Glutathione transferase (GST) A3-3 is the most efficient human steroid double-bond isomerase known. The activity with Δ5-androstene-3,17-dione is highly dependent on the phenolic hydroxyl group of Tyr-9 and the thiolate of glutathione. Removal of these groups caused an 1.1 × 10^5-fold decrease in \( k_{\text{cat}} \); the Y9F mutant displayed a 150-fold lower isomerase activity in the presence of glutathione and a further 740-fold lower activity in the absence of glutathione. The Y9F mutation in GST A3-3 did not markedly decrease the activity with the alternative substrate 1-chloro-2,4-dinitrobenzene. Residues Phe-10, Leu-111, and Ala-216 selectively govern the activity with the steroid substrate. Mutating residue 111 into phenylalanine caused a 25-fold decrease in \( k_{\text{cat}}/K_m \) for the steroid isomerization. The mutations A216S and F10S, separate or combined, affected the isomerase activity only marginally, but with the additional L111F mutation \( k_{\text{cat}}/K_m \) was reduced to 0.8% of that of the wild-type value. In contrast, the activities with 1-chloro-2,4-dinitrobenzene and phenethylisothiocyanate were not largely affected by the combined mutations F10S/L111F/A216S. \( K_v \) values for Δ5-androstene-3,17-dione and Δ5-androstene-3,17-dione were increased by the triple mutation F10S/L111F/A216S. The \( pK_v \) of the thiol group of active-site-bound glutathione, 6.1, increased to 6.5 in GST A3-3/Y9F. The \( pK_v \) of the active-site Tyr-9 was 7.9 for the wild-type enzyme. The pH dependence of \( k_{\text{cat}}/K_m \) of wild-type GST A3-3 for the isomerase reaction displays two kinetic \( pK_v \) values, 6.2 and 8.1. The basic limb of the pH dependence of \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) disappears in the Y9F mutant. Therefore, the higher kinetic \( pK_v \) reflects ionization of Tyr-9, and the lower one reflects ionization of glutathione. We propose a reaction mechanism for the double-bond isomerization involving abstraction of a proton from C4 in the steroid accompanied by protonation of C6, the thiolate of glutathione serving as a base and Tyr-9 assisting by polarizing the 5-oxo group of the substrate.

The glutathione transferases (GSTs) are generally considered to be part of the cellular defense against electrophiles and to catalyze a variety of conjugations of the tripeptide glutathione (GSH) to electrophilic centra. In general, the reactions render the electrophiles less reactive and the conjugates can be further metabolized and eventually excreted. In addition to their role as detoxication enzymes, the GSTs have been suggested to be involved in different facets of biological signaling. For example, GSTs have been implicated in the synthesis of various prostaglandins (1–3), in the 5-lipoxygenase pathway (4), and in interactions with protein kinases of signal transduction systems (5). GST A3-3 is the most recent example of GSTs with a connection to biological signaling (6). GST A3-3 efficiently catalyzes double-bond isomerizations of Δ5-androstene-3,17-dione (Δ5-AD) and of Δ5-pregnene-3,20-dione, intermediates in the biosynthesis of the steroid hormones progesterone and testosterone (6). It is noteworthy that this enzyme is expressed selectively in gonads, placenta, and the adrenal gland. These findings indicate that GST A3-3 plays a role in the production of steroid hormones.

The double-bond isomerization of Δ5-AD appears to represent an entirely different biological function than the cellular disposition of toxic agents normally associated with GSTs (7). However, there are other isomerization reactions catalyzed by GSTs. The cis-trans conversion of retinoic acid is a GST-independent reaction catalyzed by GST P1-1 (8). Zeta class GSTs are involved in the catabolic pathway of tyrosine and phenylalanine by catalyzing the GSH-dependent cis-trans isomerization of maleylacetoacetate to fumarylacetoacetate (9, 10). Rat GST A1-1 and GSTs in human liver cytosol have been shown to catalyze the tautomeration of 2-hydroxymethylfururan, a reaction that strictly requires GSH and the phenolic hydroxyl group of Tyr-9 (11). In the latter case a general base-catalyzed isomerization was proposed, in which the thiolate form of GSH serves to deprotonate the substrate and initiate the reaction. This mechanism is similar to the double-bond shift in 3-oxo steroids catalyzed by GST A1-1 (12) and GST A3-3 (6). However, in the isomerizations catalyzed by Zeta class GSTs, GSH is reversibly added as a nucleophile to the substrate rather than functioning as a base, thus representing a mechanism more closely related to GSH conjugations.

The members of the Alpha class of GSTs, A1-1, A2-2, and A3-3 are close relatives. From an evolutionary perspective GST A3-3 appears to have diverged from GST A2-2 and GST A1-1, which are the nearest neighbors on the phylogenetic tree (13). The latter enzymes have been found to function as efficient glutathione-dependent peroxidases (4). From the evolutionary branch point GST A3-3 has accumulated mutations in the active site that make it an efficient steroid double-bond isomerase. Despite the high sequence identity (88%) between GST A3-3 and GST A2-2, they differ considerably in their isomerase activities with the steroid Δ5-AD. GST A3-3 displaying a 5000-fold higher catalytic efficiency (\( k_{\text{cat}}/K_m \)). Five of the 26 residues (out of 222, including the initiator methionine) that differ between GST A2-2 and GST A3-3 are located in the hydrophobic...
substrate-binding site (H-site). In this study, we have investigated the determinants for the high steroid isomerase activity of GST A3-3, and we propose the outline of a reaction mechanism for the isomerization reaction.

