The concentrations of GSTA3 mRNA were highest in hormone-producing organs such as ovary, testis and adrenal gland. EcaGST A3-3 produced in *E. coli* has been characterized and shown to have highly efficient steroid double-bond isomerase activity, exceeding its activities with conventional GST substrates. The enzyme now ranks as one of the most efficient steroid isomerases known in mammals and approaches the activity of the bacterial ketosteroid isomerase, one of the most efficient enzymes of all categories known today. The high efficiency and the tissue distribution of EcaGST A3-3 support the view that the enzyme plays a physiologically significant role in the biosynthesis of steroid hormones.

1. Introduction

Hydrophobic exogenous and endogenous electrophiles cause damage to mammalian organisms as they react with proteins, DNA and other essential cell components that feature exposed nucleophilic centers. Glutathione transferases (GSTs) comprise a superfamily of enzymes (EC 2.5.1.18) prominently involved in detoxication processes by conjugating the polar tripeptide glutathione (γ-Glu-Cys-Gly) to hydrophobic electrophiles, thereby forming polar conjugates more easily excretable from the organism [1–3]. However, certain enzymes from some GST classes have developed alternative functions. Examples include GST P1-1 that acts as modulator of protein kinases involved in cellular signaling [4–6] and GST S1-1, known as prostaglandin D synthase that catalyzes the conversion of prostaglandin H_2 into prostaglandin D_2 [7,8].

Among the alternative GST functions a role in steroid hormone biosynthesis has emerged for the human alpha-class enzyme GST A3-3 [9,10]. The enzyme is an efficient catalyst of the obligatory double-bond isomerization of Δ^5-androstene-3,17-dione (Δ^5-AD) to Δ^4-androstene-3,17-dione (Δ^4-AD) and of Δ^5-pregnene-3,20-dione (Δ^5-PD) to Δ^4-pregnene-3,20-dione (Δ^4-PD) in the biosynthetic pathways of testosterone and progesterone, respectively (Fig. 1). Human GST A3-3 (HsaGST A3-3) is prominently expressed in steroidogenic tissues indicating its physiological role, and experiments with cell lines supported this notion [11]. However, no direct support for the involvement of the enzyme in steroid hormone biosynthesis has been available at the organism level.

To extend the characterization of alpha-class GST enzymes involved in steroid hormone isomerization to other species, homologs of human GSTA3 mRNA were cloned from pig testis and ovary [12] and bovine ovary [13]. When the porcine SscGST A2-2 and the bovine BtaGST A1-1 proteins were expressed in vitro, however, they were considerably less active as steroid isomerases than HsaGST A3-3. Specifically, SscGST A2-2 was more active than BtaGST A1-1, but it had four to five-fold lower specific activity with Δ^5-AD and 19-fold lower specific activity with Δ^5-PD than HsaGST A3-3. It is notable that rodents utilize the Δ^4 pathway of steroidogenesis [14] and apparently lack any homolog with the high isomerase activity distinguishing the human GST A3-3 enzyme.

Recently, the search for alpha-class GST enzymes with steroid isomerase activities was extended to the horse. Equine GST A3-3 (EcaGST A3-3) was discovered and its mRNA cloned from stallion testis [15]. Testosterone levels in stallion serum were suppressed in parallel with

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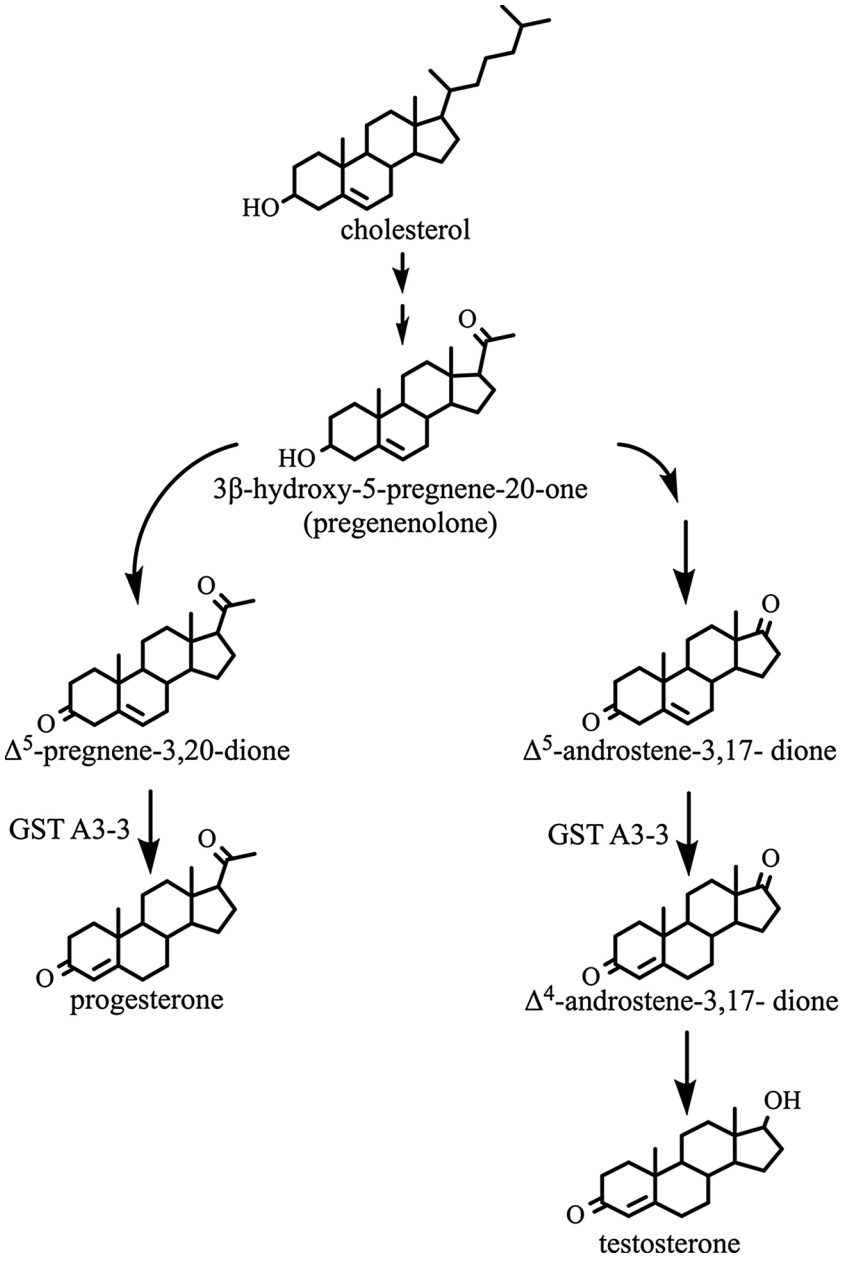

Fig. 1. Outline of the biosynthetic pathway of steroidogenesis leading to progesterone and testosterone indicating steps efficiently catalyzed by GST A3-3.

EcaGSTA3 mRNA concentrations in testes within 12 h of treatment with dexamethasone, a synthetic glucocorticoid. In addition, the Δ^5-AD isomerase activity in cytosolic extracts from testes of dexamethasone-treated stallions in vitro decreased by 50% compared to testis extracts from control stallions. This direct pharmacological evidence suggested that the horse may have an alpha-class GST enzyme with similar high steroid isomerase activity as HsaGST A3-3.

