An Efficient Antibody-Catalyzed Oxygenation Reaction

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Biological oxygen-transfer reactions are essential for the biosynthesis of steroids and neurotransmitters, the degradation of endogenous substances, and the detoxification of xenobiotics.[1] The monooxygenase enzymes responsible for these transformations require biological cofactors such as flavin, heme and nonheme iron, copper, or pterin and typically utilize NADPH for cofactor regeneration.[2] Given their biological and chemical importance, oxygenation reactions have long been targets for antibody catalysis, and in a few cases, redox-active heme and flavin-dependent antibodies have been characterized.[3] More recently, a new strategy has emerged for the generation of catalytic antibodies which utilizes unnatural, chemical cofactors.[4] We now report an antibody-catalyzed sulfide oxygenation reaction mediated by the chemical cofactor sodium periodate, with turnover numbers similar to those of the corresponding enzymatic reactions (Scheme 1).

Sodium periodate ($NaIO_4$) was chosen as the oxidant, since sulfoxide formation occurs under mild aqueous conditions with minimal overoxidation to the sulfone.[5] Furthermore, compared to the flavin and heme cofactors required by the monooxygenase enzymes, $NaIO_4$ is very inexpensive, obviating the need for cofactor recycling. Hammett σ–ρ values, solvent isotope effects, and pH-dependence studies on the oxidation of sulfides by $NaIO_4$ are consistent with a polar transition state formed by nucleophilic attack of sulfur at the periodate oxygen.[6] Consequently, antibodies were raised against aminophosphonic acid hapten **3** in order to catalyze the oxidation of sulfide **1** to sulfoxide **2**. Since hapten **3** contains a protonated amine at physiological pH, antibodies specific for **3** were expected to stabilize the incipient positive charge on sulfur present in the transition state. A phosphonic acid moiety was introduced into the hapten to provide a binding site for the periodate ion.

Hapten **3** was synthesized by reductive amination of *p*-nitrobenzaldehyde with 5-aminovaleric acid and sodium cyanoborohydride, followed by reaction with phosphorous acid in aqueous HCl/formaldehyde.[7] The hapten was conjugated to the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) through an activated *N*-hydroxysuccinimidyl ester.[8] Thirty monoclonal antibodies were generated using standard protocols and purified by protein A affinity chromatography as described previously.[9] Sulfide oxidation was assayed in 20 mM NaOAc, 50 mM NaCl, pH 5.5 assay buffer at 22.5 ± 0.2 °C by monitoring product appearance using high-performance liquid chromatography (HPLC).[10] Eight antibodies were found to accelerate the $NaIO_4$-dependent conversion of sulfide **1** to sulfoxide **2** at a significant rate above the uncatalyzed reaction. Antibody 28B4.2 showed the highest rate enhancement and was characterized in further detail.[11]

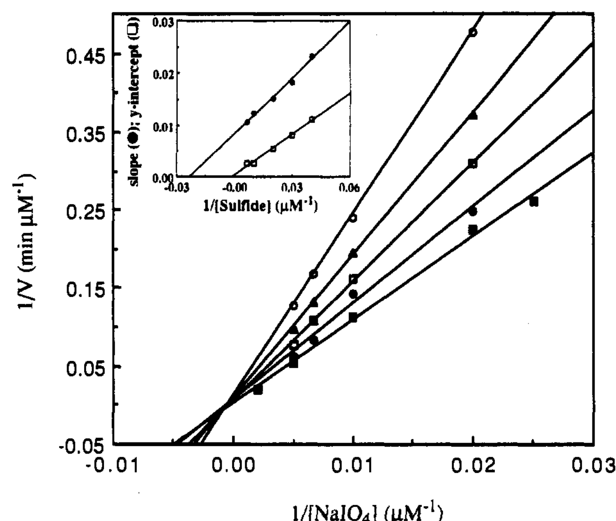

Figure 1. Lineweaver–Burk plots for antibody-catalyzed oxidation of sulfide **1**. The sulfide was held at five fixed concentrations (■ = 150 μM; ● = 100 μM; □ = 50 μM; ▲ = 33.3 μM; ○ = 25 μM), while sodium periodate was increased through at least four distinct concentrations ranging from 40 to 500 μM. Inset: Replot of the *y*-intercepts (□ = $1/V_{max,app}$) and slopes (● = $K_{m,app}/V_{max,app} \times 0.01$) of the Lineweaver–Burk plots as a function of the sulfide concentration. Analogous plots were constructed to give kinetic constants for sodium periodate.

The oxidation reaction catalyzed by antibody 28B4.2 displayed saturation kinetics consistent with a random-binding, sequential mechanism.[12] A family of Lineweaver–Burk plots was constructed at five fixed concentrations of sulfide **1**, while the $NaIO_4$ concentration was varied (Figure 1). The slopes and *y*-intercepts obtained from this analysis were replotted as a function of the sulfide or $NaIO_4$ concentration to give a true maximum velocity (V_{max}) of 49.0 μM s^{-1} and a catalytic constant (k_{cat}) of 8.2 s^{-1} (Figure 1 inset). Michaelis constant (K_m) values of 43 and 252 μM were determined respectively for **1** and $NaIO_4$. For comparison, the uncatalyzed second-order rate constant (k_{uncat}) was measured to be 8.5×10^{-1} M^{-1} s^{-1}, resulting in a rate enhancement $[(k_{cat}/K_m)/k_{uncat}]$ of 2.2×10^5. The turnover number and rate acceleration for antibody 28B4.2 reveal that this antibody is as efficient as numerous monooxygenase enzymes, including the flavin adenine dinucleotide (FAD)-dependent monooxygenases and the microsomal P-450 isozymes.[13]

Prolonged exposure of the antibody to sodium periodate at the concentrations used in this study had little effect on its specific

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(5) (a) Studies on the dissociation equilibria of periodic acid in water show that IO_4^- is the predominant oxidizing species in aqueous solution between pH 4 and 8: Crouthamel, C. E.; Hayes, A. M.; Martin, D. S. *J. Am. Chem. Soc.* **1951**, *73*, 82–87. (b) Madesclaire, M. *Tetrahedron* **1986**, *42*, 5459–5495.

