Nucleophilic Aromatic Substitution of Glutathione and 1-Chloro-2,4-dinitrobenzene in Reverse Micelles. A Model System To Assess the Transition-State Stabilization in Glutathione Transferase Catalyzed Conjugation

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Introduction

Glutathione transferase (GST; EC 2.5.1.18) constitutes one of the major detoxification enzymes in cells. The reaction mechanism of this enzyme, as implicated by kinetic, spectroscopic, and crystallographic analyses,[1] involves an essential tyrosine residue,[2] which induces ionization of the enzyme-bound glutathione (GSH), thereby lowering its pK_a value by 2−3 pH units and producing a better nucleophilic thiolate anion (GS^-). Next, the latter attacks the *ipso* carbon atom bearing the chlorine atom in 1-chloro-2,4-dinitrobenzene (CDNB), forming a Meisenheimer complex transition state. Finally, the chloride ion leaves and forms a water soluble conjugate *S*-(2,4-dinitrophenyl)glutathione [S-(DNP)GS] that is to be excreted from the cells. The rate-limiting step has been proposed at the σ-bond formation in the Meisenheimer complex.[3] Some of the factors contributing to transition-state stabilization, including the role of conserved positively charged amino acid residues in the active site have been postulated.[3,4]

In this study, a model system is described that stabilizes the transition state in the nucleophilic aromatic substitution reaction (S_NAr) and enhances the reaction rate. This system provides an exemplary model for studying the contribution of transition-state stabilization in the GST-catalyzed reaction.

Results and Discussion

The nonenzymatic conjugation between GSH and CDNB in aqueous and surfactant/isooctane reverse micellar systems is examined. The reaction in cetyltrimethylammonium bromide (CTAB) reverse micelles was found to have a second-order rate constant 799 M^{-1} s^{-1}, which is approximately 20-fold faster than the rate in aqueous solution (39 M^{-1} s^{-1}) or Triton X-100 reverse micelles (50 M^{-1} s^{-1}). On the other hand, the reaction rate in Aerosol OT (AOT) reverse micelles is slower (31 M^{-1} s^{-1}) than the rate in aqueous solution. The partition coefficients of CDNB in isooctane and aqueous phase, determined according to the procedure of Tyrakowska et al.,[5] were similar in the three micellar systems (14.74, 12.34, and 13.84 in CTAB, AOT, and Triton systems, respectively).

Since CTAB and AOT are opposite at the polar head charge and Triton is a neutral compound, the above results have prompted an investigation here of the mechanism of rate enhancement in CTAB reverse micelles. Because the hydrophobic substrate CDNB prefers the organic phase, the possible contribution to catalysis by colocalization of two substrates within the micelle might not be the major cause for the rate enhancement. The possibility of increasing GSH dissociation, which would increase the GS^- concentration in reverse micelles, was examined. The reaction was performed in buffers with various pH values. Figure 1 indicates that the reaction is base-catalyzed and GSH thiolate anion is the reactant. The pK_a value of GSH in aqueous solution was found to be 9.169 ± 0.115, which is in excellent agreement with the value (9.13) reported by Huskey et al.[6] The pK_a values of GSH in CTAB or Triton reverse micelles were found to be 9.032 ± 0.021 and 8.938 ± 0.082, respectively, which are not far from the value in aqueous solution. On the other hand, the pK_a value of GSH decreased to 8.095 ± 0.025 in AOT reverse micelles. The above results rule out the possibility of increasing GSH ionization as the major reason for the rate enhancement in CTAB reverse micelles.

Another possibility for the rate enhancement is stabilization of the transition state, which is negatively charged and is unstable due to loss of aromaticity. The polar head of CTAB is positively charged. Embedding of reactants into the CTAB reverse micelles confines the molecules into a volume-restricted vesicle with concentrated positive charges, which will stabilize the negatively charged Meisenheimer complex. We have performed the reaction in CTAB reverse micelles with various hydration degrees (represented by $[H_2O]/[CTAB]$), which was performed by maintaining the CTAB concentration constant and varying the water content of the system. In such a way vesicles with various sizes were obtained. As hydration degree decreased, the size of reverse micelles also decreased; however, the reaction rate was found to have increased (Figure 2). This correlates with our hypothesis that the transition state is stabilized in a positively charged environment. The influence of charge is more prominent in a smaller vesicle. A schematic diagram representing the GS^-–CDNB Meisenheimer complex in CTAB reverse micelles is shown in Scheme 1.