**Experimental Procedures**

Materials—1-Chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), and 2-chloro-3,5-dinitro-1,1,1-trifluoroethanol (o-CF₃-CDNB) were purchased from Sigma Chemical Co. (St. Louis, MO). Δ²-Androstene-3,17-dione and phenethylisothiocyanate were purchased from Aldrich (Milwaukee, WI). Δ²-Androstene-3,17-dione was obtained from Steretschenko (Newport, RI). All oligonucleotides were purchased from Interactiva (Ulm, Germany). Restriction enzymes were from Roche Diagnostics (Mannheim, Germany), and Pfu DNA polymerase was obtained from Promega (Madison, WI) and Novagen (Madison, WI), respectively. Escherichia coli XL1-Blue was purchased from Stratagene and E. coli BL-21(DE3) from Novagen.

Construction of a High Level GST A3-3 Expression Clone—The cDNA of GST A3 was amplified from the original expression clone pKK-DA3 (6) using PCR with the oligonucleotides A3EcoRINdeIG9T (5'-ATATGAATTCTATAAGGCGAAGTCTTTCATGATGATGATGATC-3' (the EcoRI and NdeI restriction sites are underlined and shown in boldface, respectively), A3EcoRINdeIG9T (5'-AAATTGCTGATATGATGATGATGATC-3' (the SacI site is underlined). The primer A3EcoRINdeIG9T introduces a silent G to T mutation at nucleotide position 9 in the cDNA of GSTA3 to replace a codon seldom used in highly expressed genes in E. coli. The PCR product was subcloned into the pGEM vector using EcoRI and SacI. The GSTA3 cDNA was sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (USB Corporation, Cleveland, OH) to verify that no mutations had been introduced. The GSTA3 cDNA was subsequently subcloned into the expression vector pET-21a(+) giving the expression clone pET-21a(+)/GSTA3. Approximately 65 mg of pure GST A3-3 was successfully obtained per liter of culture medium, a 40-fold increase compared with the yield obtained from the expression system previously used (6).

Mutagenesis of GST A3-3—The GST A3-3 mutants, GST A3-3/F10S, GST A3-3/L111F, GST A3-3/A216S, GST A3-3/F10S/A216S, GST A3-3/L111F/A216S, and GST A3-3/Y9F, were constructed using inverse PCR (14) with mutagenic oligonucleotides, cloned Pfu DNA polymerase, and the appropriate cDNA in the pGEM-Z vector as template. The PCR conditions used were 95 °C for 10 min followed by the cycle, 95 °C for 1 min, 54 °C for 1 min and 30 s, and 72 °C for 10 min, repeated for 35 times followed by 20 min at 72 °C. The mutagenic PCR products were sequenced and subcloned into the expression vector pET-21a(+) using the restriction sites NdeI and SacI.

Expression and Purification—The proteins were expressed from the pET-21a(+) vector in E. coli BL-21(DE3). The cells were grown to about 600 M.I. in LB broth containing 50 μg/ml ampicillin. The cells were harvested in the logarithmic phase of growth to an A₆₀₀ of 0.7, and expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG). The cells were then lysed by sonication, and the lysate was desalted on a PD-10 gel filtration column (Amersham Biosciences, Inc.). The proteins were eluted using a salt gradient. This gradient was used for the isomerization reaction.

Specific Activity Measurements—The specific activities of GST A3-3 and all the constructed GST A3-3 mutants were determined for the isomerization reaction with Δ²-AD (see Fig. 1A) and for the conjugation reaction with 1-chloro-2,4-dinitrobenzene (CDNB) and GSH (Fig. 1B). The reactions were monitored spectrophotometrically at 30 °C. The isomerization of 100 μM Δ²-AD was followed by 248 nm in 25 mM sodium phosphate buffer, pH 8.0, in the presence of 1 mM GSH. The extinction coefficient for the product Δ⁴-AD is 16,300 M⁻¹ cm⁻¹ (15). Specific activity measurements with 1 mM CDNB were performed in 0.1 M sodium phosphate buffer, pH 6.5, in the presence of 1 mM GSH as described previously (16).