In the present investigation the steroid isomerase EcaGST A3-3 from E*quus ferus caballus* has been characterized and shown to be an enzyme efficiently catalyzing the double-bond isomerization of Δ^5-AD to Δ^4-AD and Δ^5-PD to Δ^4-PD. The catalytic efficiency of the equine enzyme matches or exceeds the high activity of the counterpart HsaGST A3-3 from *Homo sapiens*, previously considered the most efficient mammalian steroid isomerase [10]. Comparisons were also made with porcine SscGST A1-1 and SscGST A2-2 from *Sus scrofa domesticus* [12] and bovine BtaGST A1-1 from *Bos taurus* [13].

2. Results

2.1. Expression of mRNA from the equine GSTA3 gene

The horse genome contains several alpha-class GST (*GSTA*) genes, and in order to distinguish the different transcripts it was necessary to determine their sequences and quantify them by RT-PCR, as previously done for the human GSTA3 mRNA [16]. As demonstrated in the case of the human gene [9,10,17] several shorter splice variants were noted in addition to the equine full length mRNA. The concentration of equine GST A3-3 mRNA was measured by quantitative RT-PCR in 15 different tissues from adult horses. The tissues included the adrenal gland, cerebrum, heart, hypothalamus, kidney cortex, liver, lung, mammary gland, ovary, skeletal muscle, small intestine, spleen, urinary bladder, and uterus. Fig. 2 presents the results, with all values normalized relative to the GSTA3 mRNA concentrations in the mammary gland. As expected, the highest levels of GSTA3 gene expression were noted in the steroidogenic tissues ovary, adrenal gland, and testis (Fig. 2a); the lowest levels were several orders below those, such as in mammary

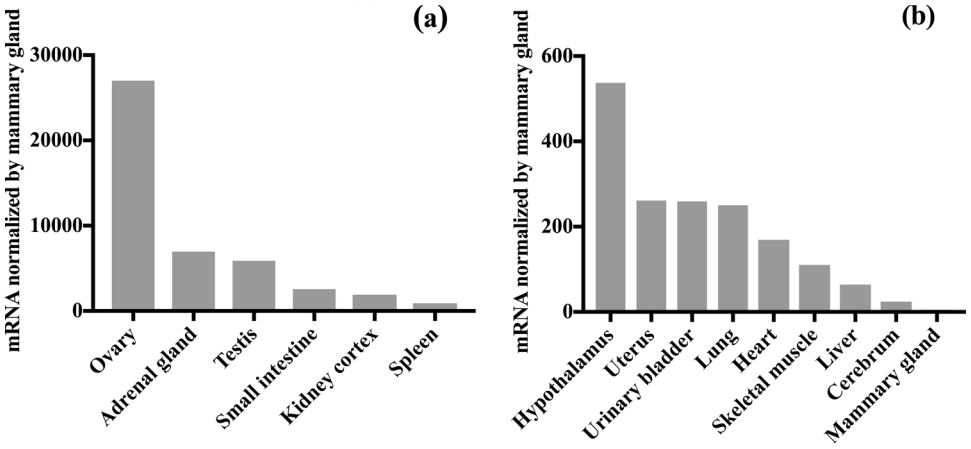

Fig. 2. A comparison of GSTA3 mRNA concentrations across equine tissues. Concentrations were measured in RNA samples from tissues from an adult stallion and a mare by using quantitative RT-PCR and normalized to the value obtained for mammary gland. Results from tissues with higher levels of expression (i.e., more than 550 times higher than in mammary gland) are shown in panel A. The relative levels of GSTA3 mRNA in tissues with lower levels of expression are shown in panel B. Values from left to right: ovary 27000; adrenal gland 6950; testis 5880; small intestine 2570; kidney cortex 1900; spleen 911; hypothalamus 537; uterus 261; urinary bladder 259; lung 250; heart 169; skeletal muscle 110; liver 64; cerebrum 24; mammary gland 1.

gland (Fig. 2b).

The full length *GSTA3* transcript in horse testis is derived from seven exons (NM001283076.1), as does the human GSTA3 mRNA. Alternative splice variants of the equine GSTA3 mRNA included one that was lacking exon 3, a second lacking both exons 3 and 4 (GenBank KC512385), and a third lacking exons 3 to 6 (GenBank KU844109). Analysis of their expression in equine testis by molecular cloning and quantitative RT-PCR demonstrated that the alternative transcripts were present in concentrations several orders of magnitude below that of the full length sequence (data not shown).

2.2. Molecular cloning of EcaGST A3 mRNA from stallion testis

Fresh testicular tissue was obtained by castration of a healthy 6-year old stallion and RNA was isolated from the parenchyma. The *GSTA3* gene is predominantly expressed in steroidogenic tissues [10,12]. In the human genome ten homologous GSTA genes and pseudogenes are clustered on human chromosome 6. Given this multiplicity of related syntenic GSTA sequences, also displayed on chromosome 20 in the horse genome [18], the *GSTA3* gene had not been identified in the horse genome assembly. Therefore, we designed primers to clone the central coding sequence of equine GSTA3 mRNA using the human GSTA3 mRNA as a template. Complementary DNA was generated using purified equine testis RNA and nested PCR by procedures described in the Materials and Methods section. The coding sequence was finally ligated into the plasmid pET-21a(+).

2.3. Expression and basic molecular properties of EcaGST A3-3

EcaGST A3-3 was heterologously expressed in the *Escherichia coli* strain BL21 (DE3) and purified to homogeneity, as described in Materials and Methods. Three L of bacterial culture yielded 26 mg pure protein.

EcaGST A3-3 is postulated to be a dimer in analogy with all other GSTs of the alpha class [19]. Each EcaGST A3-3 subunit contains 222 amino acids including the initial methionine and has the molecular mass of 24420 Da. A molar absorption coefficient of 15.93 mM^{-1} cm^{-1} and an isoelectric point of 8.58 were calculated using the ProtParam tool [20].

The deduced amino acid sequence of EcaGST A3-3 demonstrates high similarity with that of HsaGST A3-3 and of the homologous bovine enzyme BtaGST A1-1 (Fig. 3). The sequence identity between EcaGST A3-3 and HsaGST A3-3 is 80.6% with 179 identical and 28 similar positions, whereas the EcaGST A3-3 and BtaGST A1-1 sequences share 181 identical positions and have 30 similar positions, resulting in an amino acid sequence identity of 81.5%. In general, the equine amino acid sequence had the features characterizing the Alpha class enzymes [21].

The GST proteins have two binding pockets in the active site, the G-site accommodating glutathione and the H-site where the hydrophobic second substrate binds [3,22,23]. Residues of the G-site of HsaGSTA3 include Tyr9, Arg15, Arg45, Gln54, Val55, Pro56, Gln67, Thr68, Asp101, Arg131, and Phe220. All of these residues are conserved in EcaGST A3-3, HsaGST A3-3, and BtaGST A3-3 with the exception of two conservative replacements: the positively charged Lys45 in EcaGST A3-3 and BtaGST A1-1 is Arg45 in HsaGST A3-3 (Fig. 3). Residue 45 in alpha class GSTs forms an ionic bond with the glycine carboxylate of the bound glutathione molecule [23]. A similar conservative replacement is noted in position 68, where EcaGST A3-3 has Ser rather than Thr found in the other sequences (Fig. 3). Both residues commonly occur in this position and both of them form a hydrogen bond from their hydroxyl group to the α-carboxylate of the glutamyl moiety of glutathione [24]. None of these conservative G-site substitutions are expected to have any major functional consequences.

