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Conjugation of Highly Reactive Aflatoxin B_1 *exo*-8,9-Epoxide Catalyzed by Rat and Human Glutathione Transferases: Estimation of Kinetic Parameters†

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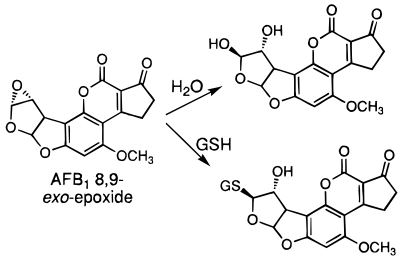
ABSTRACT: Aflatoxin B_1 (AFB_1) *exo*-8,9-epoxide, the reactive product of the hepatocarcinogen AFB_1, is stable in aprotic solvents but hydrolyzes rapidly in H_2O at 25 °C and pH 7 ($t_{1/2}$ = 1 s). However, it is also known that some glutathione (GSH) transferases can conjugate the epoxide with GSH to give the adduct in high yield. We developed an approach to estimating kinetic parameters for reactions involving this epoxide or other substrates that are unstable to H_2O. Varying concentrations of the (anhydrous) epoxide and GSH transferase were mixed and the GSH conjugates were measured. The final concentrations of product were known for each set of the starting epoxide and enzyme conentrations in a modeling approach, where the competition with the hydrolysis reaction is considered with two variables, a K for binding of the enzyme and epoxide and a rate k_2, which includes microscopic steps following complex formation and resulting in conjugate formation. The ratio k_2/K, a measure of enzyme efficiency, varied among individual recombinant GSH transferases in the the order (rat) 10-10 ≫ 3-3 > (human) M1-1 > T1-1 > A1-1 > P1-1 > A2-2, from 3×10^6 to 10 M^{-1} s^{-1}. The high ratio of M1-1 among the human GSH transferase enzymes tested is consistent with other work in which GSH−AFB_1 conjugates were not detected in hepatocytes with an M1 null polymorphism. This general kinetic approach should be applicable to estimation of kinetic parameters involved in the interaction of other unstable substrates with enzymes.

Aflatoxin B_1 (AFB_1)[1] is among the most potent carcinogens known and has a role in the etiology of human liver cancer (Qian et al., 1994). AFB_1 is produced by common fungal molds (*Aspergillus* species) but itself is relatively innocuous and must be activated to an epoxide by cytochrome P450 whereupon it becomes extremely electrophilic and reactive with DNA, protein nucleophiles, and H_2O (Busby & Wogan, 1984; Iyer et al., 1994) (Scheme 1). Interspecies variations in AFB_1 carcinogenicity appear to be reflected in differences in metabolism, particularly in terms of cytochromes P450 and GSH transferases. GSH transferase enzymes efficiently conjugate the tripeptide GSH with lipophilic electrophiles, e.g., the carcinogen benzo(*a*)pyrene diol epoxide (Robertson et al., 1986) and AFB_1 epoxides (Raney et al., 1992b).

Scheme 1: Reactions of AFB_1 *exo*-8,9-epoxide



The $t_{1/2}$ of AFB_1 *exo*-8,9-epoxide in H_2O has recently been measured to be just over 1 s at 23 °C (Johnson et al., 1996; Guengerich et al., 1996). This instability of the substrate is the primary problem in measuring rates of enzymatic conjugation (Raney et al., 1992b). Clearly, measuring the rate of formation of enzymatically formed product during the reaction lifetime poses challenges, particularly when there is no spectral change to follow. The unstable nature of AFB_1 *exo*-8,9-epoxide makes it inappropriate for rapid-quench techniques because of the hypersensitivity of the epoxide to even a small amount of H_2O, and the choice of quench solution is an issue [e.g., acid catalyzes AFB_1 *exo*-8,9-epoxide hydrolysis (Johnson et al., 1996)]. These problems

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[1] Abbreviations: GSH, (reduced) glutathione; AFB_1, aflatoxin B_1; AFB diol, aflatoxin B_1 8,9-dihydrodiol; GSH−AFB, *trans*-8,9-dihydro-8-(*S*-glutathionyl)-9-hydroxy AFB_1; HPLC, high-performance liquid chromatography.