Steady-state Kinetic Measurements—All steady-state kinetic measurements were performed at 30 °C. The isomerization activity of GST A3-3 and the GST A3-3 mutants was monitored at pH 8.0, in 25 mM sodium phosphate at a close-to-saturating concentration of GSH (2 mM). The concentration of Δ²-AD was varied between 1 and 200 μM. In addition, saturation curves with Δ²-AD were measured in the absence of GSH for GST A3-3 and GST A3-3/Y9F. The conjugating activities of GST A3-3, GST A3-3/L111F, GST A3-3/F10S/L111F/A216S, and GST A3-3/Y9F with CDNB were measured in 0.1 M sodium phosphate buffer pH 6.5 at 5 mM GSH and with phenethylisothiocyanate (PETC) (Fig. 1C) in 0.1 M sodium phosphate, pH 7.4, at 5 mM GSH as described (17). The concentrations of CDNB ranged between 25 and 1500 μM, and the concentrations of phenethylisothiocyanate were varied between 5 and 400 μM. Kinetic parameters were determined from the data using non-linear regression analysis. Catalytic center activities (kcat) were expressed per subunit (25,300 Da).

Determination of pKₐ of Tyr-9 and of the Active-site-bound Thiol of GSH—The ionization of Tyr-9 was measured as a function of pH by UV absorption difference spectroscopy (18). The intrinsic absorbance at 253 nm of 10–15 μM subunits of the different enzyme variants was measured in 0.1 M sodium phosphate buffer at pH 5.5–8.0 and 0.1 M ethanolamine/HCl at pH 8.2–9.0 at 22 °C. The spectrum of the same protein sample at pH 5.5 was subtracted from the spectra obtained at other pH values. The equation \(\Delta A = \Delta A_{\text{max}}/[1 + 10^{pK_a - \text{pH}}]\) was fitted to the data using rffit provided in the SIMFIT package (19). The effect of adding a saturating concentration of Δ⁴-AD (100 μM) or GSH (2 mM) on the pKₐ of Tyr-9 of wild-type GST A3-3 was monitored by measuring the intrinsic absorbance at 300 nm, because both molecules absorb strongly at 253 nm. To calculate the number of tyrosinates per subunit, the \(\Delta A_{\text{max}}\) and \(A_{\text{max}}\) values were divided by the subunit concentration used and the extinction coefficient determined for GST tyrosines at 253 and 300 nm, 11,000 and 2350 μM⁻¹ cm⁻¹, respectively (20, 21).

The pKₐ of GSH bound to the active site of GST A3-3 and GST A3-3/F105L/L111F/A216S was determined from spectra of 10 μM subunits in the presence of 0.5 mM GSH in the sample cuvette. Difference spectra were obtained by subtracting the spectrum for the enzyme alone at pH 5.5 and the spectra of GSH at the corresponding pH values from the spectrum of the enzyme/GSH complex. The peak at 239 nm arising from the thiolate was plotted against pH and the equation \(\Delta A = \Delta A_{\text{max}}/[1 + 10^{pK_a - \text{pH}}]\) was fitted to the data points using rffit (19). To calculate the number of ionized GSH molecules per protein subunit the extinction coefficient 5200 μM⁻¹ cm⁻¹ at 239 nm determined for the thiolate anion in the GST active site was used (22).

pH Dependence of the GST A3-catalyzed Isomerization of Δ²-AD—The isomerase activity of GST A3-3, GST A3-3/Y9F, and GST A3-3/F105/L111F/A216S with Δ²-AD in the presence of 2 mM GSH was measured at pH intervals of 0.4 pH units in the pH range 5.7–8.8. Below pH 8.0 the measurements were performed in 0.1 M sodium phosphate, and 0.1 M ethanolamine/HCl was used above pH 8.0. The concentration of Δ²-AD was varied between 1 and 200 μM.

Competitive Substrate and Inhibition Measurements—The affinity between the steroids Δ⁴-AD or Δ²-AD and wild-type GST A3-3, GST A3-3/L111F, and GST A3-3/F105/L111F/A216S was studied by inhibi-
tion experiments. When \( \Delta^A{-AD} \) was used as an inhibitor, the concentration of the substrate \( \Delta^A{-AD} \) was varied between 2.5 and 400 \( \mu \)M in 25 mM sodium phosphate at pH 8.0. The activity was measured in the absence and presence of 400 \( \mu \)M \( \Delta^A{-AD} \). The inhibitory potency of \( \Delta^A{-AD} \) was determined by means of competition experiments with the alternative substrate \( \omega{-CF}_2{-CDNB} \) using 100 \( \mu \)M \( \Delta^A{-AD} \) in 11 mM sodium phosphate, pH 6.5, at a fixed GSH concentration of 2 mM. The concentration of \( \omega{-CF}_2{-CDNB} \) was varied between 25 and 700 \( \mu \)M. The initial velocities were determined spectrophotometrically at 30 °C, and the equation describing competitive inhibition was fitted to the data by non-linear regression analysis.