On the other hand, there are more consequential variations in the amino acid residues in the H-site (Fig. 3), the subsite considered to govern the substrate specificity of the GSTs [25]. The possible implications of these differences will be elaborated in the Discussion section.

2.4. Substrate specificity of EcaGST A3-3

Specific activities of EcaGST A3-3 with the two steroid substrates Δ^5-PD and Δ^5-AD and several common electrophilic GST substrates were determined under standard conditions (Table 1). Values for HsaGST A3-3, HsaGST A1-1, SscGST A2-2, SscGST A1-1 and BtaGST A1-1, determined in previous studies [10,12,13,26–29], are included for comparison. The reaction catalyzed with Δ^5-AD is depicted in Fig. 4.

The catalytic activities of EcaGST A3-3 in the steroid isomerization reaction with Δ^5-AD and Δ^5-PD are higher overall than with the other established GST substrates. The alternative reactions include substitution with 1-chloro-2,4-dinitrobenzene (CDNB), double-bond addition with ethacrynic acid and trans-2-nonenal, transcarbamoylation with phenethyl isothiocyanate (PEITC) and sulforaphane, and reduction with cumene hydroperoxide. The specific activities of EcaGSTA3-3 with the alternative substrates are less than a few percent of the isomerization activities.

Compared to HsaGST A3-3 all specific activities of the equine enzyme are similar with the exception of the activity with CDNB which is 4.4-fold lower than that of HsaGST A3-3. This congruity of activities strongly suggests a functional similarity between these two enzymes in a physiological context. In particular, the specific activity of EcaGST A3-3 with Δ^5-AD is comparable to that of HsaGST A3-3, whereas the EcaGST A3-3 is 2.5-fold more active with Δ^5-PD. EcaGST A3-3 thereby takes the place beside HsaGST A3-3 as the most active steroid isomerase among the known GSTs.

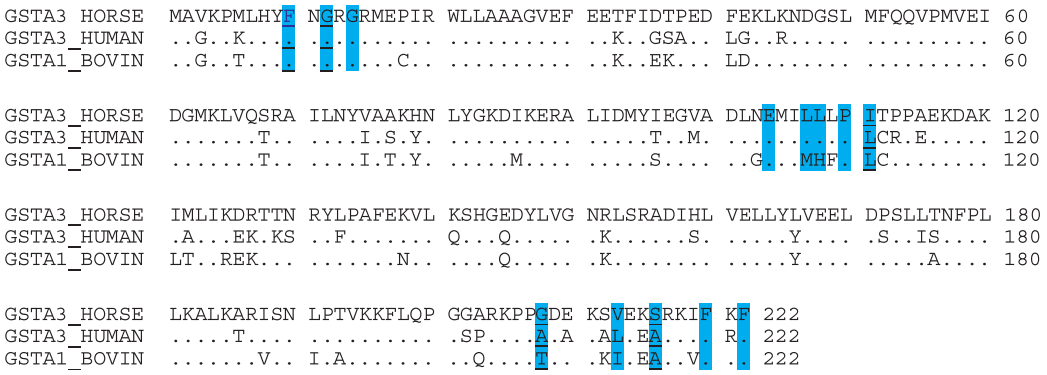

Fig. 3. Amino acid sequence alignment of equine GSTA3, human GSTA3 and bovine GSTA1. A dot indicates identity with the corresponding residue in equine GSTA3. H-site residues of human GSTA3 and corresponding residues of equine GSTA3 and bovine GSTA1 are highlighted. Equine and bovine H-site residues corresponding to those critical for steroid isomerization reaction in human GSTA3 are underlined.

Steady-state kinetic parameters for the steroid isomerase activity of EcaGST A3-3 are given in Table 2. The catalytic efficiency (k_{cat}/K_m) of EcaGST A3-3 with CDNB is merely 7% of that of HsaGST A3-3 due to the high K_m of EcaGST A3-3, which is around 7.5 times higher than that of HsaGST A3-3. Compared with HsaGST A3-3, EcaGST A3-3 has a somewhat higher catalytic constant (k_{cat}) and an almost two-fold lower K_m with Δ^5-AD, yielding a nearly two-fold higher catalytic efficiency. With Δ^5-PD, EcaGST A3-3 exhibits a 4.3-fold higher efficiency compared to HsaGST A3-3, due to a 2.5-fold higher catalytic rate constant and an almost two-fold lower K_m (Table 2). In Fig. 5 the catalytic efficiencies with the steroid substrates of the two GST A3-3 enzymes are compared with values reported for other alpha class GSTs as well as with the efficiency of the steroid isomerase function of human 3β-hydroxysteroid dehydrogenase.

2.5. Alternative thiol cofactor

The double-bond isomerization catalyzed by GSTs is strongly promoted by GSH as a cofactor [25,27]. However, the function of GSH is not that of a substrate, like in other GST reactions, but as a cofactor providing a catalytic thiol group. The alternative thiol cofactor γ-glutamyl-cysteine (γ-Glu-Cys), a naturally occurring biosynthetic precursor of GSH, was also found to support the isomerization of Δ^5-AD (Table 3). EcaGST A3-3 is overall more catalytically efficient with GSH than with γ-Glu-Cys; 6.5-fold more when the Δ^5-AD concentration is varied and concentration of the cofactor GSH or γ-Glu-Cys is kept constant.

3. Discussion

3.1. Expression of the EcaGSTA3 gene in equine tissues

The human GST A3-3 enzyme is predominantly expressed in steroid-hormone producing organs [10], and the equivalent equine enzyme is similarly abundant in ovary, adrenal gland, and testis in comparison to other tissues (Fig. 2). The levels of mRNA are used to estimate levels of gene expression without measuring protein concentrations, since specific antibodies are not available for the equine enzyme. Even some of the homologous human alpha-class GSTs cannot be distinguished by available antibodies [16]. The tissue distribution showing dramatically higher levels in steroidogenic than in other organs supports the notion that GST A3-3 plays an important role in steroid hormone biosynthesis.

Cloning and RT-PCR identified the full length GSTA3 mRNA in stallion testis along with three shorter splice variants. A similar GSTA3 transcript lacking exon 3 was identified in human placenta, and variants lacking both exons 1 and 2 or exons 2 and 3 have also been identified in human sources [9,10]. However, to our knowledge, the equine GSTA3 transcripts lacking exons 3 and 4 and exons 3 to 6 are novel discoveries.

Previously, we localized the *GSTA3* gene expression in stallion testis to the Leydig cells using in situ RT-PCR [15]. These steroidogenic cells constitute only 13% of the testis tissue [30], while male germ cells are considerably more numerous and thereby dilute the level of *GSTA3* gene expression relative to those of ovary and adrenal gland that also show high expression. For illustration of this point, when we examined GSTA3 mRNA levels in the developing testes of goats at 0, 2, 4, 6, and 8 months of age, peak levels were at 2 months of age [31]. This period in time immediately precedes the expansion of germ cells in the seminiferous tubules suggesting that the up-regulation of the expression of

Table 1
Specific activities of equine, human, porcine and bovine GST steroid isomerases.

