GSTP1-1 stereospecifically catalyzes glutathione conjugation of ethacrynic acid

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Abstract Using ^{1}H NMR two diastereoisomers of the ethacrynic acid glutathione conjugate (EASG) as well as ethacrynic acid (EA) could be distinguished and quantified individually. Chemically prepared EASG consists of equal amounts of both diastereoisomers. GSTP1-1 stereospecifically catalyzes formation of one of the diastereoisomers (A). The GSTP1-1 mutant C47S and GSTA1-1 preferentially form the same diastereoisomer of EASG as GSTP1-1. Glutathione conjugation of EA by GSTA1-2 and GSTA2-2 is not stereoselective. When human melanoma cells, expressing GSTP1-1, were exposed to ethacrynic acid, diastereoisomer A was the principal conjugate formed, indicating that even at physiological pH the enzyme catalyzed reaction dominates over the chemical conjugation.

Key words: Stereoselectivity; Glutathione *S*-transferase; Ethacrynic acid

1. Introduction

The diuretic drug ethacrynic acid, an α,β-unsaturated ketone, is known to be conjugated to glutathione (GSH), chemically as well as catalyzed by glutathione *S*-transferases (GST) [1]. Both the parent compound ethacrynic acid and the ethacrynic acid-glutathione conjugate (EASG) are potent reversible inhibitors of GSTs with I_{50} values in the range of <0.1–11 μM, and ethacrynic acid inhibits GST of the pi class by covalent binding [2]. Because of these inhibiting properties ethacrynic acid has been studied as an agent to overcome multidrug resistance against alkylating drugs, since GSTs may play a role in that phenomenon [3]. Additionally, it has recently been shown that the glutathione conjugate of EA is a very good substrate and an inhibitor for the multidrug resistance associated protein (MRP) or GS-X pump [4]. This pump plays a role in drug resistance as well. MRP was first detected in a multidrug resistant cell line [5] and transports glutathione conjugates of both endogenous and exogenous molecules [6,7].

Glutathione conjugation of α,β-unsaturated carbonyl compounds, such as ethacrynic acid, can lead to the formation of two possible diastereoisomers. Catalysis by GSTs often results in preferential formation of one of the two diastereomers [8–11]. For example the GST isoenzyme M2-2 was stereoselective for the formation of one of the diastereomers of 4-phenyl-3-buten-2-one (PBO) [12].

In view of the multiple effects of ethacrynic acid and especially of its EASG conjugate, taking into account the possible different biological effects of the diastereoisomers, in this study we investigated the stereoselectivity of the GST catalyzed conjugation of ethacrynic acid, using ^{1}H NMR spectroscopy, both using purified enzymes and in human melanoma cells.

2. Materials and methods

2.1. Materials

Ethacrynic acid was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glutathione was from Boehringer (Mannheim, Germany). Deuterium oxide was purchased from Isotec Inc. (Miamisburg, OH, USA). The racemic glutathione conjugate of ethacrynic acid (EASG) was synthesized according to Ploemen et al. [13]. GSTP1-1 and the GST of the alpha class were purified as previously described [14]. The C47S mutant was a generous gift from Dr. M. LoBello. Since EA is not a substrate for the human mu-class isoenzymes, these were not investigated [2].

2.2. Characterization of EASG diastereoisomers

The two diastereoisomeric glutathione conjugates were characterized by ^{1}H NMR spectroscopy using a Bruker AMX 500 spectrometer. A 1.5 s presaturation delay was used, a 70° pulse angle and a 2.2 s acquisition time (7575 Hz sweep width, 32 000 data points). The data were processed using an exponential multiplication of 0.5–1.0 Hz and zero filling to 64 000 data points. The ^{1}H NMR resonances of both diastereoisomers overlap in the aliphatic region and are as follows (D_2O, pD 6.1, 25°C). Isomer A/B: 0.80 (tr, 3H, $^3J=7$ Hz, ethacrynic acid CH_3), 1.53 (sex, 1H, $^2J=14$ Hz, $^3J=7$ Hz, ethacrynic acid CH_2), 1.68 (sex, 1H, $^2J=14$ Hz, $^3J=7$ Hz) ethacrynic acid CH_2), 2.04 (q, 2H, $^3J=7$ Hz, γ-Glu β-CH_2), 2.40 (tr, 2H, $^3J=7$ Hz, γ-Glu γ-CH_2), 2.74–2.86 (m, 2H, Cys β-CH_2), 2.74–2.96 (m, 2H, ethacrynic acid CH_2), 3.67 (tr, 1H, $^3J=7$ Hz, γ-Glu α-CH), 3.69 (s, 2H, Gly α-CH_2), 4.43 (q, 1H, $^3J=6$ Hz, $^3J=7$ Hz Cys α-CH), 4.67 (s, 2H, ethacrynic acid CH_2). In the aromatic region the ^{1}H NMR resonances of the two diastereoisomers are as follows. Isomer A: 6.89 (d, 1H, $^3J=9$ Hz, ethacrynic acid, aromatic CH), 7.58 (d, 1H, $^3J=9$ Hz, ethacrynic acid, aromatic CH); isomer B: 6.89 (d, 1H, $^3J=9$ Hz, ethacrynic acid, aromatic CH), 7.56 (d, 1H, $^3J=9$ Hz, ethacrynic acid, aromatic CH) (see also Fig. 1A). The HDO resonance of D_2O was put at 4.70 ppm.

