A Chromogenic Assay for Screening Large Antibody Libraries

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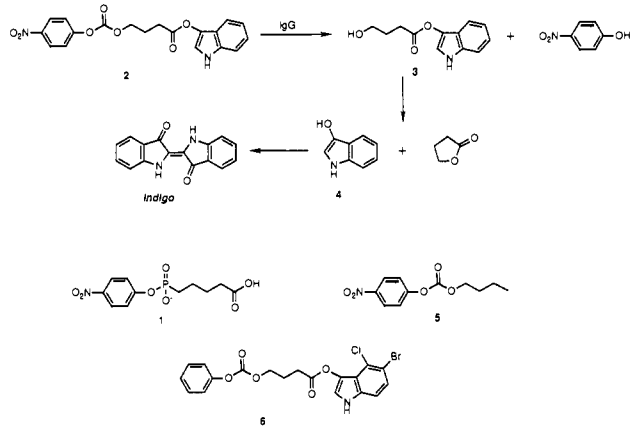
One approach to generating antibodies with increased catalytic efficiency involves augmenting "mechanism-based" hapten design[1] with direct genetic screens or selections for catalytic activity. Recent advances in the bacterial cloning and expression of antibodies make possible the generation of large libraries of antibody molecules either by directed or random mutagenesis or by combinatorial strategies.[2] We now describe the development of a facile chromogenic assay that lends itself to the screening of bacterial colonies or plaques. This scheme can be generalized to a large number of reactions for which no simple plate assay exists.

The assay was developed using the *p*-nitrophenyl phosphonate specific antibody 48G7 as a model, which has been shown to catalyze the hydrolysis of a number of nitrophenyl carbonates and esters,[3] A chromogenic substrate **2** was designed, which consists of 3-hydroxyindole (**4**) linked to the 4-nitrophenyl carbonate moiety via 4-hydroxybutyric acid (Scheme I). Antibody-catalyzed hydrolysis of the carbonate group to nitrophenol and indolylbutyric acid **3** should be followed by rapid intramolecular cyclization to give butyrolactone[4] and 3-hydroxyindole, The latter product undergoes oxidative dimerization to form the chromophore, indigo ($\lambda_{max}^{H_2O}$ = 620 nm, ϵ = 29 700),[5] Indigo is insoluble in aqueous solution and accumulates at the site of reaction (in contrast to nitrophenol which rapidly diffuses through the medium), affording high detection sensitivity,[6] The 4-hydroxybutyric acid spacer removes the chromophore from the labile bond in the substrate, *eliminating the need for incorporating the bulky indolyl group into each hapten*[7] and, as a result, the synthetic and biological limitations imposed by the chromophore, This strategy should allow the rapid synthesis of chromogenic substrates for many reactions, including the "exo" hydrolysis of ester, phosphate ester, and glycosidic bonds.

Substrate **2** was synthesized by condensation of 4-(benzyloxy)butyric anhydride[8] with 3-hydroxyindole (**4**) under basic conditions[9] to give the corresponding benzyl ether. The benzyl protecting group was removed by hydrogenolysis with palladium on carbon (EtOAc), and the resulting alcohol **3** was condensed with *p*-nitrophenyl chloroformate in the presence of 1.1 equiv of triethylamine (0 °C, CH_2Cl_2). The product, **2**, was purified by flash chromatography on silica gel (CH_2Cl_2 eluant).