RESULTS AND DISCUSSION

Choice of Mutations—Sequence alignment of the Alpha class GSTs and structural information available for the close relative GST A1-1 (23) were used to identify residues in GST A3-3 that are potential determinants for the high steroid isomerase activity. GST A2-2, which displays a lower steroid double-bond isomerase activity by three orders of magnitude as compared with GST A3-3 (6, 12), differs from GST A3-3 in 26 amino acids out of 222 (including the initiator methionine). Five of these residues are situated in the H-site as judged from the crystal structure (23). The mutations in GST A3-3 required to mimic the H-site of GST A2-2 are F10S, I12G, L111F, A208M, and A216S. The mutations F10S and A216S change the topography of the active site and introduce polar residues with hydrogen bonding potential. Because \( \Delta^A{-AD} \) is a highly hydrophobic substrate, one would expect the introduction of polar residues to lower the binding affinity by providing a less hydrophobic environment in the active site. The active site of the highly efficient \( \Delta^3{-3-ketosteroid isomerase in bacteria is very hydrophobic (24, 25) suggesting that introduction of polar residues in the active site of GST A3-3 would impair the isomerase activity. Hydrogen bonding between the serine residues, and the keto groups of the steroid may also lead to non-productive binding of the substrate. A crystal structure of GST A3-3 is not yet solved nor is any other relevant structure available for a GST in complex with a steroid such as \( \Delta^A{-AD} \). However, in all structures of GST A1-1 crystallized with a hydrophobic S-substituent on GSH in the active site, residue 111 is identified as a residue that lines the H-site with its side chain oriented toward the hydrophobic moiety (23, 26). An increase of the volume of residue 111 might affect productive binding of a large substrate through steric hindrance.

To study the role of residues 10, 111, and 216 in the enzymatic function of GST A3-3, the separate and combined mutations F10S, L111F, and A216S were constructed, introducing the amino acids of GST A2-2 in the positions of the variant residues. In other GSTs Tyr-9 has been shown to be a catalytically important residue (27, 28). The Y9F mutant was constructed to investigate the function of the phenolic hydroxyl group of Tyr-9 also in GST A3-3.

The Low \( pK_a \) Value of Tyr-9 Increases by H-site Mutations and by the Presence of GSH—The ionization of tyrosine residues in GST A3-3, GST A3-3/L111F, GSTA3-3/F10S/L111F/A216S, and GST A3-3/Y9F was monitored as a function of pH by measuring the intrinsic absorbance of the enzyme at 253 nm. The titration curves are shown in Fig. 2. Ionization of tyrosines other than Tyr-9 in the enzyme makes only minor contributions in the pH range used as shown by titration of GST A3-3/Y9F (Fig. 2). The L111F mutation in GST A3-3 increases the \( pK_a \) value of Tyr-9 from 7.93 ± 0.04 to 8.25 ± 0.08. Addition of the point mutations F10S and A216S affords an additional shift of 1 pH unit to a \( pK_a \) value of 9.2. –1.3 pH units higher than that of GST A3-3 and approaching the \( pK_a \) value of tyrosine in aqueous solution, 10.1 (29). The effect of adding a saturating concentration of \( \Delta^A{-AD} \) (100 \( \mu \)M) or GSH (2 mM) on the \( pK_a \) of Tyr-9 of wild-type GST A3-3 was monitored by measuring the tyrosine absorbance at 300 nm. This higher wavelength was chosen, because both ligands absorb strongly at 253 nm. \( \Delta^A{-AD} \) did not affect the \( pK_a \) value of Tyr-9 (data not shown), but the presence of 2 mM GSH increased the \( pK_a \) value of Tyr-9 by 1 pH unit to 8.8 ± 0.2, showing that the ionization of Tyr-9 is linked to the ionization of GSH.

The \( pK_a \) Value of GSH in the Active Site—The \( pK_a \) value of GSH bound to the active site of GST A3-3 was determined as 6.1 ± 0.1. This \( pK_a \) value was shifted to 7.1 ± 0.2 in the GST A3-3/S10F/L111F/A216S mutant. In the GST A3-3/Y9F mutant the \( pK_a \) value was increased to 6.5 ± 0.2, demonstrating that the phenolic hydroxyl group of Tyr-9 has only a minor influence on the ionization of the thiol group of GSH, which in aqueous solution has a \( pK_a \) value of 9.2 (30). The role of Tyr-9, or equivalent residues, in GSTs is generally considered to be stabilization of the thiolate of GSH in the active site by serving as hydrogen bond donor (7). In GST A1-1 the corresponding mutation afforded a similar minor increase in the \( pK_a \) value of GSH from 6.7 to 7.2 (12). This change of 0.5 pH unit corresponds to a 2.8 kJ/mol stabilization of the thiol over the thiolate form. It is notable that this is less than the energy of a fully formed hydrogen bond (31). The \( pK_a \) value of GSH bound to the active site of GST A3-3/Y9F is 2.7 pH units lower than in solution (30) showing that other groups together contribute more than the phenolic hydroxyl of Tyr-9 to lowering the \( pK_a \) of GSH. A plausible candidate is Arg-15, which in GST A1-1 lowers the \( pK_a \) of GSH (18). The \( pK_a \) of GSH was increased by 1.0 pH unit to 7.1 ± 0.2 in the GSTA3-3/F10S/L111F/A216S mutant. This may be due to an influence of the hydrophilic serines on the first-sphere interactions from Tyr-9 and Arg-15 that contribute to the lowering of the \( pK_a \) of GSH, in such a way that their thiolate-stabilizing effect is impaired.