2.3. Non-enzymatic and enzymatic conjugation

Ethacrynic acid and glutathione were mixed in equimolar quantities (5 mM) in 0.2 M potassium phosphate in deuterium oxide pD 6.1 (or pH 6.5 in H_2O). The loss of ethacrynic acid and the formation of the two diastereoisomeric glutathione conjugates were monitored by col-

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Abbreviations: EA, ethacrynic acid; EASG, ethacrynic acid-glutathione conjugate; GSH, glutathione; GST, glutathione *S*-transferase; GSTA and GSTα, alpha-class glutathione *S*-transferases; GSTP and GSTπ, pi-class glutathione *S*-transferases; Int., intermediate

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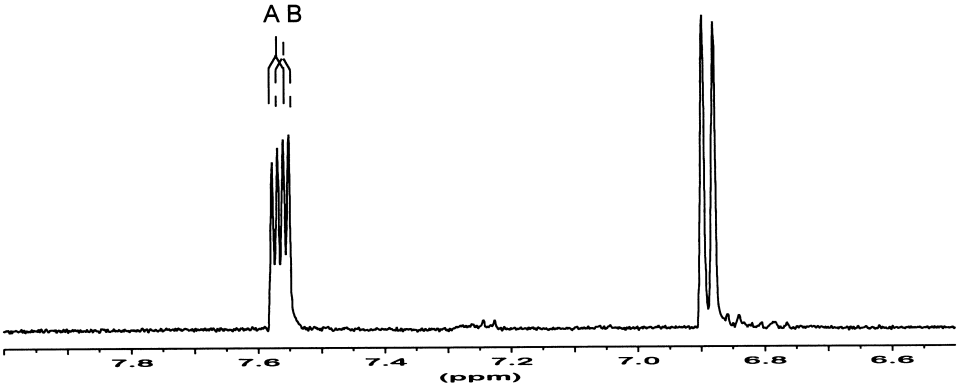
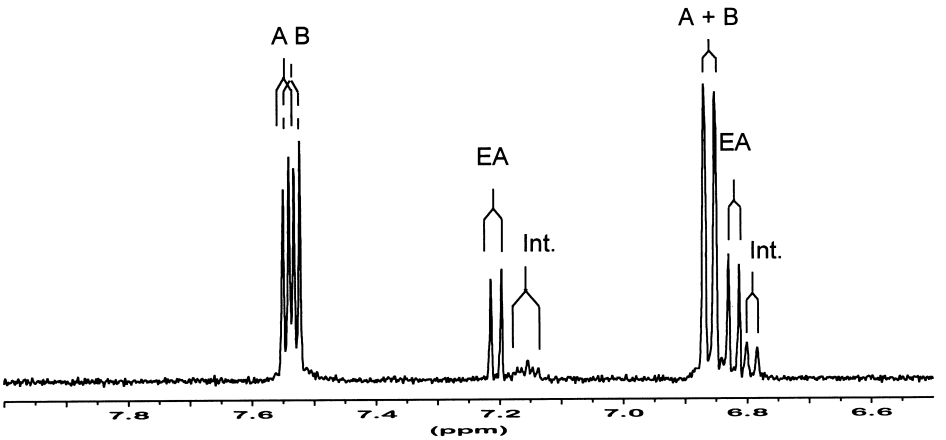
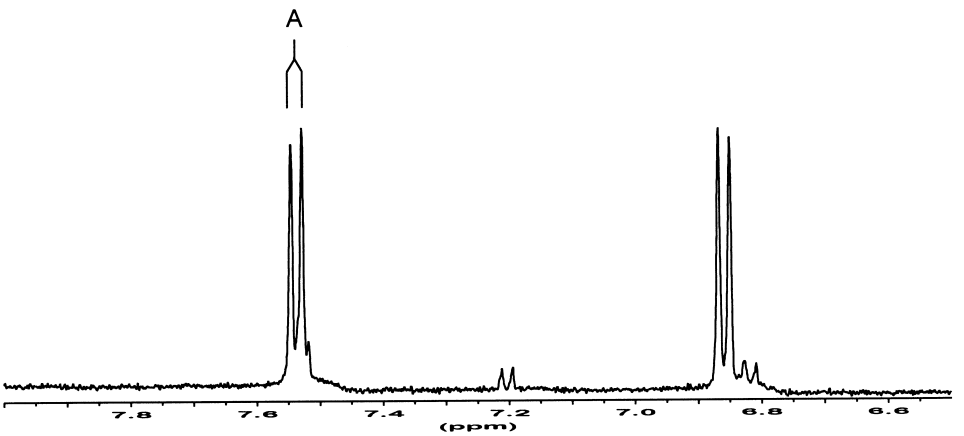
1A