Specific activity (μmol $mg^{-1}min^{-1}$)							
Substrate (mM)	GSH (mM)	EcaGST A3-3	HsaGST A3 – 3[a]	HsaGST A1 – 1[a]	SscGST A2 – 2[b]	SscGST A1 – 1[c]	BtaGST A1 – 1[d]
Δ^5-AD (0.1)	1.0	194 ± 10	197 ± 15	40	53 ± 2	3.4 ± 0.2	0.73 ± 0.01
Δ^5-PD (0.01)	1.0	92.2 ± 9.5	37.0 ± 2	3.2 ± 0.1	1.9 ± 0.1	0.19 ± 0.02	0.37 ± 0.02
CDNB (1.0)	1.0	5.20 ± 0.64	23.0 ± 2	80	14 ± 1	135 ± 3	6.22 ± 0.1
Ethacrynic acid (0.2)	0.25	0.159 ± 0.014	0.17 ± 0.01	0.2	0.056 ± 0.009	–	–
trans-2-Nonenal (0.1)	0.5	1.23 ± 0.14	2.2 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	0.045 ± 0.004	0.59 ± 0.03
PEITC (0.1)	1.0	3.30 ± 0.11	4.1 ± 0.7	1.7	13 ± 1	9.6 ± 0.2	4.56 ± 0.2[e]
Sulforaphane (0.4)	1.0	1.99 ± 0.43	4.0 ± 0.3	1.9	–	–	–
Cumene hydroperoxide (1.5)	1.0	1.85 ± 0.27	2.6 ± 0.2	10	2.2 ± 0.2	5.8 ± 0.3	3.22 ± 0.31

Values are given per homodimer. Standard deviations (SD) obtained from replicates are given where available.
[a] [10].
[b] [12].
[c] [26].
[d] [13].
[e] PEITC measured at a concentration of 0.4 mM.

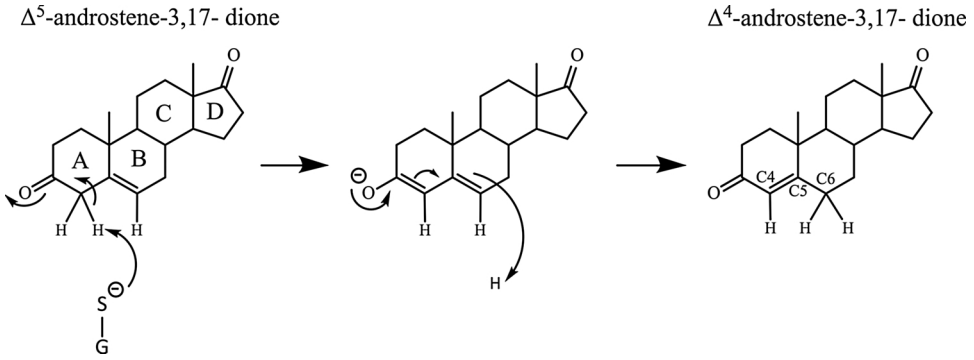

Fig. 4. The steroid double-bond isomerization reaction with the substrate Δ^5-androstene-3,17-dione catalyzed by equine GST A3-3. The enzyme catalyzes also the corresponding reaction with Δ^5-pregnene-3,20-dione.

Table 2
Steady-state kinetic parameters of equine GST A3-3 and human GST A3-3.

Substrate	EcaGST A3-3			HsaGST A3-3[a]			EcaA3-3/HsaA3-3
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	
Δ^5-AD	219 ± 7.9	13.7 ± 1.5	16000 ± 1900	204 ± 22	24 ± 4	8600 ± 800	1.86
Δ^5-PD	134 ± 9.2	9.84 ± 1.5	13600 ± 2300	54 ± 6	17 ± 3	3200 ± 220	4.25
CDNB	22.0 ± 1.2	1490 ± 140	14.8 ± 1.6	44 ± 2	200 ± 20	220 ± 20	0.07
GSH with Δ^5-AD	325 ± 12	608 ± 52	535 ± 50		110 ± 22		

Values are given per homodimer. k_{cat} and K_m are given with standard errors (SE) obtained from nonlinear regression analysis.
[a] [10].

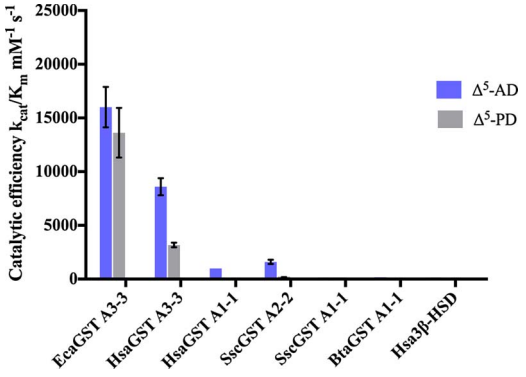

Fig. 5. Catalytic efficiencies of equine GST A3-3 with the steroid substrates Δ^5-androstene-3,17-dione and Δ^5-pregnene-3,20-dione compared to human, porcine and bovine GST steroid isomerases. Also included is human 3β-hydroxysteroid isomerase. Catalytic efficiencies (k_{cat}/K_m) with Δ^5-androstene-3,17-dione (Δ^5-AD) and Δ^5-pregnene-3,20-dione (Δ^5-PD) are given for each homodimer with standard errors (SE) where available, from left to right ($mM^{-1}\,s^{-1}$): EcaGST A3-3$_{\Delta5\text{-AD}}$ 16000 ± 1900, EcaGST A3-3$_{\Delta5\text{-PD}}$ 13600 ± 2300; HsaGST A3-3$_{\Delta5\text{-AD}}$ 8600 ± 800[a], HsaGST A3-3$_{\Delta5\text{-PD}}$ 3200 ± 220[a]; HsaGST A1-1$_{\Delta5\text{-AD}}$ 1000[b]; SscGST A2-2$_{\Delta5\text{-AD}}$ 1600 ± 200[c], SscGST A2-2$_{\Delta5\text{-PD}}$ 170 ± 20[c]; SscGST A1-1$_{\Delta5\text{-AD}}$ 20 ± 2[d], SscGST A1-1$_{\Delta5\text{-PD}}$ 16 ± 7[d]; BtaGST A1-1$_{\Delta5\text{-AD}}$ 49[e], BtaGST A1-1$_{\Delta5\text{-PD}}$ 15[c]; Hsa3β-HSD$_{\Delta5\text{-AD}}$ 28[f], Hsa3β-HSD$_{\Delta5\text{-PD}}$ 9[f].
[a][10], [b][27], [c][12], [d][26], [e][13], [f][48].

the *GSTA3* gene in Leydig cells is critical to the initiation of spermatogenesis.

3.2. The high catalytic efficiency of EcaGST A3-3 in steroid isomerization

Traditionally, GSTs have been regarded as detoxication enzymes, participating in the elimination of toxic electrophiles from the organism. However alternative functions, developed by individual GST enzymes, are emerging [32]. Alpha-class GSTs have been demonstrated to take part in steroidogenesis in several mammalian species including *Homo sapiens* [10,12,13].