Effect of Active Site Mutations for Specific Activities for Isomerization and Nucleophilic Aromatic Substitution Reactions—The specific activities of wild-type GST A3-3 and the constructed GST A3-3 mutants with the substrates \( \Delta^A{-AD} \) and CDNB are compiled in Table I. For comparison, specific activity values are also given for the homologous GST A1-1, the GST A1-1/Y9F mutant, and GST A2-2. The single mutation that produces the most pronounced difference in specific activity with \( \Delta^A{-AD} \) is the Y9F mutation, which lowers the specific

![Fig. 2. Titration of the active-site Tyr-9 of GST A3-3 and GST A3-3 mutants. Tyrosinate 9 of wild-type GST A3-3 (●), GST A3-3/L111F (○), GST A3-3/S10F/L111F/A216S (△), and GST A3-3/Y9F (□). Non-linear regression analysis of the data gave the following \( pK_a \) values for the GST A3-3 variants: GST A3-3, 7.93 ± 0.04; GST A3-3/L111F, 8.25 ± 0.08; GST A3-3/S10F/L111F/A216S, 9.18 ± 0.08. The pH dependence of tyrosinate formation in GST A3-3/Y9F is included in the graph showing that the contribution of other tyrosine residues in the protein is very low in the pH range investigated.](image-url)
activity 350-fold. However, this same mutation decreases the specific activity only ~2-fold with CDNB. In GST A1-1 the corresponding Y9F mutation decreases the specific activity with ΔS-AD and CDNB 43- and 300-fold, respectively (12). The hydroxyl group of Tyr-9 in GST A3-3 therefore seems to play a more important role in the catalysis of the double-bond isomerization than in the nucleophile aromatic substitution reaction of GSH and CDNB. In the case of GST A1-1 Tyr-9 is relatively less important for the isomerase reaction. Also the L111F mutation in GST A3-3 by itself markedly decreases the specific activity with ΔS-AD (27-fold) but affords a notable 4-fold increase in the specific activity with CDNB.

**Steady-state Kinetic Parameters for the Isomerization of ΔS-AD**—Steady-state kinetic parameters are listed in Table II for wild-type and mutated GST A3-3 variants as well as for GST A1-1 and GST A1-1/Y9F catalyzing the isomerization of ΔS-AD. Replacement of Tyr-9 by Phe in GST A3-3 lowers the kcat value 325-fold as well as the Km and the kcat/Km values 5- and 70-fold, respectively. In GST A1-1, the Y9F mutation caused a 50- and 2-fold reduction of the kcat and Km values, respectively, as well as a 20-fold decrease in kcat/Km. Thus, the effect of the Y9F mutation on the steroid isomerase reaction with ΔS-AD is more pronounced in GST A3-3 than in GST A1-1, not only in measurements of specific activities (Table I) but also under saturating conditions. The reduction of both kcat and Km (Table II) in GST A3-3/Y9F indicates increased non-productive binding, as previously concluded for GST A1-1 (12). In the absence of GSH, the Km value of GST A3-3 for ΔS-AD was increased from 45 to 310 μM. It is noteworthy that this same mutation in GST A3-3 and GST A1-1 gives rise to mutant enzymes displaying close-to-identical catalytic center activities for the isomerization of ΔS-AD (Table II). The ~10-fold difference in kcat and kcat/Km between the wild-type enzymes GST A1-1 and GST A3-3 can therefore be ascribed to differences in the contribution of the phenolic hydroxyl of Tyr-9 to catalysis. The 10-fold higher kcat/Km in GST A3-3 corresponds to an incremental transition state stabilization by 5.8 kJ/mol, as compared with the GST A1-1-catalyzed reaction, possibly because of a more suitable orientation between ΔS-AD and the hydroxyl group of Tyr-9. The isomerization reaction was greatly impaired by the L111F mutation, with kcat and kcat/Km values decreasing ~30- and 40-fold, respectively. The point mutations F10S and A216S by themselves did not affect the kcat value more than ~2-4-fold, but, combined with L111F in the triple mutant GSTA3-3/F10S/L111F/A216S, the mutations afforded a 100-fold decrease in the kcat/Km value for ΔS-AD. The effects of the active-site single mutations F10S, L111F, and A216S on the kcat value are essentially cumulative, as are their effects on the kcat/Km value. The Km values of GST A3-3/F10S, GST A3-3/F111L, GST A3-3/A216S, and GST A3-3/F10S/F111L/A216S do not differ markedly from each other and that of the wild-type enzyme. Neither the L111F mutation nor the triple mutation F10S/F111L/A216S has any marked effect on Km, suggesting that the affinity for GSH is essentially unaffected.