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are manifest across the general category of unstable substrates.

We present an approach to the estimation of conjugation rates with AFB_1 *exo*-8,9-epoxide and other unstable molecules, based upon rigorous quantitation of the rate of nonenzymatic degradation of AFB_1 *exo*-8,9-epoxide in H_2O (Johnson et al., 1996) and determination of stable hydrolysis and conjugation products formed under conditions of varying levels of enzyme and substrate. A relatively simple kinetic model is applied, where two unknowns are estimated to fit by reiteration in the context of the experimentally determined yields. Binding constants and forward rate constants were estimated for seven GSH transferases, covering the alpha, mu, pi, and theta classes. The approach should be applicable to other enzyme systems using substrates that are highly unstable in aqueous solutions.

EXPERIMENTAL PROCEDURES

Chemicals. AFB_1 8,9-epoxide was synthesized by treatment of AFB_1 (Sigma Chemical Co., St. Louis, MO) with 1.5 equiv of dimethyldioxirane, producing a mixture of *exo* and *endo* isomers in a ratio of 9:1, as judged by ^{1}H NMR (Raney et al., 1992a). The *exo* isomer of AFB_1 8,9-epoxide was purified by recrystallization from a solution of anhydrous $CH_2Cl_2/(CH_3)_2CO$ (1:1, v/v, −20 °C, 24 h).

Enzymes. *Escherichia coli* recombinant rat GSH transferase 3-3 was a gift from Prof. R. N. Armstrong (Vanderbilt Univ.). *E. coli* recombinant human GSH transferases M1-1 [allelic variant b (Widersten et al., 1996)], P1-1 (Kolm et al., 1995), A1-1 (Stenberg et al., 1992), and A2-2[2] were prepared as described. *E. coli* recombinant rat GSH transferase 10-10 was prepared as described by Hayes et al. (1994). Unlike the alpha, mu, and pi class GSH transferases, T1-1 and other theta class enzymes do not bind either the *S*-hexyl-GSH or GSH-agarose affinity matrices. Therefore, to facilitate purification, recombinant human GSH transferase T1-1 was tagged at the C-terminus with a hexahistidine tail, through ligation into a pET20 vector (Novagen). The protein was isolated by nickel-agarose affinity chromatography.[3]

The GSH transferase enzymes are all of recombinant origin and the relationship of their catalytic activities to the enzymes purified from liver is a matter of consideration (as well as the enzymes within the tissue). Further, all of these enzymes had been frozen for storage and shipping for analysis. In order to address the issue we measured marker activities after the GSH transferases had been dialyzed for use in the AFB_1 *exo*-8,9-epoxide conjugation studies. The various 1-chloro-2,4-dinitrobenzene rates, measured as described by Habig et al. (1974) and expressed in micromoles of product formed per minute per milligrams of protein, were [with reported literature values (Hayes & Pulford, 1995) in parentheses]: 3-3, 94 (58); 10-10, 79; A1-1, 77 (82 and 60); A2-2, 52 (80 and 42); M1-1, 332 (172); and P1-1, 64 (103 and 40). GSH transferase T1-1 has low catalytic activity towards 1-chloro-2,4-dinitrobenzene and we measured the conjugation of 1,2-epoxy-3-(4′-nitrophenoxy)propane instead (Meyer et al., 1991). The optimal specific activity of the enzyme is not known well (Hayes & Pulford, 1995). The particular preparation used here had a catalytic activity of 8.5 μmol of conjugate formed min^{-1} (mg of protein)$^{-1}$ when isolated in one of our laboratories (J. D. H., P. J. S.), which dropped to 3.7 after a freezing and thawing cycle. When the preparation was thawed after a shipment to another of our laboratories for analysis (F. P. G., W. W. J., Y-F. U.), the measured activity was 3.7 μmol min^{-1} (mg of protein)$^{-1}$.