Steroidogenesis begins with cholesterol and, via multiple steps, leads to production of steroid hormones such as progesterone and testosterone [33,34] (Fig. 1). One of the late steps in the synthesis of these hormones is formation of Δ^5-unsaturated 3-ketosteroids in a pyridine-nucleotide dependent reaction catalyzed by 3β-hydroxysteroid dehydrogenase [35], followed by a double-bond isomerization. The double-bond isomerization from Δ^5-PD to Δ^4-PD is the last step in progesterone biosynthesis. In the synthesis of testosterone, the double-bond isomerization from Δ^5-AD leads to the last precursor of testosterone, Δ^4-AD (Fig. 1).

3β-Hydroxysteroid dehydrogenase possesses double-bond isomerization activity, but the catalytic efficiency (k_{cat}/K_m) with Δ^5-AD reported for the dehydrogenase is 230-fold lower than that of HsaGST A3-3 [10]. We now report on an equine steroid double-bond isomerase featuring an almost two-fold more efficient Δ^5-AD isomerization and 4.3-fold more efficient Δ^5-PD isomerization than reported for HsaGST A3-3. The high catalytic efficiency demonstrated with both steroid substrates ranks EcaGST A3-3 as the most efficient steroid isomerase

Table 3
Steady-state kinetic parameters of steroid isomerization catalyzed by equine GST A3-3 with the alternative thiol cofactors γ-Glu-Cys and GSH.

Substrate	Specific activity (μmol $mg^{-1}min^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	Ratio k_{cat}/K_m GSH/γGLU-Cys[a]
Δ^5-AD (1 mM γ-Glu-Cys)	30.1 ± 2.7	22.2 ± 1.7	9.02 ± 2.5	2460 ± 700	6.50
γ-Glu-Cys (0.2 mM Δ^5-AD)		122 ± 36	4290 ± 1600	28.4 ± 13	18.8

Values are given per homodimer. Specific activity is given with standard deviations (SD) obtained from replicates. k_{cat} and K_m are given with standard errors (SE) obtained from nonlinear regression analysis.
[a] Values for GSH from Table 2.

known among mammals (Fig. 5).

The first report of the enzymatic 3-ketosteroid double-bond isomerization was published by Talalay and Wang [36]. Activity in rat liver was noted, but focus was directed to the bacterial enzyme isolated from *Pseudomonas*. The bacterial 3-ketosteroid isomerase has subsequently been extensively researched as one of the most efficient enzymes in all categories. In a recent study, the catalytic efficiency in *P. putida* was determined to be $4.1 * 10^8\ M^{-1}\ s^{-1}$ [37].

The k_{cat}/K_m values of highly efficient enzymes acting on their natural substrates are generally in the range of 10^6–$10^8\ M^{-1}\ s^{-1}$ [38]. The catalytic efficiency of EcaGST A3–3 with Δ^5–AD is $8.0 * 10^6$ and with Δ^5–PD $6.8 * 10^6\ M^{-1}\ s^{-1}$ per monomer of the dimeric enzyme. Expressed on the basis of the natural dimeric state of the enzyme the k_{cat}/K_m values are $1.60 * 10^7$ and $1.36 * 10^7\ M^{-1}\ s^{-1}$, respectively, placing the enzyme among the most efficient enzymes. These data support the view that the GST A3-3 enzyme has evolved for physiologically significant steroid isomerase activity.

3.3. Structural basis of the differences in isomerization activity among alpha class GSTs

The high steroid isomerization activity of the equine EcaGST A3-3 is not remarkable given its high amino acid sequence similarity to the highly efficient human steroid isomerase HsaGST A3-3 (Fig. 3). However, the amino acid sequence similarity is equally high between the equine and the bovine isomerases (Fig. 3). Unexpectedly, the homologous bovine GST A1-1 (BtaGST A1-1), similarly expressed in endocrine tissues such as testis, ovary, and adrenal gland, demonstrated a very modest steroid isomerase activity [13]. However, we cannot exclude the possibility that the bovine genome encodes an as yet undiscovered GST with high activity. The structural explanation of the low steroid isomerase activity of BtaGST A1-1 presumably lies in the structure of the H-site, the hydrophobic binding site of the steroid (see below). By contrast, the nine amino acids in the G-site (see Results) are functionally conserved in the above alpha-class GST proteins across the three species (Fig. 3).

The H-site is formed by thirteen amino acids (Table 4) of which five, residues 10, 12, 111, 208, and 216, have been shown to be critical for the difference in steroid isomerase activity between the efficient HsaGST A3-3 and the 5000-fold less active HsaGST A2-2 [25,39]. Some of the H-site residues of HsaGST A3-3 were mutated to those of the poorly active HsaGST A2-2 to probe their individual contribution to the steroid isomerase activity [25]. The HsaGST A3-3 mutant Ala216Ser had 90% of the wild-type k_{cat}/K_m value, the mutant Phe10Ser had 34%, and the double mutant Phe10Ser/Ala216Ser had 26% of the wild-type k_{cat}/K_m value. The mutation Leu111Phe was more detrimental giving only 4% of the steroid isomerase residual activity, and the triple mutant had a mere 0.8% of the catalytic efficiency. None of the five mutants showed a major change in K_m. These results applied to the comparison of the EcaGST A3-3 and HsaGST A3-3 suggest that Ser216 replacing Ala216 is not causing the higher efficiency of the equine enzyme. Based on the structure of the human enzyme [40], residue 216 is located below the plane of the steroid substrate and distant from the atoms in the A and B rings undergoing isomerization (Fig. 4). The additional three H-site residues 111, 208, and 213 of EcaGST A3-3 may all provide a more facile accommodation of the steroid substrate, as reflected in its lower K_m value (Table 2).

On the other hand, mutations in HsaGST A2-2 to mimic the active site with corresponding residues from HsaGST A3-3 led to a 3500-fold increase of the k_{cat}/K_m value [39].None of the H-site residues are directly implicated in the catalytic mechanism [41], but they obviously help to orient the substrate for catalysis to occur. Fig. 6 shows the tight binding of a steroid ligand in the H-site of HsaGST A3-3 as determined by X-ray crystallography [40]. Residue 208 seems to be crucial, being close to and directed towards the D-ring of the steroid. Steric interference by the bulky Met208 in HsaGST A1-1 may be a reason for this enzyme showing only 12% of the HsaGST A3-3 catalytic efficiency (Table 4). The hydroxyl group of Thr208 in SscGST A2-2 could possibly hydrogen bond to the keto group of the D-ring and lower the efficiency to 19% of the HsaGST A3-3 value, even though all other H-site residues are identical to those of the human enzyme. The much lower activity of SscGST A1-1 is probably caused by substituting Ile222 for Phe222, present in all other enzymes. Phe222 stacks with the substrate A-ring undergoing chemical transformation and shields the reactive bonds from the surrounding medium [40]. The loss of the phenyl group combined with steric hindrance caused by Met208 could explain the 80-fold lower catalytic activity of SscGST A1-1 compared with that of SscGST A2-2 (Table 4).

