Glutathione transferase mimics: micellar catalysis of an enzymic reaction

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Substances that mimic the enzyme action of glutathione transferases (which serve in detoxification) are described. These micellar catalysts enhance the reaction rate between thiols and activated halogenated nitroarenes as well as α,β -unsaturated carbonyls. The nucleophilic aromatic substitution reaction is enhanced by the following surfactants in descending order: poly(dimethyldiallylammonium-co-dodecylmethyldiallylammonium) bromide (86/14) $\gg$ cetyltrimethylammonium bromide $>$ zwittergent 3–16 (n-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate) $>$ zwittergent 3–14 (n-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate) $\approx$ *N,N*-dimethyl-laurylamine *N*-oxide $>$ *N,N*-dimethyloctylamine *N*-oxide. The most efficient catalyst studied is a polymeric material that incorporates

surfactant properties (n-dodecylmethyldiallylammonium bromide) and opens up possibilities for engineering sequences of reactions on a polymeric support. Michael addition to α,β -unsaturated carbonyls is exemplified by a model substance, *trans*-4-phenylbut-3-en-2-one, and a toxic compound that is formed during oxidative stress, 4-hydroxy-2-undecenal. The latter compound is conjugated with the highest efficiency of those tested. Micellar catalysts can thus be viewed as simple models for the glutathione transferases highlighting the influence of a positive electrostatic field and a non-specific hydrophobic binding site, pertaining to two catalytic aspects, namely thiolate anion stabilization and solvent shielding.

INTRODUCTION

Many ingeniously designed organic compounds, including metal complexes, have been synthesized to replicate the features of biological catalysts [1–11]. These enzyme mimics have been useful in the elucidation of catalytic mechanisms and have been employed in organic synthesis. Some have even been evaluated for therapeutic use [1,4].

Micellar catalysis is a well-developed field in physical organic chemistry where amphiphiles have been used to accelerate a wide array of reactions [12]. Attempts to link the pseudo-phase separation aspect of micellar catalysis with that of an enzyme mimic have not been performed systematically with regard to xenobiotic biotransformations. Since the majority of enzymic reactions of this category involve hydrophobic substrates, this approach could prove useful. Indeed, the efficiency of a cytochrome *P*-450 mimic was substantially improved when a hydrophobic polymeric support was included [3]. Furthermore, one reaction catalysed by glutathione transferases has recently been modelled in reverse micelles [13].

Glutathione transferases (EC 2.5.1.18) act on lipophilic electrophiles bound to their hydrophobic binding site, positioning the substrate in close proximity to the activated thiol in GSH [14]. By this capacity, these enzymes are involved in the detoxification of numerous carcinogenic, mutagenic, toxic and pharmacologically active compounds [15–18]. The products of the reaction, glutathione S-conjugates, are water-soluble and unreactive in the majority of cases and can be readily excreted from the body.

We show here that several cationic, zwitterionic and, in certain cases, non-ionic surfactants catalyse nucleophilic aromatic substitution reactions (Scheme 1a) and Michael addition reactions (Scheme 1b). In addition, a polymeric cationic surfactant, poly(dimethyldiallylammonium-co-dodecylmethyldiallylammonium-

ium) bromide (Copol C_{1–12}) was found to catalyse the reactions:

Scheme 1

Abbreviations used: Copol C_{1–12}, poly(dimethyldiallylammonium-co-dodecylmethyldiallylammonium) bromide; zwittergent 3–16, n-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate; zwittergent 3–14, n-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate; CDNB, 1-chloro-2,4-dinitrobenzene; CTAB, cetyltrimethylammonium bromide; CNAP, 4-chloro-3-nitroacetophenone; CMC, critical micellar concentration.

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MATERIALS AND METHODS

Materials

Glutathione, 2-mercaptoethanol, SDS and Lubrol PX were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Merck (Darmstadt, Germany). Cetyltrimethylammonium bromide (CTAB), *n*-octadecyl mercaptan and 4-chloro-3-nitroacetophenone (CNAP) were from Aldrich-Chemie (Steinheim, Germany). Tetramethylammonium chloride, *N,N*-dimethyl-laurylamine *N*-oxide, zwittergent 3–14 (*n*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate) and zwittergent 3–16 (*n*-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate) were from Calbiochem (La Jolla, CA, U.S.A.). *N,N*-Dimethyloctylamine *N*-oxide and trimethylamine *N*-oxide were from Fluka (Buchs, Switzerland). 1,3,5-Trinitrobenzene was generously given by Nobel-Chemistry (Karlskoga, Sweden). 4-Hydroxyundec-2-enal was kindly given by the late Professor H. Esterbauer (University of Graz, Austria). All other chemicals were of reagent grade and obtained from common commercial sources.