**pH Dependence of the Steroid Isomerase Reaction**—The pH dependence of kcat/Km reflects ionizations in free enzyme and free substrate (31). With GST A3-3, under conditions where GSH is present at saturating concentrations, this translates to ionizations in the enzyme-GSH complex. The pH dependence of kcat/Km of wild-type GST A3-3 is bell-shaped and has two kinetic pK values (Fig. 3A). The lower of the two pK values was calculated as 6.2 ± 0.2, which is not significantly different from the pK value, 6.1 ± 0.1, determined for the thiol of GSH in the active site, and is therefore ascribable to the ionization of GSH. Removal of the phenolic hydroxyl group of Tyr-9 results in a pH dependence profile of kcat/Km which lacks the basic limb seen with the wild-type enzyme. This suggests that the higher pK value reflects the ionization of the hydroxyl group of Tyr-9. The pK value of the basic limb in wild-type GST A3-3 was calculated to 8.1 ± 0.4, which is similar to the pK value determined for the ionization of Tyr-9, 7.93 ± 0.04. The pK value of the acidic limb of kcat/Km is increased from 6.2 ± 0.2 to 7.7 ± 0.2 by the Y9F mutation. The pK values of the pH dependence profile of kcat/Km of wild-type GST A3-3 and of the Y9F mutant show that GSH must be deprotonated and the hydroxyl group of Tyr-9 must be in its ionized form for efficient catalysis to occur. kcat/Km of GST A3-3/F10S/L111F/A216S does not show any decrease in activity at higher pH values, like GST A3-3/Y9F, and the acidic limb is shifted ~0.4 pH unit to a pH value of 6.57 ± 0.03.

The pH dependence of kcat in the present system reflects ionizations in the ternary enzyme-GSH-ΔS-AD complex. The pH dependence of kcat of GST A3-3, GST A3-3/F10S/L111F/A216S, and GST A3-3/Y9F is shown in Fig. 3B. In a log-log plot the pH dependence of kcat has a slope of ~1, indicating that a single group is titrated. The pK values of the pH dependence of kcat for GST A3-3 were determined as 7.1 ± 0.1 and 9.2 ± 0.7. The kcat value of GST A3-3/F10S/L111F/A216S shows a pH dependence similar to the wild-type with pK values of 7.2 ± 0.1 and 9.5 ± 0.2. The pH dependence curve of kcat of the GST/Y9F mutant displays only one pK value at 7.8 ± 0.2, which is very similar to the pK value of kcat/Km (7.7 ± 0.2).

**Effect of Active-site Mutations on Steroid Affinity**—KΔS-AD and KΔS-AD values of GST A3-3, GST A3-3/L111F, and GST A3-3/F10S/L111F/A216S were determined from inhibition or substrate competition experiments and are compiled in Table III. The L111F mutation causes a modest increase in the KΔS-AD but a more 10-fold increase of the KΔS-AD. Addition of the F10S and A216S mutations gives an 11-fold increase of the KΔS-AD and a 44-fold higher KΔS-AD compared with the wild-type enzyme. The difference in absolute values between the KΔS-AD and KΔS-AD may be due to the different pH values of the buffers used in the two assays for monitoring GSH conjugation to o-CF3-CDNB and isomerization of ΔS-AD. For determination of KΔS-AD, pH 8.0 was used, and for determination of the Ki value for ΔS-AD, a pH of 6.5 was used. Nevertheless, it is clear that the mutations lower the affinity for both ΔS-AD and ΔS-AD and that the relative effect of the L111F mutation is stronger for ΔS-AD. The decreased steroid isomerase activity, as well as increased K values for ΔS-AD and ΔS-AD, of the triple mutant GST A3-3/F10S/L111F/A216S compared with the wild-type enzyme, is presumably due to a perturbation of the active-site geometry. The changes in topography and decreased hydropho-
Active Site of Glutathione Transferase A3-3

TABLE II

Kinetic parameters for Alpha class GSTs and H-site mutants with Δ¹-AD

All experiments were carried out with a close-to-saturating concentration of GSH (2 mM) except for the determination of $K_m^{GSH}$ in which a close-to-saturating concentration of Δ¹-AD (250 μM) was used. $k_{cat}$ and $K_m^{GSH}$ are calculated per enzyme subunit (25,300 Da).

| Enzyme | $k_{cat}$ | $K_m^{GSH}$ | $K_m$ | $k_{cat}/K_m$ | Relative $k_{cat}/K_m$ |
|--------|-----------|-------------|-------|-------------|----------------------|
| GST A3-3 | 228 ± 9 | 45 ± 4 | 440 ± 32 | 5.0 ± 0.4 | 100 |
| GST A3-3/A216S | 146 ± 9 | 33 ± 7 | 4.5 ± 0.7 | 90 |
| GST A3-3/F10S | 98 ± 9 | 58 ± 11 | 1.7 ± 0.2 | 34 |
| GST A3-3/F10S/A216S | 67 ± 2 | 52 ± 4 | 1.30 ± 0.07 | 26 |
| GST A3-3/L111F | 67 ± 3 | 39 ± 4 | 0.29 ± 0.01 | 4 |
| GST A3-3/F10S/L111F/A216S | 2.3 ± 0.2 | 60 ± 12 | 240 ± 22 | 0.038 ± 0.004 | 0.8 |
| GST A3-3/Y9F | 0.66 ± 0.01 | 9.3 ± 0.6 | 0.07 ± 0.004 | 1.4 |
| GST A1-1 | 29.2 ± 0.8 | 58 ± 4 | 0.50 ± 0.04 | 10 |
| GST A1-1/Y9F | 0.61 ± 0.02 | 25 ± 3 | 0.024 ± 0.003 | 0.5 |
| GST A2-2 | 0.26 ± 0.01 | 250 ± 20 | 0.00104 ± 0.00009 | 0.021 |

(No enzyme) $k_{cat} = 1.6 	imes 10^{-6}$ s⁻¹

* Values are from Ref. 12.
* Value was calculated from data in Ref. 32.

