Communications to the Editor

Antibodies with Thiol-*S*-Transferase Activity

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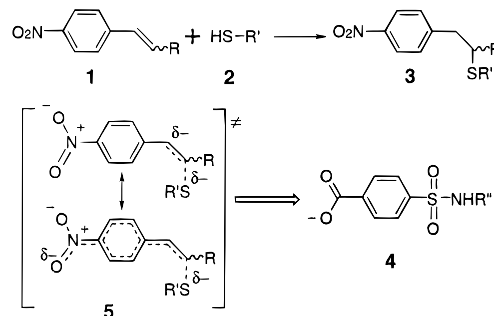
A major detoxification pathway used by aerobic organisms involves the conjugation of the tripeptide glutathione (GSH) to the electrophilic center of toxic substances.[1] This reaction is catalyzed by a class of enzymes referred to as the glutathione *S*-transferases (GST) (EC 2.5.1.18).[2,3] These enzymes activate the cysteine thiol group of GSH for nucleophilic addition to a variety of substrates, including aryl halides, α,β-unsaturated aldehydes and ketones, and epoxides. Despite the availability of X-ray crystal structures,[4,5] the mechanism whereby glutathione transferases catalyze these addition reactions remains unclear. In order to gain a greater understanding of this important biological transformation, as well as to generate new detoxification catalysts, we have asked whether antibodies can be generated that catalyze similar nucleophilic addition reactions.[6,7]

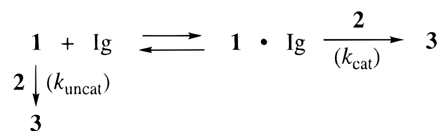
Our initial efforts focused on the addition reaction of thiol nucleophiles to the nitro-substituted styrene derivative **1** (Scheme 1). This reaction, which is typical of those catalyzed by GST, involves formation of the negatively charged transition state **5**. The carboxylate anion of hapten **4** was expected to mimic the negative charge on the aryl nitro group in this transition state. In addition, the polar character of the sulfonamide group might be expected to elicit residues in the antibody combining site capable of electrostatically stabilizing negative charge buildup on the benzylic carbon during the addition reaction. Hapten **4** was synthesized by treatment of 4-carboxylbenzenesulfonyl chloride with the desired amine. Hapten **4a** was obtained from treatment with 2-mercaptoethylamine-2-thiopyridine disulfide[8] and subsequently coupled to succinimidyl (acetylthio)acetate modified carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA).[9,10] The inhibitor **4b** was synthesized from *n*-butylamine.

Antibodies were produced by standard hybridoma technology using the KLH conjugate of **4a**.[11] Thirteen monoclonal cell lines were found to exhibit specific binding for **4**. Antibodies from these cell lines were purified by affinity chromatography on protein-A coupled sepharose and judged to be greater than 95% homogeneous by SDS−PAGE.[12] Initial screening for catalytic activity was performed with 4-nitrostyrene (**1a**) and the nucleophile, sodium 2-mercaptoethanesulfonate (**2a**), in 50 mM MOPS buffer containing 10% (v/v) DMSO at pH 8.0.[13] Product formation was monitored using high-performance liquid chromatography (HPLC). Two antibodies, mAbs 19F3.1 and 19F4.1, were found to accelerate the nucleophilic addition reaction. Addition of 0.5−1.0 mM hapten **4b** resulted in complete inhibition of catalysis, suggesting that catalysis was indeed occurring inside the antibody combining sites. No catalysis was observed when 4-nitrostyrene oxide was used as a substrate, consistent with participation of the *para* nitro group in the catalytic mechanism.

Scheme 1



Further characterization of these two antibodies was carried out with the more soluble substrate, 4-nitrocinnamyl alcohol (**1b**).[14] Catalysis followed Michaelis−Menten kinetics, and the Lineweaver−Burk plots for both antibody-catalyzed reactions are shown in Figure 1. At pH 8.0, the apparent values of k_{cat} and K_m for the formation of nucleophilic addition adduct at a fixed concentration of nucleophile **2a** (60 mM) were determined: mAb 19F3.1, $k_{cat} = 2.2 \times 10^{-3}$ s^{-1}, $K_m = 12.6$ mM; mAb 19F4.1, $k_{cat} = 2.0 \times 10^{-3}$ s^{-1}, $K_m = 12.9$ mM. Because a specific binding site for the nucleophile **2a** was not designed into the hapten (and there is no evidence for saturation by **2a**), it is reasonable to compare the k_{cat} of the antibody-catalyzed reaction with the psuedo-first-order rate constant (k_{uncat}) for the uncatalyzed reaction at high concentration of **2**:



The rate accelerations, k_{cat}/k_{uncat}, are 1.8×10^3 (19F3.1) and 1.7×10^3 (19F4.1), where $k_{uncat} = 1.2 \times 10^{-6}$ s^{-1}. In the pH range of 7.0−9.0, the values of $\log(k_{uncat})$ and $\log(k_{cat}/K_m)$ for both antibodies increase linearly with pH (slope = 0.8), consistent with nucleophilic attack by the thiolate anion of **2a**. The values of K_d for the association between hapten **4b** and the two antibodies were determined by fluorescence-quenching

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(13) Antibody-screening assays were carried out at room temperature (ca. 22 °C), in a buffer containing 10% (v/v) DMSO, 50 mM NaCl, 50 mM 3-[*N*-morpholino]propanesulfonic acid at pH 8.0, with 1.0 mM **1a**, 10.0 mM **2a**, and 2.0−10.0 μM antibody. Reactions were stopped by quenching with an aqueous solution of 50 mM maleimide and 1% trichloroacetic acid. HPLC assays were monitored at 280 nm and performed with a microsorb C-18 reverse-phase column using a gradient of 10%−100% acetonitrile in an aqueous solution of 0.1% trifluoroacetic acid. Products were identified by coinjection with authentic samples.

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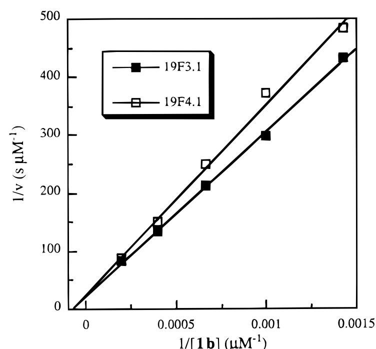

Figure 1. Lineweaver–Burk Plots for antibody-catalyzed conjugation reaction between **1b** and **2a**. Each data point represents an average of two reaction runs, which agree within 10%.

titrations[15] under the reaction conditions at pH 8.0: $K_d = 4.2$ μM (19F3.1); $K_d = 4.5$ μM (19F4.1).[16] The similarity in values of K_d for the two **4b**·mAb complexes correlate with the values of K_m and k_{cat} for substrate **1b**.

The natural glutathione *S*-tranferases exhibit high specific activity for a variety of substrates. For example, 1-chloro-2,4-dintrobenzene, 4-nitrobenzyl chloride and 1,2-epoxy-3-(4-nitrophenoxy)propane are good substrates for GST from equine liver.[17,18] The ability of this enzyme to stabilize anionic transition states involved in these reactions suggested that this GST may provide a good comparison to our catalytic antibodies. The value of k_{cat}/K_m for the reaction of substrate **1b** with glutathione, **2b**, catalyzed by this enzyme is 2.3 M^{-1} s^{-1}, which can be compared to values of 0.17 M^{-1} s^{-1} (19F3.1) and 0.16 M^{-1} s^{-1} (19F4.1) for the antibody-catalyzed reactions of **1b** with thiolate anion **2a** ($k_{uncat} = 1.2 \times 10^{-6}$ s^{-1}). However, this comparison is biased since the natural enzyme has a high apparent K_m for **1b**. The ratio of k_{cat}/K_m reported for the reaction of the isozyme 4-4′ of rat liver GST with the good substrate, 1-chloro-2,4-dinitrobenzene, is approximately 10^4 M^{-1} s^{-1} compared to a calculated pseudo-first-order rate constant for the uncatalyzed reaction of approximately 3×10^{-2} s^{-1} (60 mM GSH, pH = 8.0).[19] These comparisons suggest that with further improvements in hapten design, catalytic antibodies may prove a good source of detoxification catalysts.

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(14) Reactions were carried out at 25.0 ± 1.0 °C, in a buffer containing 10% (v/v) dioxane, 50 mM tris(hydroxymethyl)aminomethane, 50 mM NaCl, and 1.0 mM ethylenediaminetetraacetic acid at pH 8.0. The concentrations of various components are [mAb] = 10.0 μM (20 μM sites), [**2a**] = 60.0 mM, and [**1b**] = 0.7–5.0 mM. Reactions were stopped and analyzed by HPLC as described for antibody-screening reactions. Product formation was quantitated using 3,5-dimethoxybenzamide as the internal standard. Initial velocities were determined by linear fitting of the product concentration at six time points corresponding to ≤1.5% reaction completion.

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(16) Antibody fluorescence was monitored using a Hitachi F-4500 fluorescence spectrophotometer. The solution was excited at 280 nm, and fluorescence was observed at 337 nm. The concentration of antibody was 0.5 μM (1.0 μM sites), and the concentration of **4b** varied from 0–100 μM.

(17) Purchased from Sigma (G-6511) and used without further purification.

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