The synthesis of *n*-dodecylmethyldiallylammonium chloride has been described previously [19]. Copol C_{1–12} was synthesized by polymerizing 1.1 mmol of *n*-dodecylmethyldiallylammonium chloride and 6.7 mmol of dimethyldiallylammonium chloride (65 %, w/w, in 40 %, w/w, aqueous solution). After purging the solution with nitrogen for 1 h at 50 °C, 20 mg of 1 % (w/w) ammonium persulphate and a drop of EDTA solution (0.25 M) was added. The reaction mixture was heated at 65 °C for 3 h. The obtained co-polymer was purified by dialysis (Servapore dialysis tubing, 29 mm) for 3 days. The co-polymer solution was ion-exchanged against Br[–] (Fluka Dowex 1X8, 200–400 mesh) and subsequently freeze-dried. Copol C_{1–12} (86/14) was characterized by ¹H NMR [20] and the composition was consistent with the feed ratio (surfactant content was taken as 26 % and used for the calculation of catalytic constants).

Poly(dimethyldiallylammonium-co-dodecylmethyldiallylammonium) bromide (86/14) (Copol C_{1–12}, 86/14)

Feed ratio 86/14, yield 30 %, ¹H NMR δ 0.9 (CH₃), 1.15 (CH₂), 1.38 (CH₂), 1.65 (CH₂), 2.42 (CH ring, *trans*), 2.81 (CH ring, *cis*), 3.15–3.55 [m, CH₃(N), CH₂(N), CH₂ (ring *cis/trans*)], 3.94 [CH₂ (ring *cis/trans*)].

Methods

The reactions between 4-substituted 1-chloro-2-nitrobenzenes (CDNB and CNAP) and thiols were assayed in 0.1 M potassium phosphate, pH 6.5, at 30 °C at the wavelengths described [21]. The respective reactions with *trans*-4-phenylbut-3-en-2-one and 4-hydroxyundec-2-enal were followed in the same buffer by the decrease in absorbance at wavelengths described in [21] and [22]. In some experiments a 3 mm-pathlength cuvette was used to allow higher concentrations of substrate. The concentrations of the reactants and catalysts are indicated below. All experiments involved duplicate or triplicate runs and were repeated at least once. Although a more sophisticated kinetic analysis has been developed [23], apparent third-order rate constants were calculated where rates were linear in reactants as well as surfactant, to allow for the comparisons. Kinetic pK_a values with and without micellar catalysts were determined in 0.1 M glycine/NaOH and potassium phosphate buffers. Meisenheimer complex stabilization [24] was investigated at various 1,3,5-trinitrobenzene, thiol and surfactant combinations in 0.1 M potassium phosphate, pH 6.5, at room temperature.

Since a large amount of catalyst is employed, reactions with CDNB and GSH were run to completion to demonstrate actual catalysis by CTAB (50 % substoichiometric). The product spectra were identical with those obtained in the non-catalysed reaction (not shown). Two additional observations identify the reaction as subject to micellar catalysis breaks in the rate–surfactant concentration behaviour and the fact that the corresponding tetramethylammonium salt or trimethylamine *N*-oxide are not catalytically active. The critical micellar concentration (CMC) values reported for the different surfactants correlate qualitatively with the breaks in the plots (not shown). The lack of an absolute quantitative correlation can probably be ascribed to differences in buffer composition. The catalysis thus depends on the formation of micelles as microdomains.