TABLE III

Inhibition constants of Δ¹-AD and Δ⁴-AD for GST A3-3 and GST A3-3 mutants

With both inhibitors the $k_{cat}$ values of the GST A3-3/Δ-site mutants determined from the inhibition experiments did not differ significantly from that of the wild-type enzyme, demonstrating competitive inhibition.

| Enzyme | $K_i^{Δ⁴-AD}$ | $K_i^{Δ¹-AD}$ |
|--------|----------------|---------------|
| GST A3-3 | 13.7 ± 2.0 | 32.3 ± 6.9 |
| GST A3-3/L111F | 18.4 ± 2.1 | 360 ± 53 |
| GST A3-3/F10S/L111F/A216S | 151 ± 22 | 1430 ± 315 |

* Determined using 100 μM Δ⁴-AD as the inhibitor.
* Determined using 400 μM Δ¹-AD as the inhibitor.

In comparison with the isomerase reaction (Table II) the effects are limited, except for the Y9F mutation. The retention of activity in the H-site mutants demonstrates their catalytic competence. The catalytic efficiency of GST A3-3/L111F for the aromatic substitution reaction with CDNB is ~2-fold higher than that of the wild-type enzyme. The triple mutant GST A3-3/F10S/L111F/A216S displays a $k_{cat}/K_m$ value that is six times lower than the wild-type value. This is primarily an effect on the $K_m$ value, because the $k_{cat}$ value is only decreased by 30%. The effect of the mutations on the addition reaction in which GSH is conjugated with the isothiocyanate PEITC (Fig. 1C) was small, with a roughly 2-fold decrease of $k_{cat}/K_m$ of the GST A3-3/F10S/L111F/A216S mutant compared with the wild-type enzymes, largely due to an increased $K_m$ value.

Differential Effect of the Y9F and L111F Mutations on Isomerase and Nucleophilic Aromatic Substitution Activities—Stopped-flow kinetic studies of the isomerization reaction of Δ¹-AD catalyzed by GST A1-1 or GST A3-3 did not show a burst (data not shown). This suggests that a step preceding product release is rate-limiting in the isomerization reaction, for example, proton abstraction from C4 in the steroid molecule (Scheme 1). The active-site Y9F mutation in GST A3-3 drastically lowers the catalytic center activity of the steroid isomerase reaction, whereas the conjugation reaction with CDNB and GSH is decreased only 2.3-fold (Tables II and IV). This differential effect may be due to different rate-limiting steps of these two reactions.

For GST A1-1 the rate-limiting step of the CDNB reaction has been shown to be product release (33), and this is assumed to be the case also for GST A3-3. The C-terminal helix of GST A1-1, and probably the corresponding helix in GST A3-3, cannot fully close the active site unless the substrate bound is small, like CDNB. The C-terminal helix must move for release of the product, rationalizing this step as rate limiting for the reaction between GSH and CDNB. Larger substrates, such as

![Graphs](image-url)
**Table IV**

Kinetic parameters for GST A3-3 and H-site mutants with 1-chloro-2,4-dinitrobenzene and phenethylisothiocyanate as substrates

| Enzyme               | \(k_{cat}^{CDNB}\) | \(K_m^{CDNB}\) | \(k_{cat}/K_m^{CDNB}\) | \(k_{cat}/k_{cat}^{CDNB}\) | \(k_{cat}/K_m^{PEITC}\) | \(k_{cat}/k_{cat}^{CDNB}\) | \(k_{cat}/K_m^{PEITC}\) | \(k_{cat}/k_{cat}^{CDNB}\) |
|----------------------|---------------------|-----------------|------------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|-----------------------------|
| GST A3-3             | 22 ± 1              | 200 ± 20        | 110 ± 10               | 3.7 ± 0.4                   | 30 ± 9                   | 120                         | 30 ± 9                   | 120                         |
| GST A3-3/L111F       | 47 ± 2              | 180 ± 24        | 256 ± 26               | 3.3 ± 0.2                   | 41 ± 7                   | 80                          | 41 ± 7                   | 80                          |
| GST A3-3/F10S/L111F/A216S | 14.2 ± 0.6 | 810 ± 69        | 17.5 ± 0.7             | 5.3 ± 1.0                   | 110 ± 42                 | 50                          | 110 ± 42                 | 50                          |
| GST A3-3/Y9F         | 9.5 ± 0.5           | 1230 ± 140      | 7.7 ± 0.3              | ND                         | ND                       | ND                          | ND                       | ND                          |

*ND, not determined.

ethacrynic acid, protrude into the cleft between the subunits (26). \(\Delta^5\)-AD is an even larger substrate and the C-terminal helix presumably cannot fold completely over the active site when the steroid is bound. The helix may therefore be more easily mobilized, facilitating product release and making another step rate limiting for the isomerase reaction.