Enzyme Assays. GSH transferase preparations were dialyzed against 50 mM sodium phosphate buffer, pH 7.4, before use. GSH (5 mM) was mixed with GSH transferase in 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 50 μL. A GSH concentration of 20−50 μM is saturating for the various forms of GSH transferase and therefore this surplus ensures activated GS^- in the active site. Though literally random, the enzyme is effectively ordered because of the high concentrations of GSH and low K_d (Armstrong, 1991). Reactions were started by the addition of AFB_1 *exo*-8,9-epoxide [in $(CH_3)_2CO$; final concentration of $(CH_3)_2CO$ < 9%, v/v] at 23 °C and acidified with 20 μL of 2.0 M CH_3CO_2H after 15 s (Raney et al., 1992b). Mixtures were centrifuged ($3 \times 10^3 g$, 10 min) and aliquots of the supernatant were injected onto an Alltech Econosphere octadecylsilane (C18) HPLC column (4.6 × 250 mm, Alltech, Deerfield, IL). GSH−AFB and AFB_1 diol were separated using a solvent containing 43% CH_3OH in 0.10 M sodium phosphate buffer (pH 3.0), v/v, at a flow rate of 0.8 mL min^{-1}. Control reactions were run under the same reaction conditions without GSH transferase. The nonenzymatic (background) levels of conjugate were below limits of detectability (limit of detection < 0.1 μM). The amounts of GSH−AFB and AFB diol were calculated using ϵ_{362} = 21 800 M^{-1} cm^{-1} (Raney et al., 1992b; Budavari, 1996).

Kinetic Determinations. Kinetic simulations of product conjugate determinations were done with HopKINSIM software (Barshop et al., 1983; Lowe & Guengerich, 1996) run on a Macintosh PowerMac 7100 computer (Apple, Cupertino, CA), equipped with a FPU Software coprocessing simulator.

RESULTS AND DISCUSSION

Enzyme-Catalyzed Conjugation. GSH−AFB and AFB diol were determined by HPLC after incubation of varying concentrations of GSH transferase and AFB_1 *exo*-8,9-epoxide. There was a contribution to GSH−AFB conjugate formation by the GSH transferases (Figure 1). At a qualitative level, the more potent catalysts were GSH transferases 10-10, 3-3, and M1-1, whereas GSH transferases A1-1, A2-2, and P1-1 provided virtually negligible reaction enhancement over background. Human GSH transferase T1-1 was somewhat more effective than these latter three. Only rat GSH transferase 10-10 conjugated a large fraction of the AFB_1 *exo*-8,9-epoxide present in the reaction mixture. The next most capable catalyst, rat GSH transferase 3-3, conjugated ∼8% of the AFB_1 *exo*-8,9-epoxide. The low trapping efficiency is due to the extremely fast nonenzymatic rate of hydrolysis of AFB_1 *exo*-8,9-epoxide with H_2O (0.58 s^{-1}) (Johnson et al., 1996). Hence, the bulk of the pathway was rapidly toward the hydrolysis product, AFB diol, when GSH transferase 10-10 was not present in sufficient quantity.

Under these circumstances it is clearly difficult to measure or determine the catalytic parameters of the enzymes. There is no specific spectral change useful for stopped-flow kinetic

[2] Svensson, K., Widersten, M., & Mannervik, B., unpublished results; the approach is similar to that described in Stenberg et al. (1992).

[3] Sherratt, P. J., & Hayes, J. D., in preparation.