RESULTS AND DISCUSSION

The rate constants for the reactions with various surfactant and substrate combinations are given in Table 1. The surfactant headgroup usually has a major influence on the reactions catalysed. Cationic and zwitterionic, but not non-ionic, surfactants did increase the reaction rate for hydrophilic thiols, with the former being most effective. Thus a positive electrostatic field appears to be involved in catalysis, in addition to the formation of a hydrophobic pseudophase. Interestingly, with the cationic surfactant, a negatively charged thiol is more reactive than a neutral (but hydrophilic) thiol (Table 1). Consequently, it appears that a positive electrostatic field can both attract negatively charged thiol compounds and increase their reactivity. In accordance with these observations, anionic and non-ionic surfactants are not catalysts in the reactions involving hydrophilic thiols. However, non-ionic surfactants did catalyse nucleophilic aromatic substitution when an extremely hydrophobic thiol (*n*-octadecyl mercaptan) was included. The latter rate enhancement can be ascribed to an increased reactant concentration in the micellar pseudophase, perhaps in conjunction with solvent shielding (known to enhance the reactivity of thiolates [25]).

Table 1 Apparent third-order rate constants ($M^{-2} \cdot s^{-1}$) for the reaction between thiols and electrophiles catalysed by surfactants

Other thiols include CTAB/CDNB-2-mercaptoethanol ($50 M^{-2} \cdot s^{-1}$) and *N,N*-dimethyl-laurylamine *N*-oxide/CDNB-2-mercaptoethanol ($5 M^{-2} \cdot s^{-1}$). *n*-Octadecyl mercaptan was calculated as a lower limit from the activity at 0.25 % (6.7 mM) CTAB, 0.5 mM CDNB and 0.34 mM *n*-octadecyl mercaptan or 0.25 % (4.8 mM) Lubrol PX, 0.5 mM CDNB and 0.34 mM *n*-octadecyl mercaptan. The second-order rate constants for non-catalysed reactions are: CDNB-GSH ($0.015 M^{-1} \cdot s^{-1}$) and CNAP-GSH ($0.0003 M^{-1} \cdot s^{-1}$). CTAB/*trans*-4-phenyl-3-buten-2-one was calculated as a lower limit from the activity at 0.25 % (6.7 mM) CTAB, 0.1 mM *trans*-4-phenyl-3-buten-2-one and 5 mM GSH.

Surfactant/electrophile	Rate constant	
	Thiol ... Glutathione	<i>n</i> -Octadecyl mercaptan
CTAB/CDNB	700	≥ 400
CTAB/CNAP	7	
CTAB/4-hydroxy-2-undecenal	2500	
CTAB/ <i>trans</i> -4-phenyl-3-buten-2-one	≥ 10	
Zwittergent 3–16/CDNB	12	
Zwittergent 3–14/CDNB	5	
<i>N,N</i> -Dimethyl-laurylamine <i>N</i> -oxide/CDNB	7	
<i>N,N</i> -Dimethyloctylamine <i>N</i> -oxide/CDNB	0.4	
Copol C _{1–12} /CDNB	2000	
Copol C _{1–12} /4-hydroxy-2-undecenal	3000	
Lubrol PX/CDNB	0	≥ 1000

Figure 1 Meisenheimer complex spectra formed from thiols and 1,3,5-trinitrobenzene (0.75 mM) stabilized by different surfactants

Main figure: Meisenheimer complex formed in the presence of GSH (5 mM) or *n*-octadecyl mercaptan (0.34 mM) and 3.43 mM CTAB. Inset: Meisenheimer complex formed in the presence of GSH (5 mM) and 3.43 mM zwittergent 3-16 (—), zwittergent 3-14 (---) and *N,N*-dimethyl-laurylamine *N*-oxide (···) respectively.

When surfactants with increasing hydrocarbon chain lengths were tested, a positive correlation with catalytic efficiency is observed. The longer-chain surfactants are more efficient catalysts on a molar basis (Table 1). This statement holds also when it is taken into account that shorter-chain surfactants have higher CMC values and consequently a lower proportion of surfactant molecules in the catalytically competent micellar state.

In view of the fact that hydrophobically modified polyelectrolytes (polysoaps) are often more efficient catalysts than free micelles [26] and that polymers can be manipulated to achieve desirable physical forms (viscous solutions to solids), we investigated whether Copol C₁₋₁₂, a polymeric analogue of CTAB, could catalyse nucleophilic aromatic substitution as well as Michael addition reactions. Clearly this is the case (Table 1). The reaction rates are higher than those for CTAB. Since the polymeric side-chain length is actually shorter by four carbon atoms, it appears that the polymeric micelles are superior to CTAB. This finding is in accordance with previous observations on other reactions [26] and has major implications for the potential use of hydrophobically modified polyelectrolytes as catalysts.

