Formation of the 1-(*S*-Glutathionyl)-2,4,6-trinitrocyclohexadienate Anion at the Active Site of Glutathione *S*-Transferase: Evidence for Enzymic Stabilization of σ-Complex Intermediates in Nucleophilic Aromatic Substitution Reactions†

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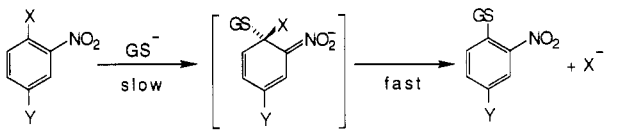
ABSTRACT: Formation of the Meisenheimer complex or σ-complex [1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate] between glutathione (GSH) and 1,3,5-trinitrobenzene (TNB) can be observed at the active sites of isoenzymes 3-3 and 4-4 of rat liver GSH transferase. The spectroscopic properties (UV–visible and CD) of the enzyme-bound σ-complex are consistent with a 1:1 complex in an asymmetric environment. Competitive inhibitors which occupy the GSH binding site (e.g., γ-L-glutamyl-D,L-2-aminomalonylglycine) inhibit σ-complex formation. The apparent formation constants of the σ-complex (M) with enzyme-bound GSH (E·GS$^-$ + TNB ⇌ E·M) at pH 7.5 are 5×10^4 M^{-1} and 7×10^2 M^{-1} for isoenzymes 3-3 and 4-4, respectively. Both values are much greater than that in aqueous solution (GS$^-$ + TNB ⇌ M), where $K_f = 28$ M^{-1}. Isoenzyme 3-3 is roughly an order of magnitude more efficient than 4-4 in catalyzing nucleophilic aromatic substitutions, a fact that appears to correlate with the ability of each enzyme to stabilize the σ-complex. The pH dependence of $K_{f(app)}$ for isoenzyme 3-3 is used to probe the ionization behavior of enzyme-bound GSH. The results are consistent with a double-ionization scheme (e.g., H$^+$E·GSH ⇌ H$^+$E·GS$^-$ ⇌ E·GS$^-$) with p*K*'s of 5.7 and 7.6, which are assigned to the thiol p*K* and the p*K* of a protonated base in the active site, respectively. Formation of the σ-complex is also observed in single crystals of isoenzyme 3-3, providing a clear demonstration of the chemical competence of the crystallized enzyme. The results are discussed with respect to catalytic efficiency and the ability of the enzyme to stabilize σ-complex intermediates in nucleophilic aromatic substitution reactions.

The glutathione *S*-transferases catalyze the addition of glutathione (GSH)[1] to a variety of electrophilic substrates and, as a result, are important in the metabolism of alkylating agents (Jakoby & Habig, 1980; Armstrong, 1987; Mannervik & Danielson, 1988). Most isoenzymic species catalyze, with varying degrees of efficiency, nucleophilic aromatic substitution reactions toward electron-deficient substrates having good leaving groups. This type of reaction is, in fact, the basis for the most commonly used spectrophotometric assay of the enzyme with 1-chloro-2,4-dinitrobenzene (Habig et al., 1974) as the electrophile.

Nucleophilic substitutions on electron-deficient aromatic molecules are generally thought to occur in solution via Meisenheimer complex or σ-complex intermediates (Miller, 1968; Bernasconi, 1980) as illustrated in Scheme I. There is strong evidence that, with many nucleophiles and leaving groups, formation of the Meisenheimer complex is the rate-limiting step in such reactions. The situation is less clear in enzyme-catalyzed additions of GSH to activated arenes. Leaving-group effects in the isoenzyme 4-4 catalyzed addition of GSH to 4-halo-3-nitro-1-(trifluoromethyl)benzenes have been interpreted to suggest that both specific base catalyzed and enzyme-catalyzed reactions have similar rate-determining steps, probably the formation of the σ-complex intermediate (Chen et al., 1988). Very little else is known about the intermediacy of Meisenheimer complexes in the enzymatic reactions, particularly those catalyzed by isoenzyme 3-3.

In an attempt to provide more direct evidence for enzyme-catalyzed formation of Meisenheimer complexes, we have investigated the interaction of 1,3,5-trinitrobenzene with the binary complexes (E·GSH) of GSH and isoenzymes 3-3 and 4-4 of rat liver GSH transferase. This highly activated arene, which does not possess a good leaving group, favors reversible accumulation of the σ-complex in the active site at relatively low concentrations of substrate. In this paper we describe the complex between 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate and isoenzyme 3-3 with respect to its spectral characteristics, the equilibrium for its formation from E·GSH and trinitrobenzene, and the pH dependence of its formation. Reversible formation of the σ-complex is also observed in crystals of isoenzyme 3-3.

Scheme I



	X	Y
1	Cl	NO_2
2	Cl	CF_3
3	F	CF_3
4	CH_3	NO_2

Experimental Procedures

Materials

Reagents. Reduced glutathione and buffer salts were from Sigma or Aldrich. 1-Chloro-2,4-dinitrobenzene and 1,3,5-

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[1] Abbreviations: GSH, glutathione; TNB, 1,3,5-trinitrobenzene; EDTA, ethylenediaminetetraacetic acid; HOMOPIPES, homopiperazine-*N*,*N*′-bis(2-ethanesulfonic acid); BHEP, 1,4-bis(2-hydroxyethyl)piperazine; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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trinitrobenzene were obtained from Eastman. 2,4-Dinitrotoluene, 4-chloro-3-nitro-1-(trifluoromethyl)benzene, and 4-fluoro-3-nitro-1-(trifluoromethyl)benzene were obtained from Aldrich. The carboxylate analogue of GSH, γ-L-glutamyl-D,L-2-aminomalonylglycine, was synthesized as described by Graminski et al. (1989).

