A Catalysis-Based Selection for Peroxidase Antibodies with Increased Activity

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Affinity-based selections involving libraries of peptides or proteins expressed on the surface of filamentous phage have proven very useful for the isolation of high affinity ligands and receptors.1 In contrast, there are relatively few examples in which phage display has been used to enhance catalytic activity, rather than binding affinity. Direct selections based on catalysis require either (1) the efficient conversion of a substrate to a reactive product which then binds covalently to the phage surface prior to diffusion out of the protein active site, or (2) an “intramolecular” reaction between a phage displayed catalyst and substrate.2 Such approaches have been used to generate DNA polymerases2b and tyrosine kinases2c with altered specificities as well as antibodies with thioesterase,2d glycosidase,2e and phosphodiesterase2d activities. Herein, we describe an activity-based strategy for selecting oxidative catalysts by phage display and apply it to an antibody with peroxidase activity.

Antibody 7G12 was raised against N-methylmesoporphyrin (NMP), which mimics the strained porphyrin substrate of the metalation reaction.3a 7G12 was found to catalyze mesoporphyrin metalation by Cu2+ and Zn2+, in the latter case with a catalytic efficiency approaching that of the natural enzyme ferrocheletase.3a The complex of 7G12 with Fe(III) mesoporphyrin IX (FeMP) also catalyzes the reduction of H2O2 with the chromogenic peroxidase substrates, o-dianisidine, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).3e While peroxidases are among the most efficient enzymes known with kcat/Km (H2O2) values of approximately 108 M−1 s−1, the corresponding value for the 7G12 Fab–FeMP complex is 274 M−1 s−1.3e

To enhance the catalytic activity of 7G12, a selection scheme was developed in which oxidation of the biotin-linked peroxidase substrate I results in a highly reactive intermediate that covalently modifies the antibody–heme complex. Active catalysts are then isolated on a streptavidin affinity support. A screen of mechanism-based inhibitors of peroxidases5 revealed that tyramine (4-(2-aminoethyl)phenol) covalently modifies the 7G12 Fab–heme complex upon oxidation of the porphyrin H2O2. The modified Fab was detected by capture on immobilized streptavidin and incubation with a goat anti mouse Fab-HRP conjugate. No Fab was detected when the acyl transferase antibody 136R Fab in a 1:10 ratio. The mixture was then incubated (sodium borate buffer, pH 9.0) with 10 nM FeMP and 2% BSA for 15 min prior to reaction with 1 mM H2O2 and 5 mM I. After reaction for an hour, phages were precipitated with PEG and washed, and the mixture was incubated on streptavidin plates for 1 h. Immobilized phages were rescued by infecting E. coli XL1 Blue cells, and subsequent plating on LB/ampicillin plates with 2% glucose. Clone identities were confirmed by Western analysis using anti Fab antibodies conjugated to horseradish peroxidase, and by capture of the 7G12-heme bearing phage (and not M13 or 136R bearing phage) on streptavidin plates upon oxidation of I in the presence of H2O2.

A model selection was carried out in which phages displaying 7G12 Fab were diluted with those displaying 136R Fab in a 1:10 ratio. The mixture was then incubated (sodium borate buffer, pH 9.0) with 10 nM FeMP and 2% BSA for 15 min prior to reaction with 1 mM H2O2 and 5 mM I. After reaction for an hour, phages were precipitated with PEG and washed, and the mixture was incubated on streptavidin plates for 1 h. Immobilized phages were rescued by infecting E. coli XL1 Blue cells, and subsequent plating on LB/ampicillin plates with 2% glucose. Clone identities were determined by colony PCR using specific primers for 7G12 Fab. This protocol yielded an almost 100-fold enrichment of the 7G12 phage over 136R phage. Importantly, the enrichment of the 7G12 phages was found to be dependent on the presence of H2O2 and I, suggesting that the selection of active phage is based on the catalytic turnover of the biotin-tyramine conjugate in a process catalyzed by 7G12 Fab displayed on the phage surface. In addition, the use of the biotin-tyramine conjugate which can be cleaved by incubation with 20 mM dithiothreitol was found to increase the recovery ratio 3 to 5-fold.

Both directed and random mutagenesis were used to generate libraries of 7G12. PCR was used to introduce random mutations (NNK) at residues Tyr496, Tyr911, Tyr94, Arg95, and Asp96, all of which appear to contact the hapten in the crystal structure of the 7G12 Fab–NMP complex3b (Figure 2). In addition, error-prone PCR was used to construct a library in which random mutations were introduced throughout the variable region. DNA sequencing revealed an average of 2.5 mutations/clone for the error-prone PCR library. Library sizes in both cases were around 5 × 106. Selections were then carried out by sequential rounds of oxidation, affinity capture, elution, and reinfecion. The directed mutant library afforded after three rounds of selection clones enriched (25%) in a Tyr496-Tyr mutation. The error-prone library did not converge on a consensus sequence even after six rounds of selection, despite a final recovery ratio of 0.2%. Therefore, a total of 5000 clones from the final round of selection were screened for peroxidase activity.

Figure 1. Scheme for catalysis-based selection of phage displayed antibody libraries.

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using an automated screening system. Twenty percent of the clones were found to have equal or higher peroxidase activity relative to wild-type 7G12 Fab. In contrast, for the naïve error-prone PCR library, less than 0.2% of the clones had activity comparable to that of the wild-type Fab.

Both the Tyr49L-Trp mutant and the 30 most active clones from the random library were expressed and purified, and their activities were determined. In general, higher levels of peroxidase activity resulted from three factors: higher expression levels, increased heme affinity resulting in a higher effective concentration of active catalyst, and higher catalytic efficiency ($k_\text{cat}/K_\text{m}$). Indeed, all of the mutants with higher peroxidase activity found in the error-prone PCR library have more than 50% of the Fab bound by heme upon isolation. In contrast, only 10–15% of wild-type 7G12 Fab is heme bound upon isolation. The most active mutants found in the error-prone PCR library were 1F11, which contains Asn53L-Lys, and Gly66-Arg mutations, and 1H4, which has Ala51-Thr, Met78-Val, Glu90-Arg mutations instead of the Tyr49L-Trp mutant nor introduction of the Tyr49L-Trp mutant, a 10-fold increase in peroxidase activity. Moreover, mutants 1F11 and 1H4 afforded 15 mg/L of the heme cofactor, and higher catalytic efficiency ($k_\text{cat}/K_\text{m}$) resulted from three factors: higher expression levels, increased heme affinity resulting in a higher effective concentration of active catalyst, and higher catalytic efficiency ($k_\text{cat}/K_\text{m}$). Indeed, all of the mutants with higher peroxidase activity found in the error-prone PCR library have more than 50% of the Fab bound by heme upon isolation. In contrast, only 10–15% of wild-type 7G12 Fab is heme bound upon isolation. The most active mutants found in the error-prone PCR library were 1F11, which contains Asn53L-Lys, and Gly66-Arg mutations, and 1H4, which has Ala51-Thr, Met78-Val, Glu90-Arg mutations instead of the Tyr49L-Trp mutant.

In summary, a novel and efficient strategy for selecting antibodies with higher peroxidase activity from phage displayed antibody libraries was developed. Previously only screen-based methods have been used to evolve enzyme peroxidases. Using this strategy, we identified mutations of antibody 7G12 that lead to significant increases in peroxidase activity, demonstrating the utility of this method for the evolution of peroxidase activities in antibodies or other protein scaffolds.

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Supporting Information Available: Material and methods, sequence of the selected mutants, and kinetic characterization of the mutants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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