Focusing on nucleophilic aromatic substitution, it could be shown that substrate reactivity (CDNB > CNAP) was reflected in a predictable manner (Table 1). Nucleophilic substitutions on electron-deficient aromatic molecules are thought to occur via Meisenheimer-complex intermediates [27]. These are negatively charged and could therefore benefit from the stabilizing effect of a positive electrostatic field juxtaposed to the micelle's hydrophobic interior. 1,3,5-Trinitrobenzene, which lacks a good leaving group, is known to reversibly form a Meisenheimer complex (Scheme 2). Complex formation with GSH and other thiols was therefore studied by visible spectroscopy to investigate the stabilizing effect of the micelles (Figure 1). As shown in Figure 1) micellar catalysts can stabilize a Meisenheimer complex. Furthermore, they appear to do so in accordance with their catalytic efficiency (cf. Table 1). The invariance with respect to the leaving group in nucleophilic aromatic substitution has been taken as evidence for an early rate-limiting step, probably preceding

Figure 2 Spectrum of the thiolate anion of GSH stabilized by CTAB

Two cuvettes, one with 0.2 mM GSH and the other with 1.6 mM CTAB solutions in 0.1 M glycine/NaOH, pH 9.5, were placed in-line in a single-beam spectrophotometer, and the baseline recorded. Subsequently, the contents (2 × 1 ml) were mixed and redistributed in the two cuvettes. The resulting spectral differences were recorded.

Scheme 2

Meisenheimer-complex formation [27]. Thus according to Hammond's postulate, stabilization of this complex would be expected to lower the energy of the activated complex and hence translate into catalytic efficiency. Since catalytic efficiency is accurately predicted, the spectroscopic method can be used as a convenient method to screen potential catalysts.

Whereas activated aromatic halocarbons are invariably man-made [28], Michael acceptors are widespread in Nature [29], as well as formed during combustion [30] and oxidative stress [31]. It is therefore highly significant that Michael addition reactions can also be catalysed. GSH conjugation to *trans*-4-phenylbut-3-ene-2-one and 4-hydroxyundec-2-enal was observed (Table 1). Other electrophilic compounds tested, e.g. cumene hydroperoxide, 1,2-epoxy-3-(4-nitrophenoxy)propane and (+/-)-anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide, did not react at measurable rates. In principle, any thiol could be used as reactant.

Although we have focused our attention on thiols that are charged, neutral and hydrophobic, the most important part of the molecule is of course the reacting sulphur atom. The thiolate form of GSH has been shown to exhibit 10⁹ times higher reactivity than its protonated form [32]. It is conceivable that the positive electrostatic field on the surface of cationic and zwitterionic micelles could stabilize the thiolate anion of GSH. The kinetic p*K*_a of GSH is 9.54 ± 0.22 and 9.26 ± 0.04, in the absence and presence of CTAB respectively (CDNB conjugation). Since the p*K*_a difference is modest, we wanted to demonstrate the stabilization of the GSH thiolate anion in an independent manner. It is possible to observe the thiolate anion by UV spectroscopy [33]. Figure 2 shows a spectrum that is

consistent with enhanced formation of the thiolate anion in the presence of surfactant.

The reactions described above involving GSH are all catalysed by glutathione transferases [14]. Glutathione transferases contain a GSH-binding site and a hydrophobic binding site for electrophilic second substrates. Although much more sophisticated binding interactions as well as specifically assisted catalysis is exhibited by the enzymes, these sites can be thought of as analogous to the surface and interior of the micelle. The following questions are of interest: (1) quantitative aspects; is the catalytic efficiency of the enzymes vastly superior to that of a micelle? (2) qualitative aspects; are the mechanistic aspects of catalysis similar? (3) what lessons can be learned from enzyme catalysis to be incorporated into the mimics to make them more efficient? (4) can the catalytic power of the enzyme mimics be put to use in detoxification in the human body or the environment?