Enzyme. Isoenzymes 3-3 and 4-4 from rat liver were purified as previously described (Cobb et al., 1983; Chen et al., 1988). Isoenzyme 3-3 was also obtained by heterologous gene expression in *Escherichia coli*. An expression vector was constructed by reassembly of the cDNA encoding subunit 3 from the 1.7-kb *Sac*II/*Hin*dIII restriction fragment of the cDNA clone pGTA/C44 (Ding et al., 1985) and a synthetic linker having *Nde*I/*Sac*II restriction sites which encoded the nine amino acids at the amino terminus. This new piece of DNA was ligated into the *Nde*I/*Hin*dIII-restricted pAS expression vector pMG27NS (Shatzman & Rosenberg, 1986). Transformation of the temperature-sensitive *E. coli* lysogen M5219 with the expression plasmid allowed heat induction of transcription and production of native isoenzyme 3-3 as 10–25% of the soluble cell protein. The enzyme, isolated by affinity chromatography and chromatography on hydroxylapatite (Cobb et al., 1983), had catalytic properties indistinguishable from those of the protein isolated from rat liver.[2] The concentrations of active sites were determined spectrophotometrically with a molar extinction coefficient for isoenzyme 4-4 of ϵ_{270}(native) = 36 700 M^{-1} cm^{-1} per active site (Graminski et al., 1989) and ϵ_{270}(native) = 37 700 M^{-1} cm^{-1} per active site for isoenzyme 3-3. The latter value was determined from the known tyrosine and tryptophan content (Ding et al., 1985) as previously described (Demchenko, 1986; Graminski et al., 1989). Enzyme was typically stored at −80 °C in 10 mM KH_2PO_4 (pH 6.7) containing 1 mM DTT and 1 mM EDTA.

Methods

Kinetic Measurements. Enzyme assays were carried out with 1-chloro-2,4-dinitrobenzene, (**1**) as the electrophilic substrate in 0.1 M KH_2PO_4 (pH 6.5) at 25 °C as previously described (Habig et al., 1974; Chen et al., 1988). Leaving-group mobility was determined with 4-chloro-3-nitro-1-(trifluoromethyl)benzene (**2**) and 4-fluoro-3-nitro-1-(trifluoromethyl)benzene (**3**) (Chen et al., 1988). Inhibition of the enzyme with TNB was performed with **1** as the variable substrate and concentrations of TNB of 0, 50, 100, and 150 μM at three fixed concentrations of GSH (0.1, 0.3, and 1.0 mM). Inhibition of isoenzyme 4-4 by TNB was done as above except [GSH] was fixed at 1 mM and the concentrations of TNB were 200, 400, and 600 μM. Attempts to observe inhibition of the two isoenzymes by 2,4-dinitrotoluene (**4**) were carried out at pH 6.5 with 1.0 mM GSH, a low concentration (100 μM) of **1**, and concentrations of **4** as high as 1.5 mM. Initial velocities were determined at least in triplicate. Initial velocity and inhibition data were analyzed with the programs HYPER, LINE, and COMP as required (Cleland, 1979).

Spectroscopic Characterization of σ-Complexes. Solutions (1.0 mL) of isoenzyme 3-3 (produced from *E. coli*) and 4-4 (from rat liver) of approximately 50 μM active sites in 0.1 M KH_2PO_4 (pH 7.5) containing 1.0 mM EDTA and 1.0 mM GSH were titrated by incremental addition of concentrated stock solutions of TNB (0.02–0.1 M) in CH_3CN. In no case did the volume of CH_3CN exceed 5% of the total volume. Reference cuvettes contained a solution identical with that of the sample but without GSH. Difference spectra were recorded between 650 and 290 nm at 25 °C on a Perkin-Elmer Lambda 4B spectrometer equipped with a double monochrometer. Circular dichroism spectra were recorded on a JASCO J-500C spectropolarimeter equipped with a DP-500N data processor. Difference CD spectra were obtained by signal averaging 16–32 scans of both the sample and reference followed by substraction of the latter from the former.

Titration of Enzyme–GSH Complexes with TNB. Formation constants for enzyme-bound σ-complexes were derived by spectrophotometric titrations of binary complexes of enzyme and GSH with TNB in buffered solutions at 25 °C under conditions of TNB in excess over the concentration of the σ-complex. This condition is satisfied when [TNB] ≫ [E·GSH]. Dithiothreitol was removed from the enzyme before titration by dialysis against 5 mM KH_2PO_4 containing 1 mM EDTA (pH 6.5) under an atmosphere of N_2. The enzyme was diluted to a final concentration of 10 μM active sites with a 0.2 M solution of the appropriate buffer to give a final buffer concentration of 0.1 M. A concentration of GSH of 1.0 mM was high enough to ensure that the enzyme was saturated with GSH under all conditions. Titrations were initiated by addition of a 1-μL aliquot of 0.1 M TNB in CH_3CN followed by additional 1-μL aliquots of a 0.05 M solution. The final concentration of CH_3CN was always less than 2% of the total volume. Solutions were buffered with Tris for pH values between 7.6 and 8.0, with KH_2PO_4 between pH 6.7 and pH 7.5, with PIPES at pH 6.6, with MES between pH 5.3 and pH 6.5, and with BHEP between pH 4.0 and pH 5.2. Enzyme concentrations were increased to 50 μM active sites, and [GSH] was increased to 2 mM at lower pH (<4.8) as the formation constant decreased. Formation constants were determined from slopes of double-reciprocal plots of $1/A_{457}$ vs 1/[TNB] as described by Benesi and Hildebrand (1949). The pH dependencies of $K_{f(app)}$ and ϵ_{app} with isoenzyme 3-3 were analyzed by the programs HOAL and WAVL, respectively (Cleland, 1979).