The bottom line is that the H-site probably governs the orientation of the steroid substrate to provide a suitable binding mode with respect to the glutathione molecule and Tyr9 of HsaGST A3-3 that serve as mechanistic devices in the catalysis [41]. Crystal structures of HsaGST A2-2 and HsaGST A3-3 show that each binds the steroid very differently. For example, in HsaGST A2-2 the double bond that undergoes isomerization is too distant for effective catalysis [40]. Nevertheless, residues far away from those contacting the substrate in the H-site may

Table 4
H-site residues of equine (EcaGST A3-3), human (HsaGST A3-3, HsaGST A1-1, HsaGST A2-2), porcine (SscGST A2-2, SscGST A1-1) and bovine (BtaGST A1-1) enzymes. $k_{cat}/K_m{}^{\Delta5-AD}$ values for each steroid isomerase are given for reference.

Position	EcaGST A3-3	HsaGST A3-3	HsaGST A1-1	HsaGST A2-2	SscGST A2-2	SscGST A1-1	BtaGST A1-1
10	F	F	F	S	F	F	F
12	G	G	A	I	G	G	G
14	G	G	G	G	G	G	G
104	E	E	E	E	E	E	E
107	L	L	L	L	L	L	M
108	L	L	L	L	L	L	H
110	P	P	P	P	P	P	P
111	I	L	V	F	L	L	L
208	G	A	M	M	T	M	T
213	V	L	L	L	L	L	I
216	S	A	A	S	A	A	A
220	F	F	F	F	F	F	F
222	F	F	F	F	F	I	F
$k_{cat}/K_m{}^{\Delta5-AD}$ ($mM^{-1}\ s^{-1}$), monomer	8000	4300[a]	500[b]	1.0[b]	800[c]	10[d]	24[e]

[a] [10].
[b] [27].
[c] [12].
[d] [26].
[e] [13].

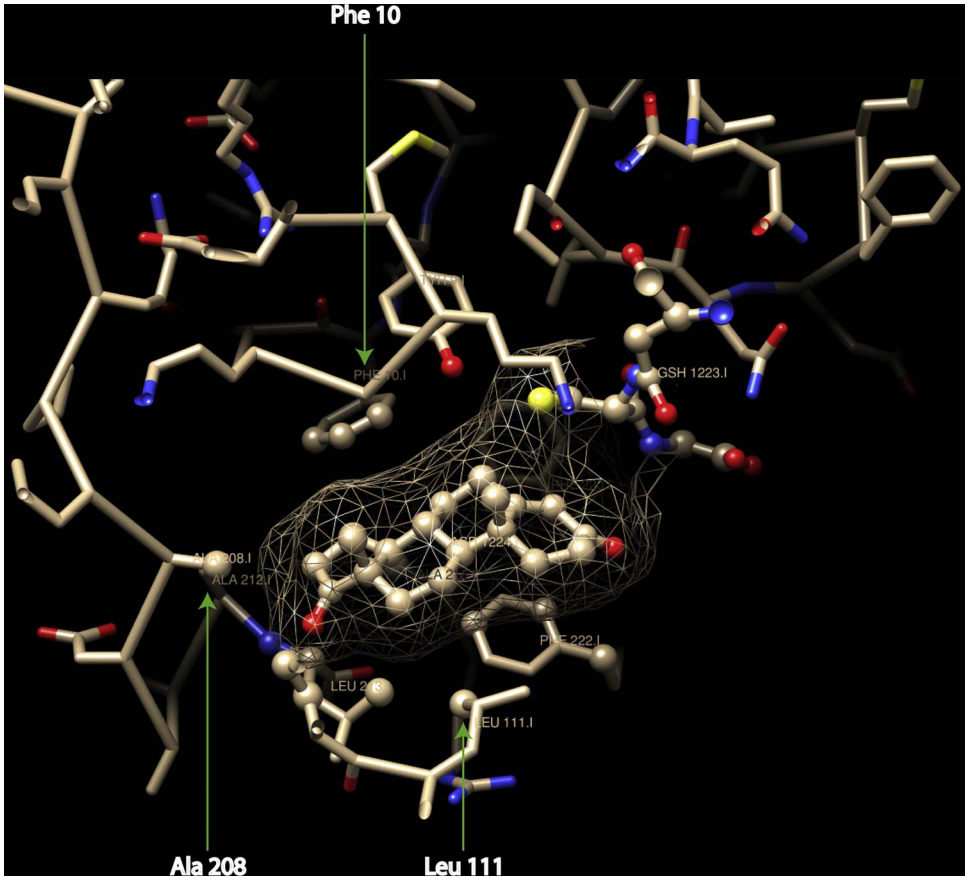

Fig. 6. Steroid binding to the H-site of GST A3-3. The figure is based on the crystal structure of the human enzyme in complex with Δ^4-androstene-3,17-dione and glutathione [40]. Atoms ≤ 4.5 Å from the steroid are shown as balls in the stick representations of the amino acid residues. Three crucial residues, Phe10, Leu111, and Ala208 are indicated by green arrows. The catalytically important sulfur (yellow) of glutathione (GSH) and oxygen (red) of Tyr9 are located above C4 and C6 of the steroid skeleton. The image was created with the UCSF Chimera package (http://www.rbvi.ucsf.edu/chimera) developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

also contribute to catalytic efficiency as shown for other GSTs [42]. However, such indirect influences in GST A3-3 remain to be found.

3.4. Alternative thiol cofactors in the steroid isomerization

The role of glutathione in the steroid isomerase activity is primarily to serve as an acid-base catalyst [27]. First, it mediates the removal of a proton at C4 of the substrate and then promotes the insertion of a proton at C6 to form the product (Fig. 4). In addition, computational studies show that the NH of the C-terminal Gly residue of the glutathione molecule forms a hydrogen bond to the oxygen on C3, thereby providing stabilization of the transient dienolate in the reaction trajectory [41]. Our experimental finding that the glutathione analog γ-Glu-Cys, which lacks Gly and thereby the NH bonding ability, gives a 10-fold lower k_{cat} than the value for glutathione (Tables 2 and 3) strongly supports this notion and suggests that the equine and human GST A3-3 enzymes are similar also in this respect. The ratio of the k_{cat}/K_m values for the two alternative thiol cofactors $k_{cat}/K_m{}^{GSH}/k_{cat}/K_m{}^{\gamma-Glu-Cys}$ = 16000 $mM^{-1}\,s^{-1}$/2460 $mM^{-1}\,s^{-1}$ = 6.5 (Tables 2 and 3) allows calculation of the ΔΔG for the incremental contribution of the Gly residue of glutathione as RTln(6.5) = 1.1 kcal/mol (4.6 kJ/mol).

In the cell, the synthesis of γ-Glu-Cys is performed by γ-glutamylcysteine ligase and constitutes the rate-limiting step of glutathione biosynthesis [3]. This immediate precursor is then coupled with Gly in the last step of the glutathione biosynthesis, which is catalyzed by glutathione synthetase. Notwithstanding its natural occurrence, the cellular levels of γ-Glu-Cys are several orders lower than the millimolar glutathione concentrations [3], and γ-Glu-Cys cannot contribute significantly to the physiological steroid isomerization reaction. The K_m value for GSH is approximately 0.6 mM (Table 2) suggesting that EcaGST A3-3 is essentially saturated with GSH in cells.

3.5. GST A3-3 in a physiological context

The present study reveals the high steroid isomerase activity of EcaGST A3-3, thereby reinforcing the notion that the GST family encompasses a versatile functional repertoire that is not restricted to detoxication. The EcaGST A3-3 and HsaGST A3-3 enzymes now rank as the most efficient steroid isomerases known in mammals, with activities approaching that of the bacterial ketosteroid isomerase, one of the most efficient enzymes known today.