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(8) Hapten **3** was purified by preparative reverse-phase HPLC (Whatman M-20 10/50 Partisil ODS-10 column, 5% to 90% acetonitrile in 0.1% aqueous trifluoroacetic acid, 8.0 mL/min), and characterized by NMR, infrared, and mass spectral analysis. Coupling to the carrier proteins afforded epitope densities of **3** (KLH) and **4** (BSA).

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(10) Reactions were initiated at 22.5 ± 0.2 °C by the addition of aqueous $NaIO_4$ to assay buffer containing the sulfide and the internal standard, *o*-nitroanisole (5% v/v EtOH, 6.0 μM antibody). HPLC assays were monitored at 260 nm and performed with a Microsorb C-18 reverse-phase column using a gradient of 15% to 100% acetonitrile in water. Products were identified by coinjection with authentic samples and quantitated against *o*-nitroanisole. Initial velocities were determined by linear fitting of the product concentration at five or more time points corresponding to <10% reaction completion.

(11) The specific activity of antibody 28B4.2 remained unchanged after further purification by anion (MONO-Q) exchange chromatography.

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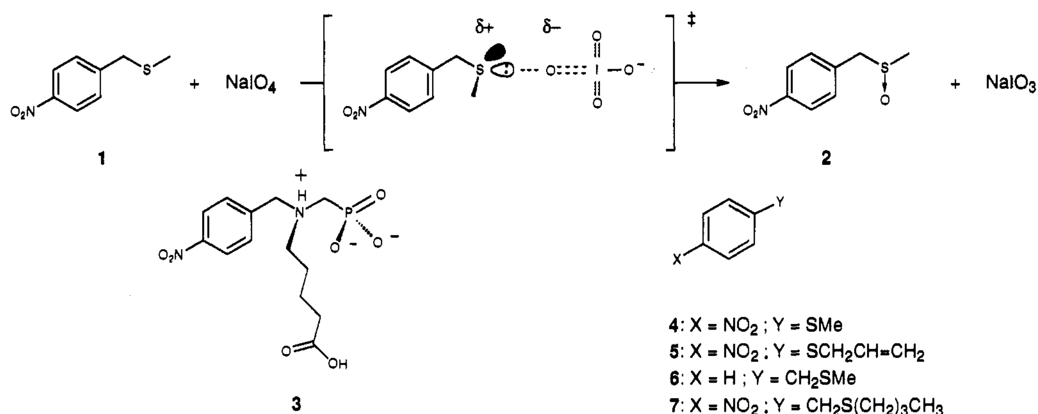
Scheme 1



activity.[14] In addition, no change in V_{max} was observed after more than 25 turnovers. The antibody-catalyzed reaction was completely inhibited by the addition of hapten **3**, consistent with catalysis occurring in the antibody combining site. Fluorescence quenching experiments performed in the reaction buffer at 22 °C afforded a dissociation constant (K_d) of 52 nM for the hapten.[15]

In order to probe the substrate specificity of the antibody-catalyzed oxidation reaction, sulfides **4–7** were also assayed. Surprisingly, antibody 28B4.2 catalyzed the oxidation of *p*-nitrothioanisole (**4**). A Lineweaver–Burk analysis of the steady-state data at high $NaIO_4$ concentration (2.0 mM) afforded an apparent catalytic constant ($k_{cat,app}$) of 13.4 min^{-1} and a Michaelis constant ($K_{m,app}$) of 36 μM.[10] Comparison with the second-order rate constant for the uncatalyzed reaction (k_{uncat} = 2.9 M^{-1} min^{-1}) reveals a rate enhancement [$(k_{cat}/K_m)/k_{uncat} = 1.3 \times 10^5$] similar to the value obtained for sulfide **1**. No catalysis was observed when benzyl sulfide **6** was used, suggesting that the nitro group plays a crucial role in antibody recognition and binding. Despite this remarkable specificity, antibody 28B4.2 tolerated a range of side chains, catalyzing the oxidation of sulfides **5** and **7** which contain allyl and *n*-butyl groups.[16] Mechanism-based inactivation of the antibody via a 2,3-sigmatropic rearrangement of the allyl sulfoxide product to generate an electrophilic allyl sulfenate was not observed.[13a,17]

Overall, these results raise the possibility of using antibodies as catalysts for regio- and stereoselective sulfide oxidations. Despite the importance of chiral sulfoxides in asymmetric synthesis and pharmaceuticals, relatively few chemical methods exist for the oxidation of prochiral sulfides to chiral sulfoxides.[18] Studies to elucidate the stereoselectivity of this antibody-catalyzed reaction are currently in progress.

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(14) For example, no change in specific activity was observed after 3 μM antibody was incubated with 200 μM $NaIO_4$ at 37 °C for 24 h.

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(16) The rate of the antibody-catalyzed oxidation of sulfide **7** was close to that observed for sulfide **1** (V_{rel} = 0.9 using 50 μM sulfide, 100 μM $NaIO_4$).

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