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The effects of mutating Tyr9 and Arg15 on the structure, stability, conformational dynamics and mechanism of GSTA3-3

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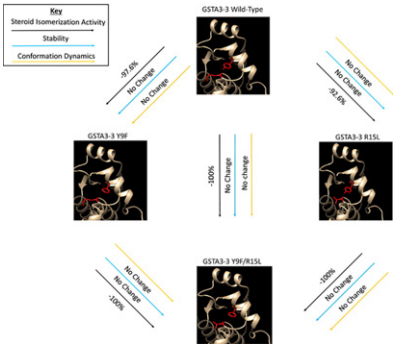
HIGHLIGHTS

- Arg15 is a critical active-site residue of GSTA3-3.
- Arg15 may be critical in differentiating substrate from product.
- Arg15 is more important than Tyr 9 for lowering the pK_a of GSH.
- Neither Y9F nor R15L mutations alter protein stability or conformational dynamics.
- Breaking the Arg15-Glu104 salt-bridge does not alter stability.

GRAPHICAL ABSTRACT



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ABSTRACT

Glutathione *S*-transferase A3-3 is the most catalytically efficient steroid isomerase enzyme known in humans, transforming Δ^5-androstene-3-17-dione into Δ^4-androstene-3-17-dione. GSTA3-3 catalyzes this reaction with ten-fold greater efficiency than GSTA1-1, its closest competitor in the Alpha class of GSTs. In order to examine the differences between Alpha class GSTs and to better elucidate the mechanism of GSTA3-3 the roles of Tyr9 and Arg15 were examined. Tyr9 is the major catalytic residue of Alpha class GSTs and Arg15 is proposed to be catalytically important to GSTA3-3 but never before experimentally examined. While the structure and stability of the Alpha class enzymes are highly comparable, subtle differences at the G-site of the enzymes account for GSTA3-3 having a ten-fold greater affinity for the substrate GSH. Y9F and R15L mutations, singly or together, have no effect on the structure and stability of GSTA3-3 (the same effect they have on GSTA1-1) despite the R15L mutation removing an interdomain salt-bridge at the active site. Hydrogen-deuterium exchange mass spectrometry also revealed that neither mutation had a significant effect on the conformational dynamics of GSTA3-3. The R15L and Y9F mutations are equally important to the specific activity of the steroid isomerase reaction; however, Arg15 is more important for lowering the pK_a of GSH. Lowering the pK_a of GSH being how GSTs catalyze their reactions. Additionally, there is evidence to suggest that Arg15 is integral to allowing GSTA3-3 to differentiate between Δ^5-androstene-3-17-dione and Δ^4-androstene-3-17-dione, indicating that Arg15 is a more important active-site residue than previously known.

Abbreviations: CD, circular dichroism; CDNB, 1-chloro-2, 4-dinitrobenzene; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; GST, glutathione *S*-transferase; HDX-MS, hydrogen–deuterium exchange mass spectrometry; ITC, isothermal titration calorimetry; PDB, Protein Data Bank; Δ^4-AD, Δ^4-androstene-3-17-dione; Δ^5-AD, Δ^5-androstene-3-17-dione.

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Enzymes are perhaps the most vital component of life and have been refined over millennia to maximize the efficiency of biological systems. The glutathione *S*-transferase superfamily (GSTs, EC 2.5.1.18) is one such group of enzymes. Divided into over 13 classes of cytosolic enzymes, the members of this protein superfamily all share a highly conserved topological fold and mechanistic role [1,2]. Common to almost all aerobic organisms [3], GSTs play a vital role in the detoxification of endogenous and exogenous compounds [1]. GSTs are dimeric enzymes composed of two subunits [4,5]. Each subunit, in turn, is composed of a highly conserved thioredoxin-like domain containing the glutathione-binding site (G-site) and a less highly conserved all alpha helical secondary domain which makes up most of the H-site (Fig. 1). The detoxification reaction involves the conjugation of a diverse range of nonpolar electrophilic compounds (which bind at the H-site) to the tripeptide co-substrate glutathione (GSH) of the enzyme, which binds at the G-site. The enzyme promotes the conjugation reaction by lowering the pK_a of GSH; thus, reducing the energetic barrier for the deprotonated GS^- anion to attack the H-site substrate [6]. The conjugated product is, in general, less reactive and more soluble and can be further metabolized and eventually excreted by the organism [5].

While it is not unexpected for proteins belonging to the same superfamily to share similar reaction chemistry, and even common mechanistic pathways [7], new research continues to bring to light additional functions of the GST enzymes. They are known to play a role in ligand transport [8–11] and to interact with the protein kinases of signal transduction pathways [12,13], amongst other functions [14]. Furthermore, in some instances, the GST enzymes are promiscuous, having the capacity to catalyze supplementary reactions in addition to their main, classical catalytic activity [7,15]. In some instances, the secondary role of GSTs becomes more important than its classical detoxification role.