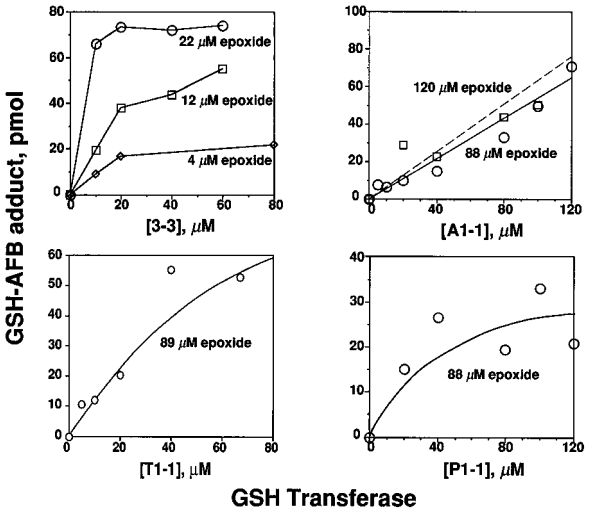
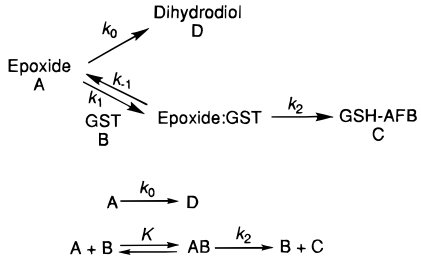


FIGURE 1: Conjugation of GSH with AFB_1 *exo*-8,9-epoxide catalyzed by GSH transferases (GSTs) 3-3, A1-1, P1-1, and T1-1. The concentration of GSH−AFB conjugate formed is shown as a function of the enzyme concentration. The concentrations of AFB_1 *exo*-8,9-epoxide used are shown on the graphs.

Scheme 2: Reactions of AFB_1 *exo*-8,9-epoxide with H_2O and GSH. GST = GSH Transferase



methods in this case. Rapid-quench kinetic methods are fundamentally problematic due to (i) hypersensitivity of the AFB_1 *exo*-8,9-epoxide when exposed to even a small amount of buffer and (ii) the need to deliver AFB_1 *exo*-8,9-epoxide in an organic solvent [e.g., $(CH_3)_2CO$], which is not very compatible with the (polyethylene) tubing used in most systems (Johnson, 1995). Both of the above methods would require considerably more GSH transferase even if they could be used, and the small amplitude differences over the small time increments would provide little more accuracy than the qualitative results presented thus far.

Rate Calculations. Mathematical kinetic modeling was employed to estimate rates and binding affinities for reactions relevant to the conjugation and hydrolysis of AFB_1 *exo*-8,9-epoxide (Scheme 2). The quantities of the typical conjugates were directly measured after reaction completion with various reagent concentrations (Figure 1), and the nonenzymatic rate of reaction with H_2O (k_o) is known in detail from previous studies (Johnson et al., 1996). The concentrations of the epoxide (A) and GSH transferase (B) are known in each assay. The quantities of GSH−AFB (C) and AFB diol (D) are determined experimentally, and the apparent affinity (K) for the GSH transferase (B) and the rate of conjugation reaction (k_2) are unknowns (Scheme 2).

Acquisition of the rates and affinities begins by providing starting values of the parameters of a mathematical model for the kinetic scheme shown and initiating calculations that provide a GSH−AFB quantity at reaction completion. An example of the two-dimensional determinations is shown

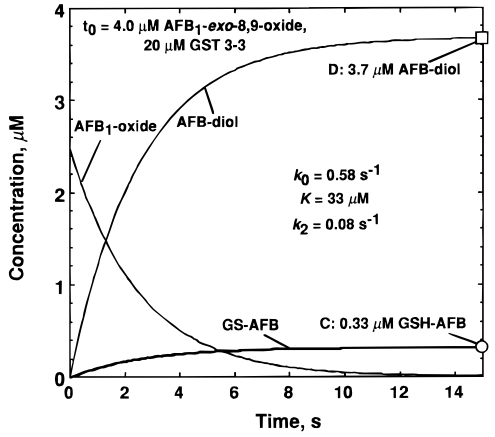


FIGURE 2: An example of mathematical model calculations that provide a product conjugate quantity at reaction completion. The kinetic parameters and kinetic equations that produce these plots are shown. See Scheme 2.