Experimental data in this study indicated that ionization of GSH does not contribute to the rate acceleration of the nonenzymatic reaction with CDNB; a similar conclusion was drawn for the enzymatic reaction.[1e] The negatively charged AOT molecules decrease the pK_a value of GSH; however, the reaction rate is decreased due to destabilization of the transition state. CTAB does not

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(2) The essential tyrosyl residue is Tyr^8 in GST-alpha, Tyr^6 in GST-mu, or Tyr^7 in GST-pi or GST-sigma.

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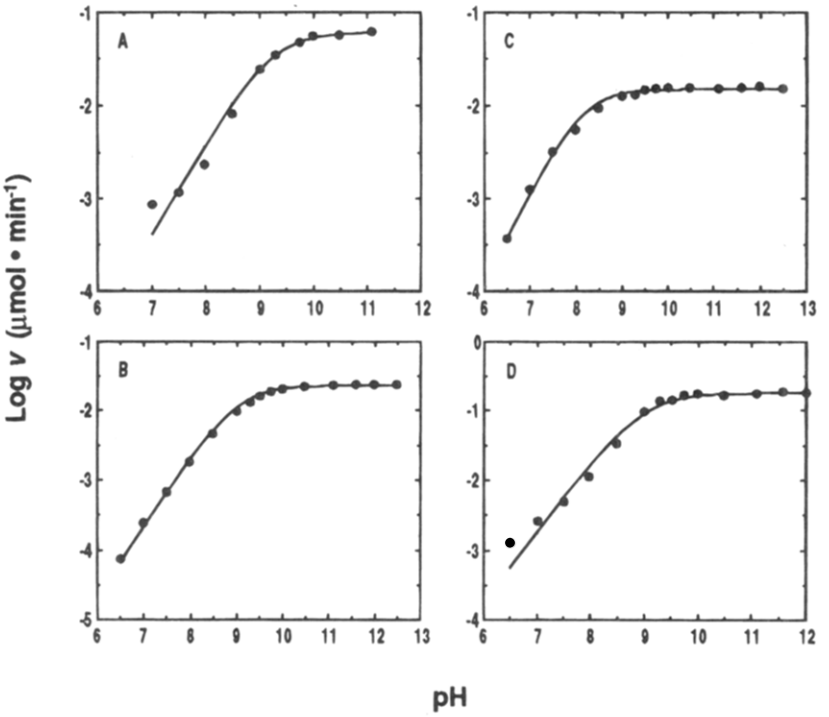


Figure 1. pH−rate profiles for the conjugation between GSH and CDNB. The reaction was performed in Bis-Tris propane (10 mM) with various pH values. Relative rates of micromoles of S(DNP)GS formed per minute were calculated. Points are experimental data and curves are computer fittings to eq 1. The [H_2O]/[surfactant] ratio was 16.65. The reactant concentrations used were [GSH] and [CDNB] both 0.1 mM in B and [CDNB] 2 mM, [GSH] 1 mM in A, C, and D. (A) Aqueous solution. (B−D) CTAB, AOT, and Triton reverse micellar systems, respectively.

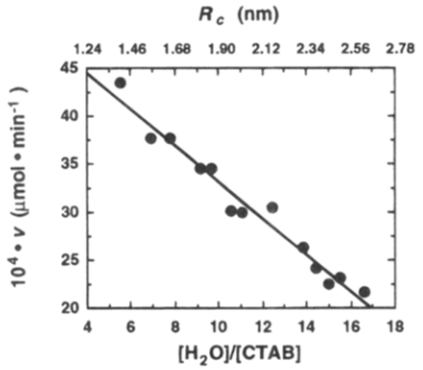
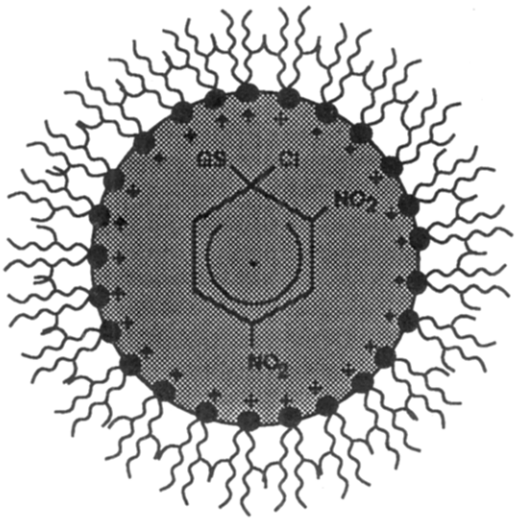