Formation of the σ-Complex in Crystals of Isoenzyme 3-3. Crystals of isoenzyme 3-3, which was isolated from rat liver, were grown as previously described (Sesay et al., 1987) except that the $(NH_4)_2SO_4$ was buffered at pH 8.0 with 10 mM Tris containing 0.01% NaN_3 and 1 mM EDTA. Crystals, about 0.2 mm on a side and 0.05–0.1 mm thick, were transferred to 25-μL drops of 72% saturated $(NH_4)_2SO_4$, 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.01% NaN_3 contained in a depression well. The drops were covered and equilibrated at room temperature. Formation of the enzyme-bound σ-complex was initiated by addition of 100 μL of a solution of 1.25 mM GSH and 0.5 mM TNB in buffered 72% $(NH_4)_2SO_4$ to give final concentrations of 1.0 and 0.4 mM GSH and TNB, respectively. Control crystals were soaked under the same condition in the presence of 0.4 mM TNB but in the absence of GSH. In order to lower the pH of crystals containing the σ-complex to <4, 100 μL of the soaking buffer was removed and replaced with 100 μL of 72% saturated $(NH_4)_2SO_4$ buffered with 50 mM HOMOPIPES (pH 3.5) containing 1.25 mM GSH and

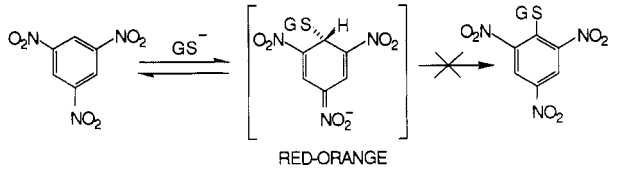
[2] Complete details of the plasmid construction, expression, and characterization of the enzyme will be forthcoming (P. Zhang and R. N. Armstrong, submitted for publication). One notable difference between the enzyme expressed in *E. coli* and that which we isolate from rat liver is that the cloned enzyme contains four Cys residues, consistent with the sequence reported by Ding et al. (1985), whereas the protein isolated from rat liver contains three Cys, a result in accord with the sequence of Lai et al. (1986). The sequences predicted by the two cDNA clones differ in two amino acids such that Asn-198 and Cys-199 in the former are replaced by Lys-198 and Ser-199 in the latter. The two isoenzymes 3-3 used in this study probably differ in the same way though no significant difference in catalytic properties is evident.

Table I: Kinetic Constants for Nucleophilic Aromatic Substitution Reactions Catalyzed by Isoenzymes 3-3 and 4-4[a]

	isoenzyme			
	3-3		4-4[b]	
substrate	k_c (s^{-1})	k_c/K_m^s (M^{-1} s^{-1})	k_c (s^{-1})	k_c/K_m^s (M^{-1} s^{-1})
1	20.0 ± 0.3	(4.3 ± 0.2) × 10^5	4.6 ± 0.2	(2.1 ± 0.1) × 10^4
2	0.64 ± 0.02	(7.9 ± 0.1) × 10^2	0.058 ± 0.014	(5.4 ± 1.0) × 10^1
3	2.5 ± 0.1	(3.5 ± 0.2) × 10^3	2.3 ± 0.8	(7.3 ± 0.7) × 10^2

[a] Kinetic constants were obtained with saturating (1.0 mM) GSH at 25 °C in 0.1 M KH_2PO_4 (pH 6.5) as previously described (Chen et al., 1988). [b] Data taken in part from Chen et al. (1988).

Scheme II



0.5 mM TNB. Color changes in the crystals were monitored by photography with a Leitz binocular microscope with an incandescent source of plane-polarized light.

Results

Kinetics. Both isoenzymes 3-3 and 4-4 catalyze the addition of GSH to activated aryl halides with reasonable efficiency. Kinetic constants for three such substrates are summarized in Table I. Three salient points should be noted. First, isoenzyme 3-3 is, in most instances, a more efficient catalyst than isoenzyme 4-4 in this class of reaction as judged by both k_c and k_c/K_m^s. This observation is consistent with previous comparisons of kinetic constants for nucleophilic aromatic substitutions catalyzed by the homodimeric proteins as well as the heterodimer (Keen et al., 1976; Danielson & Mannervik, 1985). Second, as has been noted before, the fluoro compound **3** is a considerably better substrate for isoenzyme 4-4 than is **2** by factors of 40 and 14 for k_c and k_c/K_m^s, respectively (Chen et al., 1988). Finally, k_c and k_c/K_m^s for isoenzyme 3-3 and **3** are only marginally larger (by factors of about 4) than for the chloro substrate **2**.

Inhibition of the Enzyme by Analogues of Electrophilic Substrates. In previous work it was found, somewhat surprisingly, that an isosteric analogue of **1**, namely, 2,4-dinitrotoluene (**4**), was not a reversible competitive inhibitor and could not effectively occupy the electrophilic-substrate binding site of isoenzyme 4-4 (Chen, 1987). The K_i of **4** was much greater than its solubility ($K_i \gg 1.5$ mM). A similar result is obtained with isoenzyme 3-3. This is particularly surprising given the structural similarities of **1** and **4** and the relatively low K_m^s observed for both isoenzymes and **1**.

In contrast, the electron-deficient arene trinitrobenzene is a quite good linear competitive inhibitor of isoenzyme 3-3. Under conditions of saturating GSH (0.1–1.0 mM) and pH 6.5, trinitrobenzene is a competitive inhibitor of isoenzyme 3-3 vs **1** with a $K_i = 20 \pm 3$ μM. Though no details were given, a somewhat similar result was recently reported in abstract form for a GSH transferase from *Galleria mellonella* (Clark & Sinclair, 1987). That trinitrobenzene is known to form a Meisenheimer complex with the thiolate anion of GSH in aqueous solution, albeit with a rather small formation constant (Gan, 1977), clearly suggests that inhibition of the enzyme may be due to σ-complex formation between the enzyme-bound thiolate, E·GS^-, and trinitrobenzene (E·GS^- + TNB ⇌ E·M) as illustrated in Scheme II. The situation is more complex with isoenzyme 4-4 where trinitrobenzene acts as an activator at low concentrations (<200 μM) by increasing k_c and as an inhibitor at higher concentrations by decreasing k_c/K_m. Nevertheless, a K_i of >500 μM can be estimated from the slopes of double-reciprocal plots where [TNB] ≥ 200 μM.