In summary, the results reported here add to the growing body of evidence that GST A3-3 plays a prominent role in steroid hormone biosynthesis in the horse as well as in humans. A requisite feature of the enzyme is the outstandingly high double-bond isomerase activity with the pertinent steroid substrates (Table 1 and Table 2; [10]). In human steroidogenic cell lines suppression of GST A3-3 activity by enzyme inhibitors or by siRNA downregulation were shown to impede steroid hormone production [11]. In the siRNA knockdown experiments it was shown that GSTA3 mRNA was affected without suppressing 3β-hydroxysteroid dehydrogenase mRNA [11]. Furthermore, experimental cellular modulation of steroidogenic factor-1 (SF-1) caused up- or down-regulation of GSTA3 gene expression, and SF-1 was shown to bind to the GSTA3 gene promoter [43]. In addition, coordination between the enzyme activities of 3β-hydroxysteroid dehydrogenase II, which catalyzes the oxidation of the 3β-hydroxy group immediately preceding the isomerization reaction, and GST A3-3 was demonstrated. At the tissue level GSTA3 gene transcript levels were particularly prominent in steroidogenic organs such as testis, ovary, and the adrenal gland of both horse (Fig. 2) and humans [10]. Even though it is well established that mRNA expression is not necessarily an accurate measure of the corresponding protein concentration [44] the high GSTA3 mRNA concentrations are strong indicators of an involvement in steroidogenesis. Finally, at the organism level, administration of the synthetic

glucocorticoid dexamethasone diminished testosterone production, lowered GSTA3 mRNA expression, and decreased steroid isomerase activity in testis extracts [15].

An animal model is needed to study the regulation of *GSTA3* gene expression, differential splicing, as well as enzyme activity in relation to steroid biosynthesis and various diseases. The similarities between the orthologous equine and human enzymes suggest that endocrine conditions in both species can be approached by similar pharmacological interventions targeting GST A3-3. In contrast, rodents use the Δ^4 pathway of steroid biosynthesis unlike most domestic animals and man, which use the Δ^5 pathway [14]. Rodents apparently lack a *GSTA3* gene homolog that encodes a protein with high steroid isomerase activity. Thus the discovery of the steroid isomerase GST A3-3 in the stallion testis opens new avenues to investigations in molecular endocrinology with relevance to both veterinary and human medicine.

4. Materials and methods

4.1. Molecular cloning of equine GSTA3 mRNA

A 6-year old American Quarter Horse stallion with normal semen parameters was castrated using a protocol approved by the Texas A&M University Animal Care Committee [15]. To clone the GSTA3 mRNA, RNA was isolated from testis parenchyma using TriPure reagent. The equine like the human genome encompasses numerous homologous GST genes, and the equine *GSTA3* gene had consequently not been identified in the horse genome assembly. Therefore, we designed primers to clone the central coding DNA sequence (cds) of equine GSTA3 mRNA using the human GSTA3 mRNA sequence as a substitute (GenBank accession no. NM_000847.4). Complementary DNA was generated using 1 μg testis RNA, Superscript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA), and oligo-dT_{20} and random octamer primers [15]. The initial PCR used two GSTA3 primers: sense (5′-AAG CCCAAGCTTCACTACTTCAATGGA) and antisense (5′-CTGGTTTTCAG GGCCTTCAG) and Takara ExTaq DNA polymerase (Clontech Laboratories, Mountain View, CA). The PCR program included thirty cycles of 95 °C denaturation for 15 s, 50 °C annealing for 1 min, and 74 °C elongation for 1 min. Then, secondary PCR used 10% of the initial PCR product with nested primers: sense (5′-CGAGGCCGGATGGAGCCTAT) and antisense (5′-CAAGGTAGAGAAGTTCCACCAGGTG). The DNA band of expected size (450 bp) was gel purified, TA-cloned into pCR 2.1 (Invitrogen, Carlsbad, CA) and sequenced.

Based on this horse GSTA3 sequence of the central cds, we designed primers for 5′ and 3′ RACE in order to obtain 5′ and 3′UTR sequences, respectively [45]. The commercially available 5′ RACE System for Rapid Amplification of cDNA Ends (v.2) and 3′RACE System for Rapid Amplification of cDNA Ends (v.1) from Invitrogen were used. For RACE cloning of GSTA3 mRNA, reverse transcription was performed, as above but with an antisense GSTA3 primer for 5′ RACE (5′-GGCAACGTAGT TGAGAATGGCTCTG) or oligo-dT_{20} for 3′ RACE. The 5′RACE cDNA products were C-tailed. Nested PCR was performed as described above. The first PCR for 5′ RACE used an antisense GSTA3 primer (5′-GCTCT GCACCAGCTTCATCCCATC) and "InvAAP" (5′-GGCCACGCGTCGACTA GTACGGGIIGGGIIGGGIIG), with the underlined sequence complementary to the C-tails. The second PCR reaction amplified cDNA from 10% of the first PCR reaction. The second PCR for 5′ RACE used a nested antisense GSTA3 primer (5′-GCGTCGACCATTGGCACTTGCTGG AACATCAAACT) and "InvAUAP" (5′-GGCCACGCGTCGACTAGTA), with the Sal I cloning sites underlined. The first PCR for 3′RACE used a GSTA3 sense primer (5′-ACAGGCTGAGCAGGGCTGACAT) and oligo-dT_{20}, while the second PCR used the sense GSTA3 nested primer (5′-ACATTCACCTGGTGGAACTTCTCTACTAT) and oligo-dT_{20}. Products were gel purified, cloned and sequenced as above.

These 5′ and 3′ UTR sequences were used to design primers to clone the entire cds of the horse GSTA3 mRNA. Reverse transcription was performed as described above but with oligo-dT_{20} and random octamer primers [15]. The first PCR reaction was performed as above but with sense (5′ − GGAGACTGCATCATGGCAGTGAAGCCCATG) and antisense (5′ − CTTCAGAAGATCGGTCCTGCCTG) primers. The second PCR used nested primers bearing restriction enzyme sites for cloning (underlined); 5′ − GCGAATTCATGGCAGTGAAGCCCATGCTTCACTAC TTCAATGG and 5′ − CGCTCGAGCTGGGGGCCAGGCCTGCTTTGTCAA AACTT. The PCR band and the plasmid pET-21a(+) (EMD Millipore, Billerica, MA) were digested with EcoRI and XhoI, gel purified and ligated. Plasmid minipreps were sequenced to confirm that the entire GSTA3 coding sequence (GenBank accession no. KC512384.1) was present and in frame with the T7-tag on the N-terminus and the natural stop codon of GSTA3 mRNA.

