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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.[2a] Such approaches have been used to generate DNA polymerases[2b] and tyrosine kinases[2c] with altered specificities as well as antibodies with thioesterase,[2d] glycosidase,[2e] and phosphodiesterase[2f] 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 Cu^{2+} and Zn^{2+}, in the latter case with a catalytic efficiency approaching that of the natural enzyme ferrocheletase.[3a−d] The complex of 7G12 with Fe(III) mesoporphyrin IX (FeMP) also catalyzes the reduction of H_2O_2 with the chromogenic peroxidase substrates, *o*-dionisidine, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).[3e] While peroxidases are among the most efficient enzymes known with k_{cat}/K_m (H_2O_2) values of approximately 10^7 M^{-1} s^{-1},[4] 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 **1** 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 peroxidases[5] revealed that tyramine (4-(2-aminoethyl)phenol) covalently modifies the 7G12 Fab−heme complex upon oxidation in the presence of H_2O_2. The modified Fab was detected by capture on immobilized streptavidin and incubation with a goat anti mouse Fab-HRP conjugate. No Fab was captured when the acyl transferase antibody 136R[6] (which lacks oxidative activity) was used as a control. Tyramine is known to undergo rapid condensation with phenolic side chains on protein surfaces upon oxidation by hydrogen peroxide in a process catalyzed by the enzyme peroxidase.[7a] Further, biotin-linked tyramine molecules have previously been shown to biotinylate protein[5] conjugated to peroxidase enzymes.[7b−d]

To carry out the selections, the Fab fragment of 7G12 was displayed on M13 phage as a fusion to the coat protein g3p using the pComb3H vector.[8] Expression of the Fab on phage was 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 **1** in the presence of H_2O_2.

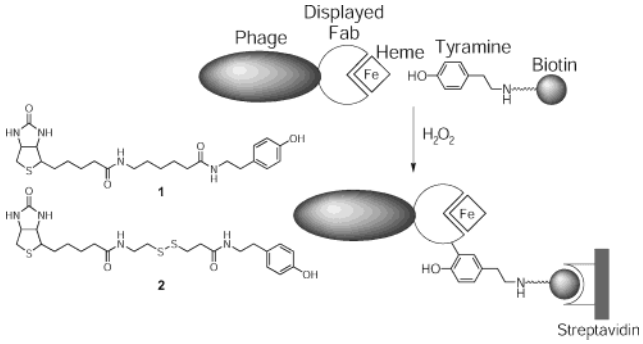

Figure 1. Scheme for catalysis-based selection of phage displayed antibody libraries.

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 H_2O_2 and 5 μM **1**. 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 H_2O_2 and **1**, 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 **2** 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 $Tyr49^L$, $Tyr91^L$, $Tyr94^L$, $Arg95^H$, and $Asp96^H$, all of which appear to contact the hapten in the crystal structure of the 7G12 Fab−NMP complex[3b] (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 5×10^7. Selections were then carried out by sequential rounds of oxidation, affinity capture, elution, and reinfection. The directed mutant library afforded after three rounds of selection clones enriched (25%) in a $Tyr49^L$Trp 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

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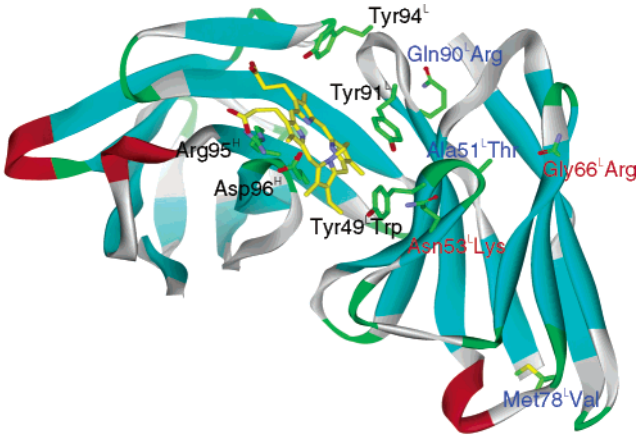

Figure 2. Crystal structure of 7G12 Fab−hapten NMP complex[3b] (Protein Data Bank: 3FCT) showing the active site residues Tyr49^{L}, Tyr91^{L}, Tyr94^{L}, Arg95^{H}, and Asp96^{H} as well as the Asn53^{L}Lys and Gly66^{L}Arg mutations in 1F11 (red) and the Ala51^{L}Thr, Met78^{L}Val, and Gln90^{L}Arg mutations from 1H4 (blue).

using an automated screening system.[9] 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 Tyr49^{L}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_{cat}/K_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 Asn53^{L}Lys, and Gly66^{L}Arg mutations, and 1H4, which has Ala51^{L}Thr, Met78^{L}Val, Gln90^{L}Arg, Val110^{L}Ala, Asn137^{L}His, and Phe139^{L}Tyr mutations. 1F11 and 1H4 have approximately 50-fold higher catalytic activity than does wild-type Fab at the same protein concentration. Both mutants have a K_m for H_2O_2 similar to that of 7G12; 1F11 and 1H4 have k_{cat} values of 123 and 97 s^{-1}, respectively, as compared to a k_{cat} of 6 s^{-1} for wild-type Fab with *o*-dionisidine as the reducing agent. Moreover, mutants 1F11 and 1H4 afforded 15 mg/L of purified protein, in contrast to 3−4 mg/L for 7G12 Fab. For the Tyr49^{L}Trp mutant, a 10-fold increase in k_{cat}/K_m (3×10^3 M^{-1} s^{-1}) over wild-type 7G12 was found, while the expression level remained unchanged from that of the wild type. Approximately 30% of the Tyr49^{L}Trp mutant was heme bound upon isolation. Unfortunately, neither shuffling of the 30 most active mutants from the error-prone library with the Tyr49^{L}Trp mutant nor introduction of the latter mutant into 1H4 or 1F11 led to further increases in activity. Thus, it appears that it is difficult to evolve 7G12 by additive increases in catalytic efficiency from the individual mutations isolated here.

The catalytic cycle for heme-containing peroxidases involves oxidation of the native peroxidase with H_2O_2 to yield the active enzyme.[10a] The radical cation in the iron(IV)oxo porphyrin ring can be delocalized onto proximal protein side chains, as is the case with cytochrome *c* peroxidase in which the radical cation delocalizes onto the indole ring of Trp191.[10b] Because Tyr49^{L} makes direct contact with the porphyrin ring through π-stacking interactions in the 7G12 Fab−NMP complex (Figure 2), the Tyr49^{L}Trp mutation may similarly help stabilize the radical cation on the porphyrin ring and lead to higher peroxidase activity. The Asn53^{L}Lys mutation in 1F11 may directly affect the π-stacking interactions between Tyr49^{L} and the porphyrin ring of the heme cofactor, which may play a major role in stabilizing intermediates formed during the peroxidation reaction. The other mutation in 1F11, Gly66^{L}Arg, is on the β strand opposite the Asn53^{L}Lys mutation and may also play a role in orienting the newly introduced Lys53^{L} for better packing with Tyr49^{L}. The Gln90^{L}Arg mutation in CDR L3 of 1H4 may change the conformation of L3 and affect the packing between Tyr91^{L} and the heme cofactor. The Ala51^{L}Thr mutation is located on CDR L2 and may affect binding between Tyr49^{L} and the heme cofactor. The other four mutations in 1H4 are located far from the cofactor binding site and may contribute to increased expression levels of the Fab 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.[11a−d] 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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