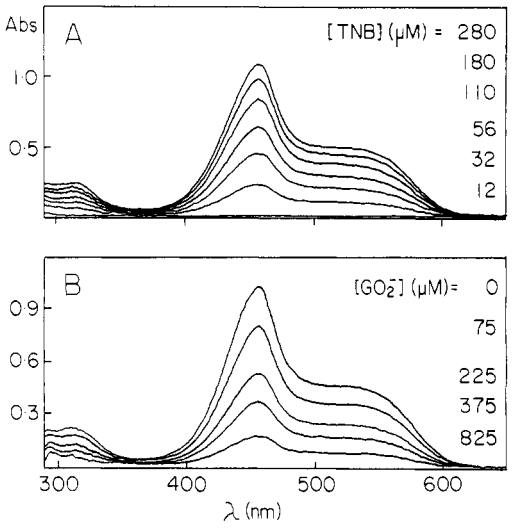


FIGURE 1: (A) Difference spectrophotometric titration of 50 μM E·GSH (isoenzyme 3-3) with trinitrobenzene at pH 7.5. (B) Competitive removal of σ-complex formed in the presence of 50 μM enzyme, 470 μM GSH, and 500 μM trinitrobenzene by addition of increasing concentrations of γ-L-glutamyl-D,L-2-aminomalonylglycine ($(GO)_2^-$).

Spectroscopic Evidence for the Enzyme-Bound σ-Complex. Incremental addition of trinitrobenzene to solutions containing modest concentrations (10–50 μM) of the binary complex of enzyme and GSH results in the progressive formation of a species that is red-orange in color. The appearance of the species is instantaneous. Formation of the complex can be monitored by UV–visible difference spectroscopy as illustrated in Figure 1A. The visible spectrum with an absorption maximum at 457 nm and a shoulder at 520 nm is typical of that expected for a 1:1 complex between a thiolate anion and trinitrobenzene. For example, the absorption spectrum for the 1:1 Meisenheimer complex of ethylthiolate and trinitrobenzene in methanol exhibits a maximum at 460 nm (ϵ_{460} = 28 500 M^{-1} cm^{-1}) with a shoulder at 550 nm (Crampton, 1968). Moreover, solutions of GSH (1 mM) and trinitrobenzene (5 mM) at pH 8.0 show absorption bands at 465 and 550 nm (data not shown). It should be noted, however, that formation of the σ-complex in solution is, under most conditions, negligible compared to that occurring on the enzyme surface. Furthermore, no evidence for a σ-complex between the enzyme and trinitrobenzene in the absence of GSH could be found.

The addition of the potent competitive inhibitor γ-L-glutamyl-D,L-2-aminomalonylglycine that occupies the GSH binding site (Graminski et al., 1989) results in a considerable decrease in the amount of σ-complex observed as illustrated in Figure 1. This result is obviously expected if efficient formation of the σ-complex requires GSH to be bound at the active site of the enzyme.

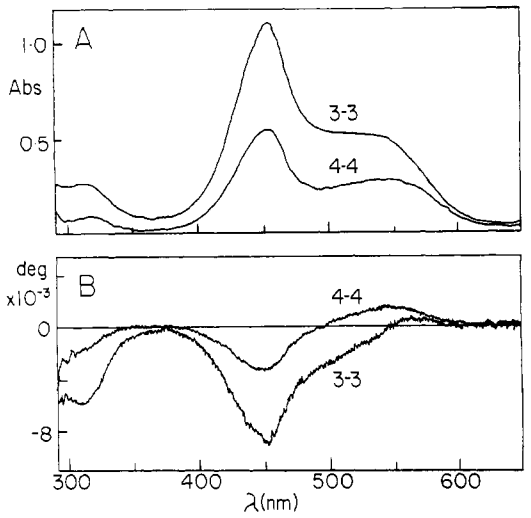


FIGURE 2: (A) Difference UV–visible spectra of enzyme-bound σ-complexes with isoenzyme 3-3 (40 μM E·M) and isoenzyme 4-4 (20 μM E·M). Spectra were recorded at 25 °C in 0.1 M Tris and 1 mM EDTA (pH 7.5) containing 1 mM GSH. The concentration of isoenzyme 3-3 was 51.6 μM, and the total concentration of TNB was 277 μM. The concentration of isoenzyme 4-4 was 50.5 μM with a total [TNB] = 1.07 mM. (B) Difference circular dichroism spectra of 40 μM E·M for isoenzyme 3-3 obtained with signal averaging of 16 scans and of 20 μM E·M for isoenzyme 4-4 with signal averaging of 32 scans. Conditions are identical with those in (A).

Titration of the binary complex of GSH and isoenzyme 4-4 also results in formation of an enzyme-bound σ-complex with slightly different spectral features from those observed for isoenzyme 3-3 as shown in Figure 2. In this instance absorption bands appear at 457 and 544 nm. The 25-nm difference in the position of the long-wavelength band suggests that the microenvironments for the trinitrocyclohexadienate chromophores in the active site of each enzyme are different.