A B
7.8
7.6
7.4
7.2
7.0
6.8
6.6
(ppm)

1B


A B
EA
Int.
A + B
EA
Int.
7.8
7.6
7.4
7.2
7.0
6.8
6.6
(ppm)

1C


A
7.8
7.6
7.4
7.2
7.0
6.8
6.6
(ppm)

1D

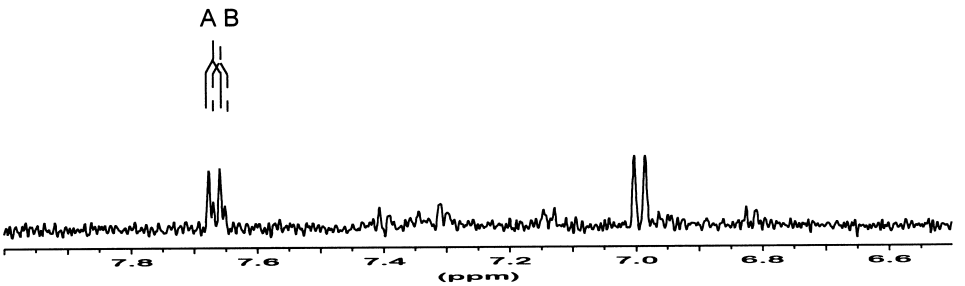

A B
7.8
7.6
7.4
7.2
7.0
6.8
6.6
(ppm)

Table 1
Stereoselectivity of GSTP1-1, GSTP1-1 mutant C47S and human alpha-class GST for the conjugation reaction between glutathione and ethacrynic acid, depicted as percentage diastereoisomer of the total amount of conjugate formed, corrected for the contribution of the chemical reaction

GST class	Range enzyme concentration (μM) used	Diastereoisomer A (%)	Diastereoisomer B (%)
GSTP1-1	20–50	100	0
GSTP1-1/C47S	10–30	100	0
GSTA1-1	20–120	66	34
GSTA2-2	30–150	50	50

lecting a series of ^{1}H NMR spectra at regular time intervals and measuring the changes in integrals of selected resonances.

The enzymatic reactions of glutathione with ethacrynic acid were carried out at 25°C in 0.2 M potassium phosphate in deuterium oxide pD 6.1. Equimolar amounts of compounds GSH and EA (5 mM) and various concentrations of GST were mixed and formation of the two diastereoisomers was followed by NMR analysis. GSH was always added prior to ethacrynic acid. Incubations were conducted with 6.5, 22.6 and 47.6 μM GSTP1-1; 11.2 and 29.8 μM C47S mutant of GSTP1-1. For the alpha-class GSTs, 24 and 120 μM GSTA1-1, and 28.8 and 144 μM GSTA2-2 were used. The stereoselectivity data of the enzyme catalyzed reaction were corrected for the contribution of the chemical reaction by subtracting the amount of diastereoisomers formed in the chemical assay from the amount that is formed in the enzyme catalyzed reaction. Generally the time dependent formation of the EASG diastereoisomers was linear up to 12 min in both the chemical and the enzyme catalyzed reaction. The rate of the chemical reaction always amounted to less than 40% of the overall rate detected in the enzyme containing incubations, with this percentage decreasing with the increasing amount of enzyme added. Incubations were performed at this pD 6.1, because, although GSTs show optimum activity at pH 7.6, chemical conjugation and deconjugation rates and aerobic oxidation of GSH are reduced at this pH and thus maximum effect of the enzyme can be expected [15].