(Figure 2), in which the amount of AFB_1 *exo*-8,9-epoxide decreases and the amounts of AFB diol and GSH−AFB increase with time. Consecutive sets of rationally estimated kinetic parameters (which diminish discrepancies with experimental values) are iteratively attempted until the calculated product quantity at reaction completion matches the experimentally measured amount. These kinetic parameters are then tested for compliance with another set of reagent (A and B) concentrations for which there is experimentally determined GSH−AFB data. Iterations of decreasing incremental changes are attempted for the various experimental conditions until convergence is reached on a set of kinetic parameters concordant for all. If one set of concentrations (enzyme and substrate) were used there would be numerous sets of these two parameters that would give the experimental value. Using a variety of concentrations of both reagents is imperative to obtaining a unique set of kinetic parameters. In other words, an incorrect set of parameters that will give the amount of conjugate for a set of reagents will not represent a distinctly different set of reagent concentrations. This systematic approach was employed for rat GSH transferases 3-3 and 10-10 and human GSH transferases M1-1, A1-1, A2-2, P1-1, and T1-1. In general, the agreement to the experimental data was sensitive to 5% changes in the kinetic parameters and very sensitive to 10% changes, with a more precise agreement and sensitivity for the more active enzymes. The curves produced from the model are shown to fit reasonably well with the experimental values (Figure 3). Due to the binding step and slower step(s) subsequent, this relationship is necessarily hyperbolic. In several cases, within the set of data for a particular isozyme, the data is above and below the curve generated by the model for a particular isozyme. This is error resulting from aspects of the experimental method, i.e., reagent preparations, volumetric transfer, conjugate product assays, etc. The resulting kinetic parameters are presented in Table 1. The ratio of k_2/K (molarity^{-1} seconds^{-1}) is shown in the last data column as a convenient representation of relative efficiency and an approximation of the second-order rate constant.

Relevance of Conjugation Rates of GSH Transferase. The two rat GSH transferase enzymes exhibit good ability to catalyze conjugation, especially the potent GSH transferase 10-10, which competes very well with nonenzymatic chemi-

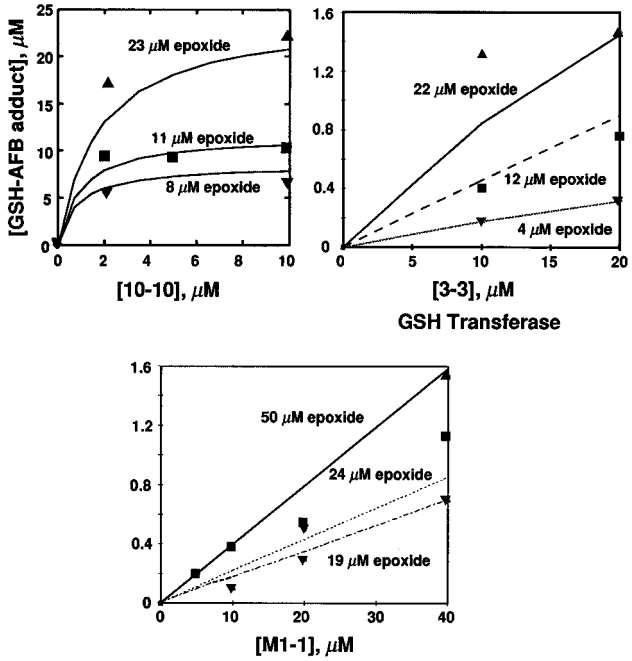


FIGURE 3: Conjugation of GSH with AFB_1 *exo*-8,9-epoxide catalyzed by GSH transferases 3-3, 10-10, and M1-1. The amount of GSH−AFB conjugate formed is shown as a function of the enzyme concentration. The concentrations of AFB_1 *exo*-8,9-epoxide used are shown on the graphs. The points represent the experimental data and the lines are fits to the parameters estimated from the model simulations (Table 1).