Circular Dichroism Spectra. The asymmetric nature of the environments of the chromophores in the two enzyme-bound σ-complexes is obvious from their difference circular dichroism spectra (Figure 2). Although the 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate chromophore exhibits a featureless CD spectrum between 650 and 300 nm in aqueous solution (pH 9.0) (data not shown), the two enzyme-bound complexes show very pronounced induced CD transitions in this region. Both have strong negative transitions centered at 450 and 300–310 nm. The induced circular dichroism is quite different above 450 nm where the complex with isoenzyme 3-3 shows a negative shoulder between 500 and 530 nm while the complex with isoenzyme 4-4 exhibits a positive transition centered at about 550 nm.

Formation Constants for σ-Complexes. Formation constants and extinction coefficients for the σ-complexes between E·GS⁻ and trinitrobenzene can be determined by spectrophotometric titration under conditions where the titrant (either TNB or E·GS⁻) is in sufficient excess over the concentration of the σ-complex (E·M) formed. In this limit the absorption intensity is a hyperbolic function of the titrant concentration (in this case TNB) which can be analyzed in double-reciprocal form (eq 1) (Benesi & Hildebrand, 1949), where A is the

$$1/A = (1/K_{f(app)}\epsilon[E\cdot GS^-])(1/[TNB]) + 1/\epsilon[E\cdot GS^-] \quad (1)$$

absorbance of the E·M complex, $K_{f(app)}$ is the apparent formation constant for the equilibrium (E·GS⁻ + TNB ⇌ E·M), and ϵ is the extinction coefficient for E·M. Plots of $1/A_{457}$ vs 1/[TNB] for isoenzyme 3-3 at pH 7.5 gave a $K_{f(app)}$ = (5.1 ± 0.1) × 10^4 M^{-1} and ϵ_{457} = 26 800 ± 100 M^{-1} cm^{-1}. The

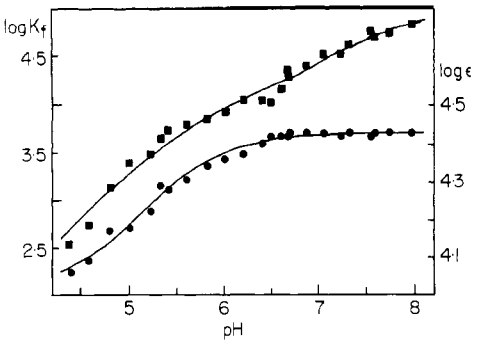
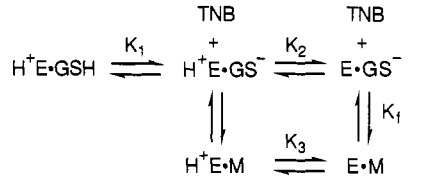


FIGURE 3: pH dependence of log $K_{f(app)}$ (■) and log $\epsilon_{(app)}$ (●). Experimental points are shown. Solid lines are fits of the experimental data for log $K_{f(app)}$ to eq 2 with K_f = (9.60 ± 1.33) × 10^4 M^{-1}, pK_1 = 5.74 ± 0.17, pK_2 = 7.60 ± 0.31, and pK_3 = 6.69 ± 0.31 with the program HOAL and for log $\epsilon_{(app)}$ to eq 3 with ϵ_1 = 10 200 ± 200 M^{-1} cm^{-1}, ϵ_2 = 26 900 ± 100 M^{-1} cm^{-1}, and pK = 5.36 ± 0.15 with the program WAVL.

Scheme III



formation constant in the case of isoenzyme 4-4 is considerably smaller, $K_{f(app)}$ = (7.0 ± 0.2) × 10^2 M^{-1} and ϵ_{457} = 27 000 ± 600 M^{-1} cm^{-1}. Values for ϵ are calculated per subunit or active site, assuming only one σ-complex per active site. Two points are worthy of note. First, the extinction coefficients for the enzyme-bound σ-complexes are in good agreement with those found for other thiolate anions and trinitrobenzene (vida supra) (Crampton, 1968). Second, the formation constants for the σ-complex between the enzyme-bound thiolate, E·GS⁻, and TNB are considerably (10 to 10^3) larger than that reported for aquated GSH where K_f = 28 M^{-1} for GS⁻(aq) + TNB ⇌ M (Gan, 1977).

pH Dependence of σ-Complex Formation. Given that trinitrobenzene almost certainly reacts only with the thiolate anion (GS⁻ or E·GS⁻), it is, in principle, possible to use the reaction as an indicator of the concentration of E·GS⁻ and hence of the ionization behavior of the thiol of enzyme-bound GSH under equilibrium conditions. Inasmuch as the apparent K_f for σ-complex formation is a function of [E·GS⁻], protonation of the thiolate should result in a decrease in $K_{f(app)}$. The large K_f makes isoenzyme 3-3 an ideal candidate for this purpose.

The pH dependence of $K_{f(app)}$ for σ-complex formation at the active site of isoenzyme 3-3 is illustrated in Figure 3. It is clear that the pH dependence is more complex than that expected for a single ionization (e.g., E·GSH ⇌ E·GS⁻ + H⁺). The experimental data do satisfy an ionization scheme involving two reactive thiolate species as illustrated in Scheme III. The pH dependence for $K_{f(app)}$ derived from Scheme III is given by eq 2, where H is the hydronium ion concentration

$$\log K_{f(app)} = \log [K_f(1 + H/K_3)/[1 + (H/K_2)(1 + H/K_1)]] \quad (2)$$

(H^+ in Scheme III), K_f is the pH-independent formation constant for the Meisenheimer complex, and K_1, K_2, and K_3 are the acid-dissociation constants shown in Scheme III. The best fit of the data to eq 2 gives values for K_f = (9.6 ± 1.3) × 10^4 M^{-1}, pK_1 = 5.7 ± 0.2, pK_2 = 7.6 ± 0.3, and pK_3 = 6.7 ± 0.3. Unfortunately, at pH <4.5 the enzyme underwent a