2.4. Cell studies

Human melanoma cancer cells (IGR-39), containing a high amount of GSTP1-1 [16], were provided by the Dr. Daniel den Hoed Kliniek (Rotterdam, Netherlands). IGR-39 melanoma cells were cultured in RPMI 1640 medium (Gibco, Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum, 50 mg/l gentamicin, at 37°C in a humid atmosphere containing 5% CO_2. For the experiments 50×10^4 cells/ml were plated onto a 75 cm^2 cell culture flask (Costar, Cambridge, MA) and cultured overnight. Cells were exposed for 2 h to 50 μM ethacrynic acid in Hanks' balanced salt solution (HBSS without phenol red from Gibco), supplemented with $NaHCO_3$ (final concentration 0.35 g/l). After 2 h HBSS was removed and immediately frozen to −80°C. Cells were trypsinized, resuspended in 10 ml of PBS and immediately frozen to −80°C. Samples were freeze dried, dissolved in 0.5 ml ^2H_2O, pD 6.1 and analyzed by ^{1}H NMR.

3. Results

The most relevant section of a typical NMR spectrum of the EASG diastereoisomeric mixture is shown in Fig. 1A. Fig. 2 presents the structures of ethacrynic acid, an intermediate glutathione conjugate and the relevant parts of the two diastereoisomers of EASG. The addition of the sulfur of glutathione to the β-carbon (C10), followed by protonation of the negatively charged Cα center (C9) thus formed, results in generation of a chiral α-carbon C9. Because the proton most likely originates from the solvent or from a pool of protons that readily exchanges with the solvent [9,10], protonation can occur on either site of the carbon.

In the ^{1}H NMR spectrum two pairs of doublet resonances arising from the protons in the aromatic moiety of the two diastereoisomers can be readily observed around 7.5–7.6 ppm. These resonances can be used to detect and quantify the two diastereoisomers. From the results presented in Fig. 1A it can be derived that, upon chemical synthesis of EASG, equal amounts of A and B are formed, since the ratio of the peak areas is 48:52.

From spectra obtained at increasing time intervals upon addition of EA to a 5 mM GSH solution the chemical formation of the glutathione conjugates could be followed in time. A spectrum of the incubation at one hour is shown in Fig. 1B. After 1 h, product A and product B were formed in a ratio of 46:54. About 24% of the parent compound ethacrynic acid is still present as well as 9% of an intermediate (peaks are observed around 7.13 and 6.77 ppm; Fig. 1B). A considerable amount of this intermediate is formed during the conjugation reaction, and it is most likely the enol tautomer of the glutathione conjugate, as presented in Fig. 2. However, the compound was not further identified in the present study.

Fig. 1C presents the ^{1}H NMR spectrum of an incubation of glutathione and ethacrynic acid in the presence of 47.6 μM GSTP1-1. The spectrum clearly shows that the GSTP1-1 catalyzed reaction between EA and GSH is stereoselective and results in formation of diastereoisomer A. At the highest concentration GSTP1-1 tested (47.6 μM), 87% of the formed EASG was diastereomer A, leaving 13% product B and about 7% of free ethacrynic acid after 40 min. The enzyme catalyzed conjugation reaction was corrected for the contribution of the chemical conjugation reaction, by subtracting the amount of diastereoisomers formed in the chemical reaction from the amount formed in the enzyme catalyzed reaction, in the linear part of the reaction. It appeared that no diastereoisomer B was formed by the enzyme catalyzed conjugation. Thus, GSTP1-1 is 100% stereoselective for formation of diastereoisomer A. In the enzyme catalyzed reaction only 18% of the EA added to the reaction mixture is present as the intermediate form after 1 min, rapidly decreasing to 14% after 2 min and to 0% after 12 min. When the formation of the intermediate conjugate is corrected for the chemical reaction, it appears that the amount of intermediate formed during the enzyme catalyzed reaction is essentially the result of the chem-