The GST A3-3/L111F mutant displays higher \(k_{cat}\) and \(k_{cat}/K_m\) values than the wild-type enzyme for the conjugation reaction between CDNB and GSH (Table IV). This may be due to a more open H-site increasing the rate of product release in this mutant. The small increase in the \(pK_a\) of Tyr-9 afforded by the L111F mutation corroborates the notion of a more open active site in this mutant.

**Comparisons with the Bacterial \(\Delta^5\)-3-Ketosteroid Isomerase—\(\Delta^5\)-3-Ketosteroid isomerase from the soil bacterium Pseudomonas testosteroni catalyzes the isomerization reaction of \(\Delta^5\)-AD and other \(\beta,\gamma\)-unsaturated 3-oxo steroids to their conjugated isomers at a near diffusion-controlled rate (1.6 \(\times 10^7\) M\(^{-1}\) s\(^{-1}\)) (34). The catalytic efficiency is 30-fold higher than that of GST A3-3 saturated with GSH. In the postulated reaction mechanism for the bacterial enzyme an aspartate (Asp-38) transfers a proton from C4 to C6 in the steroid. A tyrosine residue (Tyr-14) and a second aspartate (Asp-99) stabilize the dienolate intermediate on the reaction pathway by hydrogen bond formation to the 3-oxo group of the substrate. Removal of these three residues lowers the catalytic efficiency by nine orders of magnitude to a value similar to that of GST A3-3/Y9F in the absence of GSH. Thus, the protein scaffolds of the two structurally distinct enzymes have steroid binding sites with similar intrinsic catalytic properties.

**Composing an Active Site Catalyzing Steroid Double-bond Isomerizations—**In the absence of GSH, the GST A3-3 scaffold lacking the active-site phenolic hydroxyl group gives a rate enhancement three log units over the non-enzymatic double-bond isomerization of \(\Delta^5\)-AD (Fig. 4). Mutation of Arg-15 in the active site of the related GST A1-1 causes a marked decrease in its steroid isomerase activity (18). This suggests that also in GST A3-3 the Arg-15 residue, directly or via a water molecule, promotes deprotonation of the steroid and formation of the dienolate intermediate. Addition of the phenolic hydroxyl group (replacing Phe-9 with Tyr-9 in the active site) increases the reaction rate 3240-fold, indicating that this group has an important catalytic function per se and does not just stabilize the thiolate. Supplementing the active site with the cofactor GSH (2 mM) affords an additional 34-fold rate enhancement. The effect obtained by the combined addition of GSH and the phenolic hydroxyl group is a 1.1 \(\times 10^5\)-fold increase of the isomerase activity. In total, the rate enhancement provided by the complete active site of GST A3-3 is 1.2 \(\times 10^8\), which is
similar to the value for triose phosphate isomerase and other highly efficient enzymes (31). It is noteworthy that the sum of the individual contributions of GSH and the phenolic hydroxyl group is 7.6 kJ/mol larger than their combined contribution (calculated from the difference in free energy, $\Delta G = -RT \ln 20$), indicating energetic linkage of the two functional groups in catalysis.

Possible Reaction Mechanisms for the GST A3-3-catalyzed Isomerase Reaction—Two functional groups in GST A3-3 have been shown to be important for efficient catalysis of the isomerization of $\Delta^3$-AD: the hydroxyl group of Tyr-9 and the thiolate of GSH. The pH dependence of $k_{cat}$ and $K_m$ indicates that GSH must be deprotonated and Tyr-9 unionized for efficient catalysis. In the reaction mechanism of the steroid double-bond isomerization, a proton is abstracted from C4 and is transferred to C6, presumably via a dienolate intermediate (35). An outline of the reaction mechanism is shown in Scheme 1. R2-OH functions as a base abstracting a proton from C4 of $\Delta^3$-AD, and a dienolate intermediate is formed. R1-OH represents a group promoting dienolate formation by donating a hydrogen bond.

The product $\Delta^4$-AD arises by addition of a proton at C6, assisted by R3-BH. In the non-enzymatic reaction of the double-bond isomerization, a hydroxide ion functions as the base (32). This is probably also the case in the enzyme-catalyzed reaction in the absence of both GSH and the phenolic hydroxyl of Tyr-9 (Fig. 4). Thus, R2-OH replaces the hydroxide ion, and R2-OH might still be a hydroxide ion, but R1-OH now represents a thiolate of GSH with a $k_{cat}/K_m$ value, which is $\sim 10$ times lower than that for GST A3-3 but still 500 times higher than for GST A2-2 (6). GST A1-1 and GST A3-3 differ in three H-site residues, but in both enzymes they have similar hydrophobic side chains, in contrast to the polar series GST A2-2. Residues 12, 111, and 208 are Ala, Val, and Met in GST A1-1, corresponding to the GST A3-3 residues Gly, Leu, and Ala, respectively. Evidently, small alterations in the H-site can have profound effects on the catalytic activity and substrate specificity of the enzyme, even though the main contributors to the high catalytic efficiency are the functional group of Tyr-9 and GSH.

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