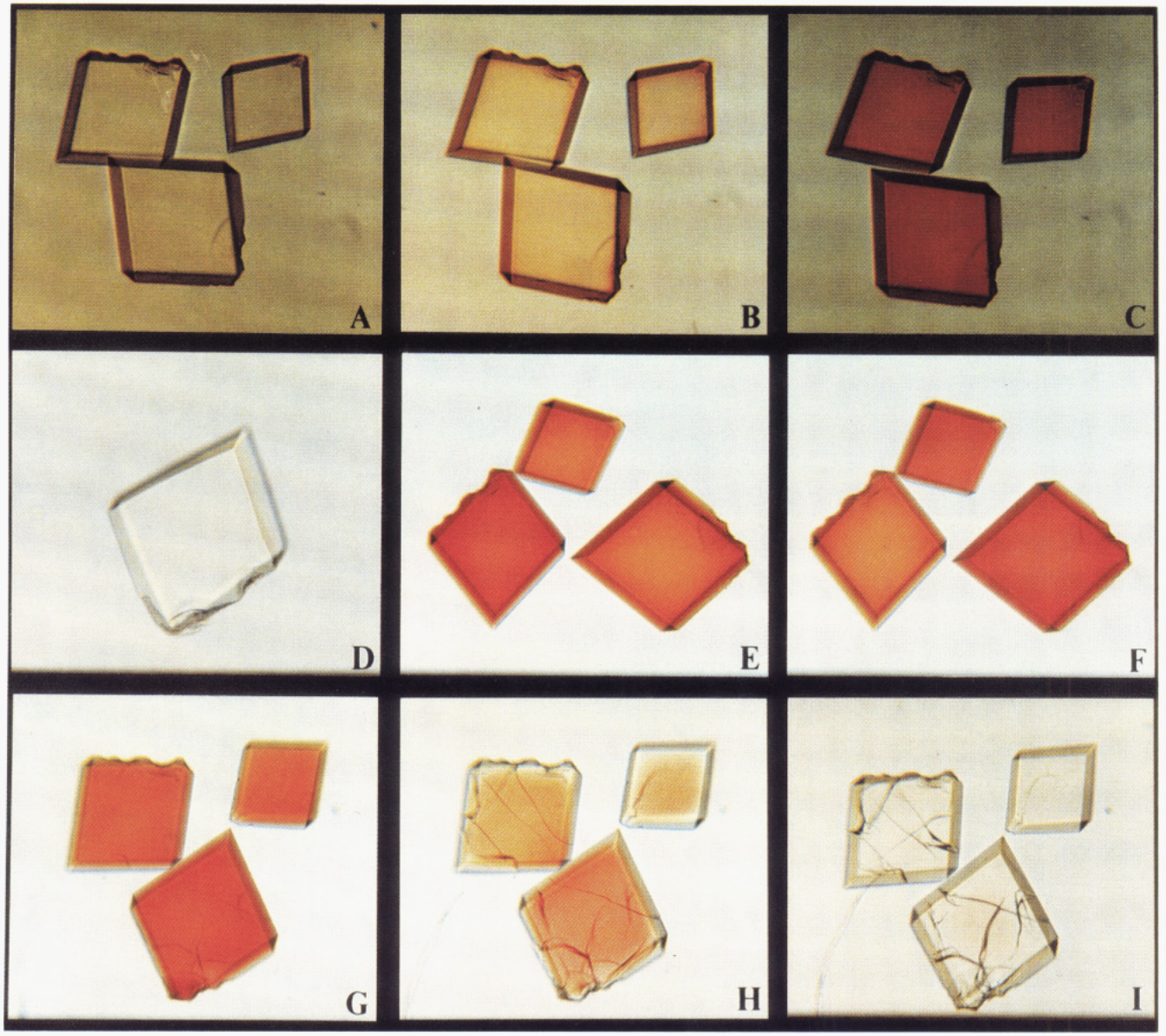


FIGURE 4: σ-Complex formation in single crystals of isoenzyme 3-3 from rat liver as viewed by color photography with an incandescent source of plane-polarized light. Panels A–C are photographs taken 1, 5, and 30 min after addition of 1 mM GSH and 0.4 mM trinitrobenzene at pH 8.0. Panel D was taken 6 h after addition of 0.4 mM trinitrobenzene at pH 8.0 in the absence of GSH. Panels E and F illustrate the pleochromism of the crystals after being soaked for 2 h in GSH and trinitrobenzene. In panel E the plane of polarization is parallel (0°) with respect to the vertical axis of the photograph, and in panel F the polarizer has been rotated 95°. Panels G–I were taken 1, 6, and 12 min after adjustment of the pH to 3.5 in the presence of 1 mM GSH and 0.4 mM trinitrobenzene.

time-dependent precipitation, making measurements below that pH suspect. It is clear, nonetheless, that more than a single ionization is responsible for the pH dependence of $K_{f(app)}$.

Somewhat surprisingly, the apparent extinction coefficient for the σ-complex also exhibited a dependence on pH. Since the extinction coefficient is obtained by extrapolation of [TNB] to infinity (eq 1), it is obvious that any variation in ϵ should not be due to a decrease in complex concentration but rather must reflect a pH-dependent distribution between two or more Meisenheimer complexes (e.g., Scheme III) that have different ϵ. The pH dependence of log $\epsilon_{(app)}$ is shown in Figure 3 and is described by eq 3 for the case of two σ-complexes, where

$$\log \epsilon_{(app)} = \log \frac{\epsilon_1 H + \epsilon_2 K}{K + H} \qquad (3)$$

ϵ_1 and ϵ_2 are the extinction coefficients for the protonated and unprotonated σ-complex and K is the acid dissociation constant for the group involved in the perturbation in ϵ. The best fit of the data to eq 3 yields values of $\epsilon_1 = 10\,200 \pm 200$ M^{-1} cm^{-1}, $\epsilon_2 = 26\,900 \pm 100$ M^{-1} cm^{-1}, and p$K = 5.4 \pm 0.2$. It is interesting to note that if the decrease in $\epsilon_{(app)}$ with decreasing pH were due to the two σ-complex species illustrated in Scheme III, then the pK should be equal to pK_3. This is clearly not the case.