Figure 2. Effects of [H_2O]/[surfactant] ratio on the conjugation between GSH and CDNB. The [H_2O]/[surfactant] ratio was adjusted by varying the water amount while the CTAB concentration was kept constant. The core radius (R_c) of the inner cavity of the reverse micelles is shown on the top scale.

affect the dissociation of GSH; however, the transition state is stabilized and provides a 20-fold acceleration of the reaction rate. Both π-complex and radical anion-radical pair charge-transfer complexes have been identified for the reaction of OH^- and CDNB in aprotic solvents by hydrogen exchange and NMR spectroscopic analysis.[7] It has been observed that aprotic solvents accelerate the nucleophilic aromatic substitution of thiolate anions even for unactivated substrates.[8] The polar aprotic solvent tetraglyme (2,5,8,11,14-pentaoxapentadecane) accelerates the reaction, presumably by better solvating sodium counterions and reducing ion pairing.[9] A proposed S_NAr reaction mechanism for the GSH and CDNB in reverse micelles is shown in Scheme 2. Our present experimental results are consistent with this mechanism with a rate-limiting electron transfer step.

Scheme 1. Schematic Model Showing GS^-−CDNB Meisenheimer Complex in CTAB Reverse Micelles. The Polar Head of the CTAB Molecule Is Drawn into the Water Pool (Gray Area) according to Martinek et al.[13] The Electron Transfer Steps May Be Facilitated in This Reverse Micellar System



The reaction mechanism shown in Scheme 2 might also apply to GST. Detoxification enzymes like glutathione transferase are characterized as sluggish enzymes with wide substrate specificities. GST catalyses essentially unidirectional reactions capable of reacting with a broad spectrum of xenobiotics that cells might encounter. As demonstrated above, the nonenzymatic reaction has a measurable rate, which provides one of the few reactions appropriate for comparison with the enzymatic reaction. In the mechanism shown in Scheme 2 the Meisenheimer

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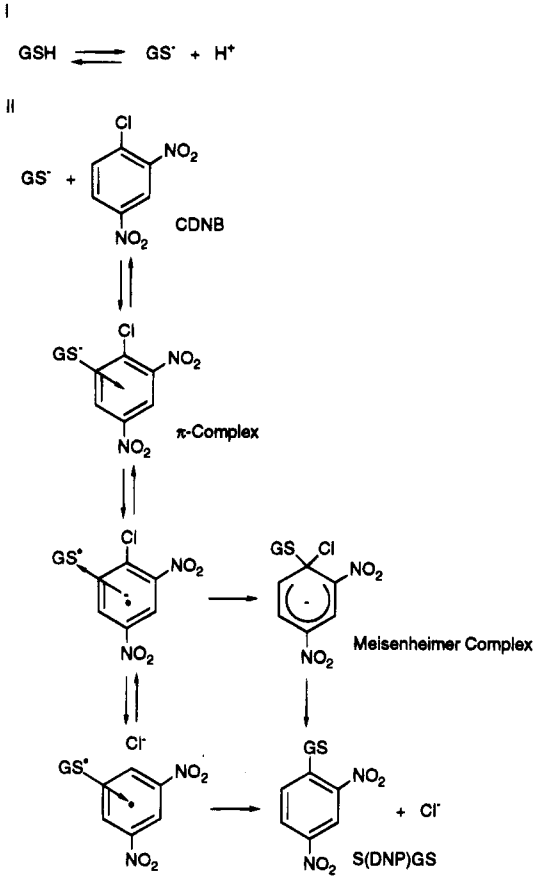
Scheme 2. Proposed Chemical Mechanism for the Base-Catalyzed Nucleophilic Aromatic Substitution of GSH and CDNB in Reverse Micelles. Step I: Ionization of GSH To Give the GS^- Thiolate Anion. Step II: Attacks of GS− on the *ipso* Carbon To Form the S(DNP)GS Conjugate through π-Complex and Meisenheimer Complex Intermediates and Radical−Radical Anion Electron Transfer Complexes



complex is not a maximum on the potential surface. The electron transfer may or may not resemble the Meisenheimer complex. If it does, the electron transfer will differ from the complex, having at the least an elongated S−C bond. Barriers for electron transfer are largely due to reorganization of the reactant solvent shells to accommodate product ion formation. In the vesicle wall of the reverse micelle or in the active site of GST, the thiolate anion would become a better reducing agent due to desolvation, while positive charges located adjacently to the aromatic substrate would facilitate charge transfer.