Scheme I



The rate of hydrolysis of substrate **2** was assayed by monitoring nitrophenol release (λ_{max} = 405 nm, ϵ = 15 480) spectrophotometrically at 37 °C in a 50 mM NaCl/50 mM Tris, pH 8,2 buffer containing 0,5% Triton X-100.[10] The antibody-catalyzed reaction demonstrated saturation kinetics, and a Lineweaver–Burk analysis of initial rates afforded kinetic constants K_m (**2**) and k_{cal} (**2**) of 274 μM and 0.31 min^{-1}, respectively. These values are comparable to those for 4-nitrophenyl butyl carbonate **5** (k_{cat} = 1,8 min^{-1}, K_m = 251 μM) and methyl 4-nitrophenyl succinate (k_{cat} = 0.66 min^{-1}, K_m = 274 μM). The half-life for the cyclization reaction leading to indolyl release is less than 5 min in reaction buffer at 37 °C. The bimolecular rate constant for oxidative dimerization of 3-hydroxyindole to indigo was reported to be 3.1 × 10^{-3} M^{-1} s^{-1} in gelatin-stabilized 0.5 M Na_2PO_4 buffer, pH 8.0 at 25 °C.[11]

Antibody-catalyzed hydrolysis of **2** resulted in the formation of 0.3 equiv of indigo per equivalent of nitrophenol.[12] Indigo formation was found to be dependent upon antibody catalytic activity, In the absence of antibody, no indigo formation was detected. The antibody-dependent formation of indigo was inhibited by the nitrophenyl phosphonate transition-state analogue **1** to which the antibody was generated. In addition, the benzyl ether of indolyl butyric acid **3** did not form detectable indigo when incubated with the antibody, indicating that the indigo formation from **2** is not due to direct antibody-catalyzed hydrolysis of the indolyl ester, consistent with the known specificity of the antibody. Because indigo precipitates when formed on an agar plate, this method allows assays to be carried out over a period of several days (typical of most colony/plaque plate screens), In contrast, nitrophenol (as well as a fluorescent anthranilyl derivative of phenylalanine) could not be detected after approximately 12 h on a 1,7% agar plate due to extensive diffusion. Finally, this approach has also been applied to catalytic assays of an antibody (7D4)[3] that catalyzes the hydrolysis of alkylphenyl carbonates (using the 5-bromo-4-chloro-3-indolyl substrate **6**), a reaction in which no visible chromophore is generated.

We have described a facile, rapid approach for generating chromogenic substrates which can be generalized to a considerable number of antibody-catalyzed reactions.[13] This scheme allows for a simple qualitative assay of catalytic activity rather than hapten binding activity at the stage of antibody generation and isolation, allowing large numbers of antibodies to be assayed. This methodology is currently being applied to the screening of 48G7 mutants for increased catalytic efficiency,

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(12) Indigo formation was assayed spectrophotometrically (λ_{max} = 620 nm; ϵ = 29 700) after 1 h at 37 °C in reaction buffer containing 5.9 μM antibody.

(13) In many cases the indolyl group can be introduced into the substrate at the position corresponding to carrier protein in hapten.

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Ligand Photodissociation and Recombination Dynamics of Ferrous Cytochrome *c* Peroxidase at Alkaline pH

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The dynamics associated with ligand photodissociation and ligand binding provide an avenue through which information concerning conformational interactions involving the active site of heme proteins may be obtained. To date, most time-resolved spectroscopic investigations have involved exogenous π-acceptor ligands such as CO, O_2, and NO.[1-3] In fact, until very recently hexacoordinate low-spin hemes with strong-field σ-donor ligands were considered to be largely nonphotolabile. Magda and coworkers[4] have, however, demonstrated that both cytochrome *c* and cytochrome b_5 exhibit photodissociation of nitrogenous ligands on a very fast (<100 ps) time scale. Photodissociation of a σ ligand has also been implicated in the photodynamics of the cytochrome a_3 distal heme pocket in cytochrome *c* oxidase.[5] This study characterizes the dynamics of the low-spin alkaline form of ferrous cytochrome *c* peroxidase[6] (CCP) subsequent to heme photodissociation.