σ-Complex Formation in Single Crystals of Isoenzyme 3-3. The availability of single crystals of isoenzyme 3-3 (Sesay et al., 1987) and a colorimetric half-reaction that can be reasonably expected to reflect potential catalytic activity provides a rare opportunity to directly visualize the catalytic competence of an enzyme in a single crystal. When soaked with a mixture of GSH and trinitrobenzene at pH 8.0, crystals of isoenzyme 3-3, which initially contain the product inhibitor (9*R*,10*R*)-9,10-dihydro-9-(*S*-glutathionyl)-10-hydroxyphenanthrene (Chen et al., 1986), undergo a time-dependent change in color from clear to red-orange (Figure 4, panels A–C) as the inhibitor is replaced in the active site by the σ-complex between GSH and trinitrobenzene. The color change is considerably more rapid at room temperature than at 4 °C, being complete

at 23 °C in roughly 3 h. Prolonged soaking of crystals with TNB in the absence of GSH produces no color change in the crystals as shown in Figure 4, panel D.

Not surprisingly, the crystals containing the σ-complex are pleochromic. That is, they exhibit slightly different colors depending on the orientation of the crystal axes with respect to the direction of plane-polarized light. Two such orientations are shown in Figure 4 (panel E) in which one crystal is red with the other two orange. Rotation of the plane of polarization by about 95° causes just the opposite coloration (Figure 4, panel F). The pleochromism is a clear indication that the chromophore has a specific orientation or, at least, a restricted set of orientations with respect to the molecular architecture of the protein.

The σ-complex in the crystal manifests equilibrium behavior similar to that seen in solution. For example, soaking crystals containing the preformed σ-complex in a solution of the competitive inhibitor γ-L-glutamyl-D,L-2-aminomalonylglycine results in a slow fading of the color (data not shown). Furthermore, adjustment of the pH of the crystals containing the σ-complex to ≤4 results in the rapid (within minutes) decay of the color along with severe cracking of the crystals as shown in Figure 4, panels G–I. If the pH is adjusted back to neutrality, the bleaching is reversed (data not shown), though the cracking, obviously, is not.

Discussion

Catalysis and σ-Complex Intermediates. It is, perhaps, informative to compare the catalytic properties of isoenzymes 3-3 and 4-4 and their abilities to stabilize σ-complex intemediates. In previous work (Chen et al., 1988) we suggested, on the basis of leaving-group mobilities (F^- vs Cl^-), that formation of a σ-complex intermediate was at least partially rate limiting in the isoenzyme 4-4 catalyzed addition of GSH to 1-halo-2-nitro-4-X-benzenes. Comparison of kinetic results with **2** and **3** presented in this work (Table I) suggest that isoenzyme 3-3, which in general shows greater efficiency in nucleophilic aromatic substitutions, is somewhat less sensitive to the nature of the leaving group. It is plausible that this is due to the ability of this isoenzyme to lower the activation barrier for σ-complex formation to such an extent that other steps in the reaction, such as product formation or release, begin to become kinetically significant.

The fact that **4**, an isosteric, hydrophobic analogue of **1**, does not bind effectively ($K_i \gg 1.5$ mM) to the active site of either isoenzyme 3-3 or isoenzyme 4-4 strongly suggests that the observed values of K_m^s for **1** (50 and 200 μM) do not reflect the equilibrium constants for formation of Michaelis complexes with this highly activated substrate. Rather, it is more likely that K_m^s reflects, to a significant extent, buildup of another species in the steady state. It is our hypothesis that, with *highly activated* substrates, this species is the σ-complex intermediate formed from a simple encounter complex between $E{\cdot}GS^-$ and the substrate and that any Michaelis complex formed is kinetically insignificant. This view appears to be consistent with the very effective inhibition of the enzyme by trinitrobenzene.

The competitive inhibition of both isoenzymes by trinitrobenzene is most reasonably attributed to reversible formation of a dead-end σ-complex between the electron-deficient arene and enzyme-bound GSH. That the enzyme is not effectively inhibited by structural analogues of **1** (e.g., **4**) and that there is no detectable σ-complex formation with the enzyme in the absence of GSH argue against the involvement of either a direct noncovalent or covalent interaction between trinitrobenzene and the enzyme in the inhibition. If the inhibition is due entirely to σ-complex formation, then the reciprocal of the inhibition constant should be identical with the apparent formation constant [$1/K_i = K_{(app)}$]. This is approximately the case with both isoenzymes 3-3 and 4-4. However, the values for $1/K_i$ at pH 6.5 are about 3-fold higher than $K_{f(app)}$ in both instances. The reason for and significance of this discrepancy are under investigation. In addition, the basis for the activation of isoenzyme 4-4 is not known, but does not appear to be related to σ-complex formation.

Isoenzyme 3-3 appears to have a particular capacity to stabilize the σ-complex between GSH and trinitrobenzene. A fair comparison of the two nucleophiles, GS^-(aq) and $E{\cdot}GS^-$, can be made with the pH-independent formation constants for the complexes (M and E·M) from these two species and trinitrobenzene. The two formation constants of 28 M^{-1} for GS^-(aq) and 9.6×10^4 M^{-1} for $E{\cdot}GS^-$ suggest the enzyme provides a stabilization of about 4.8 kcal/mol. It should be remembered that GS^- is the more basic thiolate and should, in principle, be more likely to add to trinitrobenzene. However, β_{nuc} values for such additions appear to be quite low (<0.2) (Chen et al., 1988). Therefore, differences in K_f for GS^- and $E{\cdot}GS^-$, due strictly to the basicity of the nucleophile, are expected to be modest. Whether the much more favorable equilibrium in the active site of the enzyme is due entirely to a smaller rate constant for decomposition of E·M is not clear. The preequilibrium kinetics of complex formation is under investigation.