An example of this behavior is the Alpha class of GSTs [16,17]. All the enzymes in this class are, in addition to their classical role, able to catalyze a steroid isomerization reaction. Specifically, they catalyze the isomerization of the β,γ double bond of Δ^5-androstene-3,17-dione (Δ^5-AD) to the α,β isomer Δ^4-androstene-3,17-dione (Δ^4-AD). This reaction is critical in metabolic pathways that produce steroid hormones such as testosterone and progesterone from cholesterol. However, the Alpha GSTs perform each catalytic role with widely varying levels of efficiency. GSTA3-3 displays the highest isomerase activity in the alpha class; ten-fold greater than GSTA1-1 and 5000-fold greater than GSTA2-2 [18]. Inevitably though, as GSTA3-3 has evolved to optimize its isomerase activity, its efficiency regarding its classical detoxification role has diminished. GSTA3-3 also has the lowest activity in its class for to the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to GSH which is representative of its ability to act as a detoxification enzyme [18]. These significant differences in the capabilities of the enzymes exist despite sharing >80% sequence identity. In fact, it has already been shown that just five point mutations in the H-site of the enzyme accounts for the majority of these differences [18].

GSTA3-3 is the most efficient steroid isomerization enzyme in the Alpha class and a major player in steroid isomerization in humans. It has, therefore, has attracted significant interest and research as a potential therapeutic drug target in the treatment of diseases characterized by excessive steroid hormone production such as polycystic ovary syndrome, Cushing's syndrome, congenital adrenal hyperplasia and some cancers of the sex organs [19]. Rational drug design would be greatly aided by a thorough understanding of the reaction mechanism of GSTA3-3. Additionally, knowing the differences that exist between the GST enzymes is important in avoiding any unwanted side-effects.

Nevertheless, there are many questions regarding the reaction mechanism. Currently, there are two major competing viewpoints regarding the reaction mechanisms (Fig. 2) [19–22], which have left several details unresolved. These are: (i) does the reaction proceed through a dienolate intermediate and, if so, how is it stabilized? (ii) what is the role of the GSH cofactor? (iii) what is the role of Tyr9? (iv) are there any other active site residues important to catalysis? In regards to the first question, more recent evidence has suggested that Scheme 1 may also proceed via an enforced concerted mechanism with the putative intermediate existing for only a short time (less than ~10^{-13} s) [19,23]. In order to address the questions above, this study examined the enzymatic properties of the wild-type, Y9F, R15L and Y9F/R15L GSTA3-3 enzymes. Arg15 was chosen to be examined as it has long been suspected to play a catalytic role, lowering the pK_a of GSH, as it does in GSTA1-1 [24,25]. However, the Arg15 residue of GSTA3-3 has as yet, never been experimentally examined.

Many aspects of GSTA3-3, such as stability and substrate affinities, have also gone un-investigated. Instead researchers have inferred information about GSTA3-3 from our knowledge of the rest of the GST Alpha class. Given the highly conserved sequence identity and topological fold within the Alpha class, such inference is justified. However, given the evolutionary divergence between GSTA3-3 and the rest of the GST Alpha class, there may be significant differences which have gone unnoticed. In support of this supposition, it is worth noticing that despite all the Alpha class GSTs sharing an identical G-site, GSTA3-3 is reported to

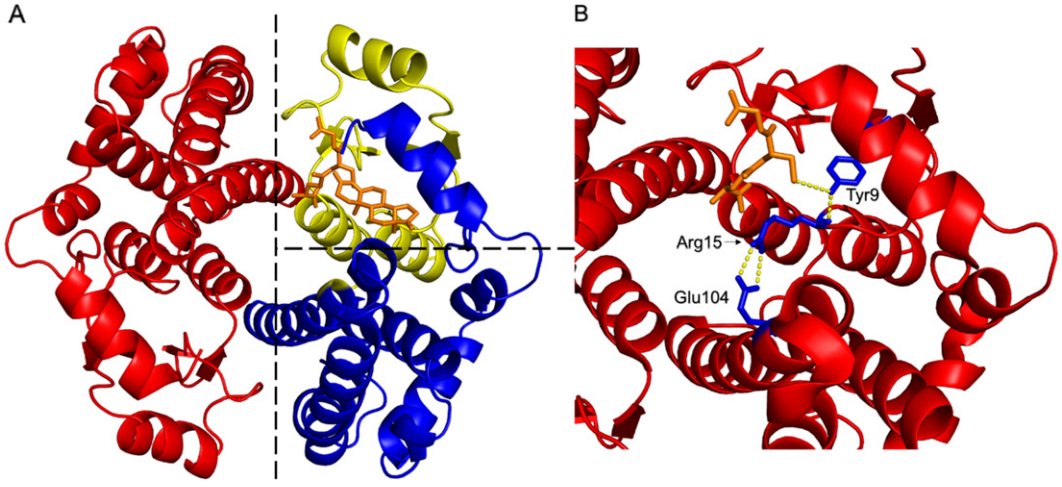

Fig. 1. The crystal structure of the homodimer hGSTA3-3. (A) A complete GSTA3 subunit is shown in red, the second subunit is divided into domain 1 (yellow) and domain 2 (blue). The substrate/co-factor GSH and product Δ^4-AD (orange) is shown bound at the active site. The domain interface and the axis of symmetry at the subunit interface are signposted. (B) Highlighting the active site. GSH (Orange) and the residues Tyr9, Arg15 and Glu104 (blue) are shown as stick models. The hydrogen bonds between the hydroxyl group of Tyr9 and the Sulphur atom of GSH and between the hydroxyl group and Arg15 are shown. Additionally, the salt bridge formed between Arg15 and Glu104 is shown. Image generated from the PDB file 2VCV using the program PyMol (www.pymol.sourceforge.net).