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Fig. 1. A: Typical part of a ^{1}H NMR spectrum of the ethacrynic acid glutathione conjugate (EASG). EASG was synthesized according to Ploemen et al. [13] and dissolved in D_2O. The distinction between the two diastereoisomers can be seen from the pair of doublet resonances arising from the aromatic moiety around 7.55 ppm. B: Part of a spectrum of the chemical conjugation reaction of EA and GSH. C: Part of a spectrum of the conjugation reaction of EA and GSH catalyzed by 47.6 μM GSTP1-1. D: Part of a spectrum of the medium from IGR-39 melanoma cells exposed to ethacrynic acid. Cells were exposed to 50 μM ethacrynic acid for 2 h and medium was collected, freeze dried, dissolved in ^2H_2O and analyzed by ^{1}H NMR. Spectral processing: LB: −3.00 Hz; GB: 0.1; PC: 1.0; WDW: GM.

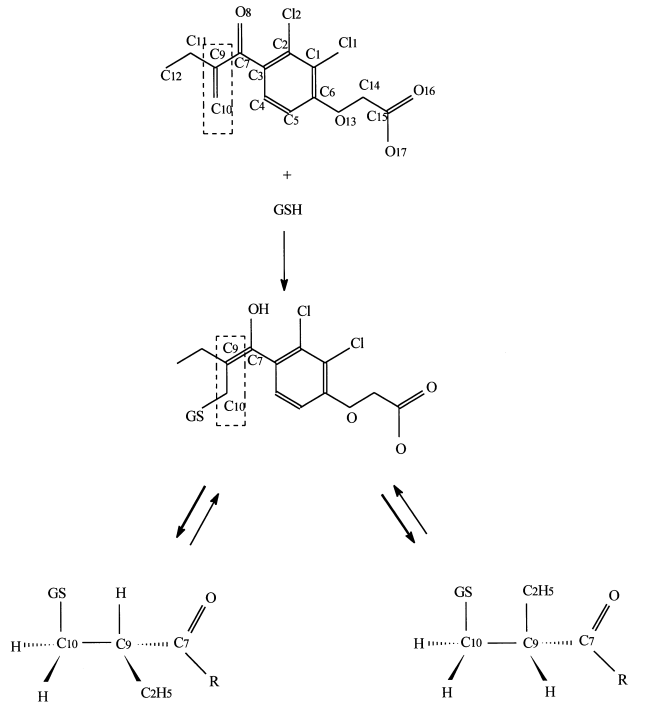

Fig. 2. Schematic representation of EA (above), the intermediate EASG and the important parts of the two diastereoisomers of EASG. The numbering in the figure is taken from Lamotte et al. [28]. As we do not know the absolute stereochemistry of A or B, no designation is given.

ical reaction. No tautomerization of diastereoisomer A could be detected in the time course of the experiments (data not shown).

Table 1 summarizes the stereoselectivity data for GSTP1-1 and other isoenzymes tested in various concentrations, corrected for the contribution of the chemical reaction. At low concentrations of enzyme the reactions were dominated by the chemical reaction; at higher concentrations of GST the enzyme catalyzed reaction became dominant and stereoselectivity could be registered. The GSTP1-1 mutant C47S was used to study the effect of the cysteine 47 residue on the stereoselectivity of the enzyme toward the reaction of EA and GSH. The reaction catalyzed by this mutant (C47S) showed equivalent stereochemistry as GSTP1-1. The isoenzymes of the α-class both catalyze the glutathione conjugation of ethacrynic acid. Only GSTA1-1 showed stereoselectivity, in favor of product A, but the formation of diastereoisomer B was catalyzed as well, although to a lesser extent.

To investigate whether the stereoselectivity of EA-GSH conjugation observed in vitro with purified enzymes would also be relevant for EA-GSH conjugation in whole cells, where pH values are generally above 6.1, IGR-39 human melanoma cells were exposed to ethacrynic acid and the EASG, excreted into the medium, was analyzed for its diastereoisomeric content. Fig. 1D shows a typical ^{1}H NMR spectrum of the medium after 2 h of exposure to EA. The chemical shift of the conjugates in the spectrum is slightly different from the spectrum of the purified EASG. This is the result of the high salt concentration, resulting from the HBSS. However, spiking the samples with purified EASG and EA showed that the proton resonances arise from the ethacrynic acid-glutathione conjugates. From Fig. 1D it appears that diastereoisomer A of EASG is the principal conjugate present in the medium of EA exposed IGR-39 cells.