It is interesting to speculate on the possible relationship between the relative catalytic efficiencies of isoenzymes 3-3 and 4-4 and their ability to stabilize the σ-complex between GSH and trinitrobenzene. In most instances isoenzyme 3-3 is roughly an order of magnitude more efficient than 4-4 at catalyzing nucleophilic aromatic substitutions as judged by k_c/K_m^s. It might then be argued that the ability of the enzyme to stabilize a σ-complex is related to its ability to stabilize the transition state for formation of a σ-complex intermediate. The transition state must, in some sense, resemble the σ-complex. Comparison of $K_{f(app)}$ at pH 7.5 reveals that isoenzyme 3-3 stabilizes the 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate anion about 70-fold more effectively than does isoenzyme 4-4. This is equivalent to a 2.5 kcal/mol difference in the stabilization energy provided by the two enzymes. The catalytic efficiency of isoenzyme 3-3 would benefit by a factor of 10 if just half of this difference is realized in the transition state for formation of the complex. These observations are consistent with the notion that the ability of a particular isoenzyme to stabilize the transition state for a nucleophilic aromatic substitution reaction is related to its capacity to stabilize the σ-complex. The structural basis for this difference is not understood.

Ionization Behavior of the Binary Enzyme–GSH Complex. The formation of the σ-complex between enzyme-bound GSH and trinitrobenzene provides a sensitive indicator of the ionization behavior of the binary complex of isoenzyme 3-3 and GSH under equilibrium conditions. The pH dependence of $K_{f(app)}$ is consistent with the existence of two thiolate anion species ($H^+E{\cdot}GS^-$ and $E{\cdot}GS^-$ of Scheme III) capable of reaction with trinitrobenzene or, presumably, any other substrate. Although previous kinetic studies with isoenzyme 4-4 have indirectly implicated an active site base with a conjugate-acid $pK \geq 7.5$ (Chen et al., 1988), the results here with isoenzyme 3-3 appear to be the first, direct experimental indication that a protonated base ($pK_2 = 7.6$; Scheme III) may exist in the active site of a GSH transferase complexed with GSH. More importantly, the pH dependence of $K_{f(app)}$ demonstrates that

the pK of enzyme-bound GSH is about 5.7 or roughly 3 log units below its pK in aqueous solution. This observation is in reasonable agreement with very recent, direct spectrophotometric titration experiments with isoenzyme 4-4, which have revealed that the pK of enzyme-bound GSH is ≤6.6 (Graminski et al., 1989). However, the latter experiments which involved direct titration of the thiolate anion at 239 nm by UV-difference spectroscopy showed only a single ionization (pK = 6.4–6.7) in the region of pH 5–8 (Graminski et al., 1989). It is likely that this may be due to the relatively low sensitivity of the direct titration and not to the absence of a second ionization in isoenzyme 4-4.

At present the best evidence is that the pK of the thiol of enzyme-bound GSH is 5.7 with isoenzyme 3-3 (this work) and 6.6 with isoenzyme 4-4 (Chen et al., 1988; Graminski et al., 1989). Whether the difference in pK is due to differences in the active sites of the two enzymes or related to the reliability of the different experimental techniques used is not certain. The detection of a second ionizing group (pK_2 = 7.6) tempts one to speculate that the activation of the thiol of GSH in the active site occurs through a zwitterionic pair involving GS^- and a protonated base. The supposition that this protonated base is the imidazolium side chain of an active site histidine residue, as is common in enzymes with essential thiolate groups, remains to be established.

The pH dependence of $\epsilon_{(app)}$ for the σ-complex leads to the conclusion that there is an additional group in the enzyme-bound Meisenheimer complex that exhibits a pK = 5.4 and that has a marked effect on ϵ. Inasmuch as there is no significant change in $\epsilon_{(app)}$ until the pH <6.5, it would appear that the two σ-complexes illustrated in Scheme III ($H^+E{\cdot}M$ and $E{\cdot}M$) which are related by pK_3 = 6.6 have approximately the same ϵ. The loss of chromophore intensity at low pH must be due to protonation of another group in the enzyme-bound σ-complex, perhaps on the chromophore but more likely on the protein. Thus, the species with ϵ_{457} = 10 200 M^{-1} cm^{-1} can be written as $H^+E{\cdot}MH^+$ or $H^+EH^+{\cdot}M$.

In spite of several unanswered questions it is clear that the spectroscopic properties of the σ-complex between GSH and trinitrobenzene are useful as a probe of the acid–base chemistry in the active site of GSH transferase. The general utility of this "half-reaction" in understanding the catalytic diversity of different isoenzymes is under further investigation.

Chemistry in the Crystal. The chromogenic half-reaction between GSH and trinitrobenzene in single crystals of isoenzyme 3-3 provides a rather dramatic demonstration of the chemical competence of the crystallized enzyme. Moreover, it is expected that several other benefits may accrue from this observation. First and foremost is the opportunity to obtain X-ray diffraction data on a model for what appears to be a key intermediate in the enzyme-catalyzed addition of GSH to aromatic substrates, the σ-complex or Meisenheimer complex. Furthermore, it may in fact be posible to derive some knowledge concerning the orientation of the chromophore with respect to the crystal axes by single-crystal absorption spectroscopy. Finally, the complex (or its disappearance) can be used as a simple visual monitor of the movement of products or substrate analogues into the active site of the enzyme in single crystals.

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