In conclusion, we describe in this Note a reverse micellar system prepared by dissolving a surfactant with a positively charged polar head in isooctane that mimics the active site of glutathione transferase and thus provides a model system to assess the transition-state stabilization factor in the enzymatic reaction. We believe that this novel approach should provide a medium to delineate the reaction mechanism of glutathione transferase.

Experimental Section

Materials. AOT [Sodium bis(ethylhexyl) sulfosuccinate)], Bis-Tris, Bis-Tris propane, CDNB, CTAB, GSH, and Triton X-100 were purchased from Sigma. Isooctane (2,2,4-trimethylpentane) and *n*-hexanol were obtained from E. Merck. Other chemicals used were described previously.[10]

Preparation of Reverse Micellar Stock Solution. Stock AOT reverse micellar solution (0.24 M) was prepared by mixing AOT (10.67 g, 24 mmol) in isooctane (100 mL) until a clear solution was obtained. For CTAB reverse micelles (0.24 M), the composition was CTAB (8.75 g, 24 mmol), isooctane (86 mL), *n*-hexanol (12 mL) (as cosurfactant), and Bis-Tris buffer (2 mL, 200 mM, pH 6.5). *n*-Hexanol and a minimum amount of aqueous solution were essential for dissolving CTAB in isooctane. For Triton reverse micelles (0.24 M), it consisted of Triton X-100 (14.49 mL, 24 mmol), *n*-hexanol (12 mL), and isooctane (73.51 mL). The solutions were used within 24 h of preparation.

Reaction between Glutathione and 1-Chloro-2,4-dinitrobenzene in Reverse Micelles. A stock solution of CDNB was prepared in acetonitrile. Aliquot amounts of the above solution were put into test tubes. After evaporation of acetonitrile by dry air, stock reverse micellar solution was added and the tube was stoppered until use. To the stock reverse micellar solution (2.5 mL, already containing the desired CDNB concentration) was added GSH aqueous solution (30 μL) to give an overall concentration of 0.1 or 1.0 mM as cited in the figure legend. The final $[H_2O]$/[surfactant] ratio of 16.65 was obtained by the addition of Bis-Tris buffer (120 μL, 200 mM, pH 6.5) and isooctane (350 μL) to make a final volume of 3 mL. The conjugation reaction was followed by monitoring the change in absorbance at 340 nm using a Perkin-Elmer Lambda 3B spectrophotometer. A molar absorption coefficient of 9.6×10^3 M^{-1} cm^{-1} for the conjugate[11] was used in calculation. We found that the extinction coefficient of the conjugate in four solvent systems is the same. The relative reaction rate was expressed as μmol S(DNP)GS formed per min under the specified experimental conditions.

Reaction of GSH and CDNB in Reverse Micelles with Various pH Values. The reaction between GSH and CDNB in aqueous solution and various reverse micellar systems was performed in Bis-Tris propane (10 mM) at pH values between 6.5 and 12.5. The $[H_2O]$/[surfactant] was maintained at 16.65. The pH−rate data were analyzed with the nonlinear regression program SigmaPlot (Jandel Scientific, San Rafael, CA) by fitting to eq 1 to estimate the pK_a value of GSH

$$\log Y = \log\{C/(1 + [H^+])/K_a\} \quad (1)$$

where Y is the value of reaction rate measured at any pH value, C is the pH-independent value of Y, and K_a represents the dissociation constant for GSH.

Reaction of GSH and CDNB in CTAB Reverse Micelles with Various $[H_2O]$/[CTAB] Ratios. The conjugation between GSH (0.5 mM) and CDNB (1 mM) in Bis-Tris (4 mM, pH 6.5) was performed in CTAB reverse micelles. The CTAB concentration was maintained constant at 200 mM. The $[H_2O]$/[surfactant] ratio was adjusted by varying the water amount that varied the dimensions of the vesicles.

The core radius (R_c) of the inner cavity of CTAB reverse micelles was estimated by the following empirical equation (eq 2) using data of Vos et al.[12]

$$R_c\,(\text{nm}) = 0.11([H_2O]/[\text{CTAB}]) + 0.8 \quad (2)$$

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