Table 1: Rates of GSH Transferase-Catalyzed Conjugation of GSH with AFB_1 *exo*-8,9-epoxide[a]

GSH transferase	k_2 (s^{-1})	K (μM)	k_2/K (M^{-1} s^{-1})
	rat		
10-10	3.2	1	3×10^6
3-3	0.08	30	3×10^3
	human		
M1-1	0.055	30	1.7×10^3
T1-1	0.015	70	2×10^2
P1-1	0.002	20	10^2
A1-1	0.009	100	9×10^1
A2-2	0.001	100	10

[a] Kinetic rate constants were estimated from iterative simulations of experimental determinations at various substrate and catalyst concentrations. The estimates are limited to ≤2 significant digits here.

cal hydrolysis and conjugates ~95% of the AFB_1 *exo*-8,9-epoxide before it can hydrolyze (Figure 3). GSH transferase 3-3 has been shown to be an effective conjugator, both here and in an earlier study (Raney et al., 1992b). The human enzymes are poor catalysts for this reaction except for the GSH transferase M1-1. Conclusions regarding the roles of individual GSH transferase from these results (Table 1) are, of course, based upon the assumption that the catalytic activities of these recombinant enzyme preparations reflect those in human tissue. The relevance of the estimated rates is also determined by the levels of individual GSH transferases in human liver, and the interindividual variation is known to be considerable (van Ommen et al., 1990). In general, though, levels of P1 and T1 seem to be very low in liver, levels of M1 are moderate, and the alpha class enzymes, including A1 and A2, are present at nearly an order of magnitude higher than the mu enzymes (van Ommen et al., 1990; Hayes et al., 1989; Ketterer et al., 1991). Therefore, even though A1-1 has a lower activity than M1-1 (Table 1), the effective difference may be less when the amounts of the enzymes are considered. It appears, therefore, the GSH transferase M1-1 may be relevant to the genotoxicity of AFB_1 (Langouët et al., 1995). To date, epidemiological studies have not given a clear answer as to whether or not the M1 null phenotype is a contributing factor in aflatoxin related liver cancer (McGlynn et al., 1995; Liu et al., 1991).

Conclusions. The described method of kinetic rate determinations provides a useful approach to assessing enzymatic rates for a substrate which is short-lived or very unstable in H_2O. This method works by matching results from a mathematical model of the kinetic scheme and proposed kinetic constants with experimentally determined product quantities. Measurements at reaction completion are possible even when the substrate is abating very fast. In this case there is not a spectral distinction between hydrolysis and conjugation, so direct observation of conjugation is not possible. Various concentrations of substrate and catalyst must be used. For some time it has been known that the existence of the ultimate carcinogen AFB_1 *exo*-8,9-epoxide in H_2O is very short and the relative roles of GSH transferases in conjugating this genotoxin have been assessed, therefore, only qualitatively at best (Raney et al., 1992b). The general approach should be applicable to substrates of other enzymes that are stable in apolar solvents but highly unstable in H_2O [e.g., certain strained rings and electrophiles]. In addition to several other unstable members of the epoxide family there are a number of other examples of short-lived biological intermediates. For instance, leukotriene A_4, a potent immunologic mediator, is a very short-lived (hydrolysis $t_{1/2} \approx 6$ s) (Fitzpatrick et al., 1982) substrate for both leukotriene C_4 synthase and leukotriene A_4 hydrolase. The plant growth hormone allene oxide is a substrate for allene oxide cyclase (Hamberg, 1988) and exists briefly before hydrolysis and racemic rearrangement (hydrolysis $t_{1/2} \approx 9$ s) (Brash et al., 1988; Song & Brash, 1991). The prostanoids thromboxane A_2 and prostacyclin can be enzymatically converted to 2,3-dinor-thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, respectively (Chiabrando et al., 1992), although they are rapidly hydrolyzed to thromboxane B_2 and 6-keto-prostaglandin $F_1\alpha$ (hydrolysis $t_{1/2} \approx 30$ s) (Smith et al., 1991). The application of the approach developed here may be useful with systems such as these.

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