The heme pocket of CCP is specifically designed to catalyze the reduction of H_2O_2 utilizing electrons derived from cytochrome *c*. In addition, the local heme environment is conformationally sensitive to pH,[7-9] temperature,[10] and mutagenesis,[7a,11] Recently

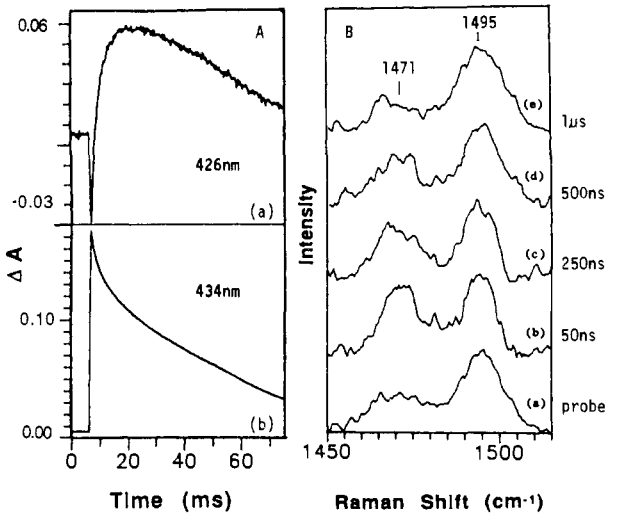

Figure 1. Transient absorption (A) and time-resolved resonance Raman (B) spectra of ferrous CCP in 100-200 mM Tris-HCl, pH = 8.50. (A) Pump laser: output of a Lambda Physik FL 3002 dye laser (at 480 nm) pumped by a Lambda Physik LPX210i excimer laser. Probe light: xenon arc lamp (~75 W) at 426 nm (a) and 434 nm (b). Transient spectra obtained in Soret region are the average of 50 shots. (B) the ν_3 (spin-state-sensitive band) region. Pump laser: Nd:YAG (Quanta-Ray DCR-II) with $\sim 4 \times 10^8$ W/cm^2 at samples (560 nm). Probe laser: output ($\sim 3 \times 10^7$ W/cm^2) of Molectron DL-24 dye laser excited by Molectron UV-24 N_2 laser, excited at 436 nm, which is close to the isosbestic point of 5c/hs and 6c/ls Soret bands of CCP(II). The spectra are the average of several scans with a rate of 10 cm^{-1} min^{-1}. Detailed descriptions of the instrumentation and sample preparation have been published elsewhere.[8,12] in detail; (a) probe only; (b) 50-ns time delay; (c) 250 ns; (d) 500 ns; (e) 1 μs.

it has been demonstrated that the equilibrium hexacoordinate low-spin ferrous form of CCP is photolabile in the pH range 7.8-9.0,[3,7,8] The time-resolved resonance Raman results presented in Figure 1B demonstrate that the photodissociation of a ligand occurs rapidly (<10 ns) and with a high yield.

The photodissociation and ligand recombination are easily followed by monitoring the time evolution of the spin-state-sensitive ν_3 band (Figure 1B). The pentacoordinate high-spin band (appearing at 1471 cm^{-1}) is generated within the 10-ns pump pulse and persists on a time scale of 500 ns. Geminate ligand recombination then occurs, and the transient species reverts back to the hexacoordinate low-spin species (observed by the increase in signal at 1495 cm^{-1}). It is likely that under alkaline conditions the distal His-52 binds to the heme Fe,[7,8] and it is this ligand which is photodissociated by pump laser pulses. The time-resolved resonance Raman data indicate that the recombination of the σ ligand in alkaline CCP(II) is quite slow ($t_{1/2} \sim 500$ ns), relative to the <50-ns half-life observed for geminate recombination in the photodissociation of HbCO or HbO_2[2] and <100 ps for ligand recombination of cytochrome *c*.[4] An endogenous ligand would not be expected to be removed from close proximity to the heme by photolysis. Thus the slow recombination kinetics most likely result from activation energetics rather than ligand mobility away from the heme. We speculate that structural dynamics within the distal heme pocket may play a significant role in these recombination energetics.

Subsequent transient absorption measurements of the alkaline form of CCP(II) indicate that ligand recombination initiates relatively slow and complex conformational dynamics in the protein (Figure 1A). Heme ligand photodissociation is indicated by the

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