4.2. Determination of GSTA3 mRNA concentrations in various horse tissues

Quantitative RT-PCR was performed to compare GSTA3 mRNA concentrations between testis and 14 different normal tissues collected from adult horses. Reverse transcription was done as described in the previous section, but with 10-fold less input RNA. PCR was performed with POWER SYBR Green Master Mix (ABI) and GSTA3 primers (sense 5′ − ACATCCACCTGGTGGAACTTCTCTACCTT and antisense 5′ − CTGGTTTTCAGGGCCTTCAG) in triplicate, as described previously [15]. Similarly, concentrations of 18S rRNA (HUGO symbol RN18S1) were measured for use as a normalizer. The 18S rRNA primers were sense 5′ − GCGCCGCTAGAGGTGAAAT and antisense 5′ − CATTCTT GGCAAATGCTTTCG and the reverse transcription reactions were diluted 1:65,000 to get the C_ts in the range of those of GSTA3 mRNA. The PCR amplification efficiencies of the GSTA3 and 18S rRNA amplicons were assessed as described by [31]. The efficiencies were similar and both exceeded 90%.

4.3. Heterologous protein expression and purification

The expression clone EcaGSTA3-pET-21a was introduced into *E. coli* BL21 (DE3) by means of heat shock electroporation at 42 °C. The transformed *E. coli* was grown overnight on agar plates containing 50 μg/mL ampicillin at 37 °C. Isolated colonies were grown overnight in lysogeny broth [46] [LB: 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl] with the same ampicillin concentration in a shaking incubator at 200 rpm, 37 °C. The culture was diluted 70-fold and grown to A_{600} = 0.55. Protein expression was induced by addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.5 mM. After 3 h of growth in a shaking incubator at 200 rpm, 37 °C, the cells were harvested by 15 min centrifugation at 7000*g*.

Cells were lysed by ultrasonication [Vibracell High intensity UltraSonic Liquid Processor VCX 130, converter model CV18, amplitude 080, 5 × 20 s (Sonics & Materials, Inc; Newtown, CT)] and centrifuged 1 h at 27,000*g*. EcaGST A3-3 was purified from the supernatant by affinity chromatography using GSH-Sepharose (GE Healthcare Life Sciences). The purification procedure was as follows: the column was first equilibrated with binding/washing buffer (10 mM Tris-HCl pH 7.8, 0.2 M NaCl, 0.2 mM DTT and 1.0 mM EDTA). After application of the lysate, the column was washed repeatedly with the same buffer. EcaGST A3-3 was eluted with 20 mM glutathione in binding/washing buffer and dialyzed overnight against 3 L dialysis buffer (10 mM Tris-HCl pH 7.8, 0.2 mM DTT and 1.0 mM EDTA) and again against 2 L dialysis buffer during the day. The purity of the dialyzed protein was checked with SDS-PAGE.

4.4. Kinetic measurements

Substrates were purchased from Sigma-Aldrich, with the exception of Δ^5-AD and Δ^5-PD, which were obtained from Steraloids Inc. (Newport, RI). All substrates except GSH were dissolved in organic solvents (ethanol, methanol, or acetonitrile). The final solvent concentration in the reaction system was kept at a maximum of 5% (v/v).

Specific activities were determined using the following conditions: 0.1 mM Δ^5-AD or 0.01 mM Δ^5-PD with 1 mM GSH in 25 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 2% methanol; 1 mM CDNB with 1 mM GSH in 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 and 2% ethanol; 0.2 mM ethacrynic acid (EA) with 0.25 mM GSH in 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 and 2% acetonitrile; 0.1 mM trans-2-nonenal (Non) with 0.5 mM GSH in 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 and 2% acetonitrile; 0.1 mM phenethyl isothiocyanate (PEITC) with 1.0 mM GSH in 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 and 2% acetonitrile; 0.4 mM sulforaphane (SR) with 1.0 mM GSH in 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 and 2% acetonitrile; 1.5 mM cumene hydroperoxide (CHP) with 1.0 mM GSH, 0.1 mM NADPH and 0.3 unit/mL glutathione reductase in 100 mM Na_2HPO_4/NaH_2PO_4 pH 7.0 and 2% acetonitrile; 1.0 mM γ-glutamyl-cysteine with 0.1 mM Δ^5-AD in 25 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 2% methanol.

To determine the steady-state parameters, rate saturation curves were obtained using the following conditions: Δ^5-AD varying between 0.003 mM and 0.1 mM with 1 mM GSH in 25 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 2% methanol; Δ^5-PD varying between 0.002 mM and 0.02 mM with 1 mM GSH in 25 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 2% methanol (due to low solubility it was not possible to further increase the concentration of Δ^5-PD); CDNB varying between 0.05 mM and 1.5 mM with 5 mM GSH in 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 and 5% ethanol; GSH varying between 0.02 mM and 1.5 mM with 0.2 mM Δ^5-AD in 25 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 2% methanol; Δ^5-AD varying between 0.003 mM and 0.1 mM with 1 mM γ-glutamyl-cysteine in 25 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 2% methanol; γ-glutamyl-cysteine varying between 0.2 mM and 1.5 mM with 0.2 mM Δ^5-AD in 25 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 and 2% methanol.

Due to the high activity of EcaGST A3-3 with steroid substrates the enzyme had to be diluted to nanomolar concentrations. With these low concentrations, adsorption of the enzyme to the inner walls of various test tubes caused irreproducible activity and kinetic measurement results [47]. After numerous trials with tubes from different manufacturers, Sarstedt micro-tubes 1.5 mL EASY-CAP (ref. no. 72.690.550) were found not to adsorb the enzyme and generated consistent results, and were subsequently used.

The reaction rates were monitored spectrophotometrically at 30 °C using a Shimadzu UV-2501 PC spectrophotometer, Shimadzu Inc. All experiments were performed twice with triplicate measurements in each data point. Molar absorption coefficients used for calculations were: $\varepsilon_{248} = 16.3\ mM^{-1}\ cm^{-1}$ for Δ5-AD, $\varepsilon_{248} = 17.0\ mM^{-1}\ cm^{-1}$ for Δ5-PD, $\varepsilon_{340} = 9.6\ mM^{-1}\ cm^{-1}$for CDNB, $\varepsilon_{270} = 5.0\ mM^{-1}\ cm^{-1}$ for EA, $\varepsilon_{225} = -19.2\ mM^{-1}\ cm^{-1}$ for Non, $\varepsilon_{274} = 8.89\ mM^{-1}\ cm^{-1}$ for PEITC, $\varepsilon_{274} = 8.0\ mM^{-1}\ cm^{-1}$ for SR, $\varepsilon_{340} = -6.2\ mM^{-1}\ cm^{-1}$ for CHP. Protein concentration was determined by means of the Bradford assay.

4.5. Data analysis

Steady-state kinetic parameters were determined by fitting the Michaelis-Menten equation to the data points using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

4.6. Accession numbers

The following accession numbers were used:

EcaGSTA3 coding sequence (GenBank accession no. NM_001283076.1), alternative splice variant of EcaGSTA3 mRNA lacking exons 3 and 4 (GenBank accession no. KC512385), alternative splice variant of EcaGSTA3 mRNA lacking exons 3 to 6 (GenBank accession no. KU844109), HsaGSTA3 mRNA (GenBank accession no. NM_000847.4), and amino acid sequences for: EcaGST A3 (UniProtKB accession no. M9ZT87), HsaGST A3 (UniProtKB accession no. Q16772), HsaGST A1 (UniProtKB accession no. P08263), HsaGST A2 (UniProtKB accession no. P09210), SscGST A1 (UniProtKB accession no. F1S7D3), SscGST A2 (UniProtKB accession no. Q29057) and BtaGST A1 (UniProtKB accession no. Q28035).

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