On a weight basis, the glutathione transferases perform about 10–700 times better than the catalyst CTAB in the standard CDNB assay for the enzyme. That is, at 0.5 mM CDNB and 5 mM GSH the reaction rate is 0.3 $\mu\text{mol}/\text{min}$ per mg of CTAB, whereas the enzymes have activities in the 2–200 $\mu\text{mol}/\text{min}$ per mg range. This appears as a rather modest catalytic advantage. However, saturating conditions are of course chosen for the enzyme assay, whereas first-order conditions in each of the reactants as well as the micellar catalyst are obtained with the surfactant. The apparent second-order rate constant (k_{cat}/K_m) divided by the concentration of the second substrate yields an apparent third-order rate constant for the enzyme reaction that can be compared with the apparent third-order rate constant of the micellar catalysts. In this comparison the enzymes are $\geq 10^4$ times more efficient. Consequently, there is the potential and a need for improvement of synthetic surfactant catalysts.

It is an interesting coincidence that natural compounds, known as biosurfactants (formed by micro-organisms), have been investigated regarding their potential to increase the efficiency of microbial degradation of soil pollutants [34] (functioning as solubilizing agents). Consequently biosurfactants, which are biodegradable [34], should be investigated with regard to their catalytic properties.

Glutathione transferases are highly selective for GSH, whereas micellar catalysts can probably use most thiols. As mentioned, we could show a modest decrease of the kinetic pK_a (by approx. 0.3 unit) of the GSH thiol by CTAB. This value is an order of magnitude lower than the pK_a decrease (approx. 3 units) obtained for the enzyme-bound thiol. This is a highly significant aspect of glutathione transferase catalysis [33].

Meisenheimer-complex stabilization is a characteristic of the enzyme as well as of the micellar catalyst. The degree of stabilization does correlate with the catalytic efficiency. It is interesting that the shapes of the Meisenheimer-complex spectra show variations with different glutathione transferases (Figure 3) [35,36] and also with different thiols and CTAB (Figure 1). The more hydrophilic GSH yields a spectral shape characterized by a valley between two peaks, whereas the hydrophobic *n*-octadecyl mercaptan yields a spectrum comprising one major peak with a shoulder at higher wavelength. These differences can be interpreted as primarily reflecting the degree of solvent shielding of the Meisenheimer complex. Since solvent shielding has been discussed as a contributor to the catalytic efficiency of glutathione transferases [14], it now seems that the shape of the Meisenheimer-complex spectra can be used as an experimental tool to probe the active site of the enzymes and can be correlated with enzyme structure. The published spectra (Figure 3) [35,36] show variation between the extremes exemplified by the different thiols and the micellar catalyst (Figure 1). For instance, one could argue that

Figure 3 Comparison of Meisenheimer-complex spectra between GSH and 1,3,5-trinitrobenzene stabilized by different isoforms of glutathione transferase

μGST , rat microsomal glutathione transferase; 3-3 and 4-4; rat cytosolic glutathione transferase 3-3 and 4-4 respectively from refs. [35,36].

the microsomal glutathione transferase may have a more open active site architecture by these criteria. A less strict specificity for the thiol substrate in this case agrees with this suggestion [37].

The hydrophobic binding sites of glutathione transferases accommodate a large variety of substances. The interior of micelles should of course behave even less specifically.

A major goal for future studies involves increasing the efficiency and versatility of polymeric catalysts. These could be used to detoxify reactive electrophiles in our environment. Persistent organic compounds are a more serious environmental problem, however. Mimicking the strategy of living organisms entails the combination of an activating step that can functionalize relatively inert compounds (such as aliphatic and polycyclic aromatic hydrocarbons) and an ensuing detoxification step that couples water-soluble groups to these functionalities. The activating step is usually carried out by cytochrome *P*-450s, whereas glutathione transferases belong to the class of detoxification enzymes. It is thus highly interesting that a zeolite-encaged iron complex in a polymeric membrane that mimics cytochrome *P*-450 reactions has been described [3]. If the catalysts characterized here can be incorporated into the same polymer, whole reaction sequences could be performed. One could also envision that such copolymers could be used as sensors to estimate the environmental burden of seemingly innocuous pollutants that can undergo transformation to reactive intermediates.

This work was supported by the Swedish Cancer Society, the Swedish Natural Science Research Council and Funds from the Karolinska Institute. J.B.F.N.E. gratefully acknowledges financial support from the Dutch Foundation for Technical Sciences (STW).

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