4. Discussion

In the present paper it was shown for the first time that the conjugation of ethacrynic acid and glutathione catalyzed by GSTP1-1 stereospecifically forms one of the diastereoisomers of the glutathione conjugate (EASG). Chemical conjugation results in formation of a mixture of both diastereoisomers with a slight, but not significant preference for diastereoisomer B (ratio A:B = 46:54). From the human GSTs of the alpha class, only GSTA1-1 appeared to be stereoselective and to form diastereoisomer A preferentially. The fact that only GST A1-1 seems to be stereoselective in the conjugation of ethacrynic acid is quite remarkable as GSTA1-1 and GSTA2-2 only differ in 11 amino acids, three of which are in the hydrophobic binding site (as reviewed in [17]).

Human GSTM1a-1a has a very low specific activity [2], so it was impossible to test its EA conjugation to an extent significantly above the rate of the chemical background reaction in this study. Furthermore, Ploemen et al. [2] already showed that rat GST3-3 was not stereoselective towards EA conjugation.

The GSTP1-1 mutant, C47S, was demonstrated to be stereoselective towards EA conjugation in the same way as GSTP1-1, so it can be concluded that the cysteine 47 residue does not influence the stereoselectivity of the enzyme. This result is in line with previous conclusions that the amino acid does not influence the catalytic mechanism [18].

Since the crystallization of the GST isoenzymes, numerous studies have been performed, characterizing the three-dimensional structures of GST isoenzymes complexed with substrates and/or GSH (conjugates) [19–24]. For both GSTA1-1 [23] and GSTP1-1 [24] a three-dimensional structure of the enzyme in complex with EA and an EASG diastereoisomer has been described and deposited in the Brookhaven PDB databank. However, for both these GST-EASG complexes arbitrarily only one of both EASG diastereoisomers is depicted in complex with the enzyme. The experimental electron density is reported to be not sufficiently well defined to be conclusive as to whether the EASG bound to the enzyme can be identified as the *R* or *S* diastereoisomer. As a consequence the authors have apparently arbitrarily added only one of the two diastereoisomers to their pdb file. However, it is interesting to notice that in the pdb file of GSTA1-1 the *R*-form is included whereas the GSTP1-1 pdb file contains the *S*-form. This is remarkable taking into account the results of the present study showing that both isoenzymes actually form the same diastereoisomer.

For the glutathione conjugation of 7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, Hu et al. [25] suggest that the enantioselectivity of GSTP1-1 may be predicted by the structure of the active site. For the glutathione conjugation of ethacrynic acid, such a prediction on the basis of a crystal structure will be more difficult. This becomes clear when looking in detail at the possible mechanisms for glutathione conjugation of ethacrynic acid as a means to find a clue to whether the *R* or the *S* isomer is the product to be expected. The diastereoisomeric products are formed by the protonation of C9 of the ethacrynic acid-glutathione intermediate, generating a chiral center, in principle after the initial

attack of the sulfur atom from glutathione (Fig. 2). It is very interesting to notice that only one diastereoisomer is actually formed as the proton is usually assumed to arise from the solvent or from a pool of protons that readily exchanges with the solvent [9]; the enzyme thus determines the direction of this protonation.

As is shown in Fig. 1B, an intermediate compound is formed during the formation of the actual conjugates, which is most likely the enol tautomer of the glutathione conjugate. The fact that this intermediate is only detected in the chemical conjugation reaction implies that in the enzyme catalyzed reaction the protonation of the C9 takes place in the active site of the enzyme.

All experiments in this study were performed at pD 6.1 for reasons described above. This leaves a question about the implications of stereochemistry in vivo. At physiological pH the GSTP1-1 catalyzed rate of EASG formation is only 1.1-fold higher than the non-enzymatic rate [26]. However, when human melanoma cells were exposed to ethacrynic acid for 2 h, diastereoisomer A was the principal conjugate present in the medium. This might be the result of either the stereoselective formation of EASG by GSTP1-1, present in the cells, and/or the stereoselective transport out of the cells by transport pumps, i.e. the multidrug resistance associated protein (MRP). Results from a recent study [27] describing non-stereoselective transport of prostaglandin A_2-glutathione conjugates by MRP indicate that very likely no stereoselective transport will occur for the EASG diastereoisomers. Stereoselectivity thus plays a role in cellular systems and further research on this topic would be very valuable.

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