Peroxidase Activity in Heme Proteins Derived from a Designed Combinatorial Library

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Received April 6, 2000

We previously reported the design of a library of de novo proteins targeted to fold into 4-helix bundles.[1] The library was created using a "binary code" strategy in which the sequence locations of polar and nonpolar amino acids were specified explicitly, but the identities of these side chains were varied extensively. Combinatorial diversity was made possible by the organization of the genetic code: Positions designed to contain polar amino acids were encoded by the degenerate DNA codon NAN, which codes for Lys, His, Glu, Gln, Asp, or Asn. Positions designed to contain nonpolar amino acids were encoded by NTN, which codes for Met, Leu, Ile, Val, or Phe (N represents a mixture of DNA nucleotides). We subsequently reported that approximately half the sequences in our initial library[1] bind heme.[2] The enormous diversity of sequences within the binary code library presents an opportunity for the isolation of de novo heme-based enzymes. We now establish the catalytic potential of the binary code proteins by demonstrating that several of the de novo heme proteins function as peroxidases.

Natural peroxidases such as horseradish peroxidase (HRP) catalyze the reduction of hydrogen peroxide or alkyl peroxides to water or alcohol, respectively. The peroxidase mechanism is described by the following steps:[3]

$$\mathrm{E} + \mathrm{H_2O_2} \xrightarrow{k_1} \text{compound I} + \mathrm{H_2O} \qquad (1)$$

$$\text{compound I} + \mathrm{AH_2} \xrightarrow{k_2} \text{compound II} + \mathrm{AH}\bullet \qquad (2)$$

$$\text{compound II} + \mathrm{AH_2} \xrightarrow{k_3} \mathrm{E} + \mathrm{AH}\bullet \qquad (3)$$

E represents the ferric resting state of the heme enzyme. Compound I is an intermediate two oxidation states above the resting state, and compound II is one oxidation state above the resting state. AH_2 is the reducing agent. Two commonly used reducing agents are 2,2′,5,5′-tetramethyl-benzidine (TMB) and 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS). These reagents become colored upon oxidation, thereby allowing peroxidase activity to be monitored spectraphotometrically.

To search for novel peroxidases in our library of heme proteins, we developed an activity screen that does not require purification of the de novo proteins from cellular contaminants. Samples were prepared by a rapid freeze/thaw protocol[4] shown previously to produce protein samples of sufficient quality for various studies including NMR spectroscopy[5a] and H/D exchange kinetics.[5b] Freeze/thaw samples from 37 binary code proteins[6] were screened by monitoring oxidation of TMB. The results (Figure 1) indicate that several of the de novo proteins display activity significantly above the controls.

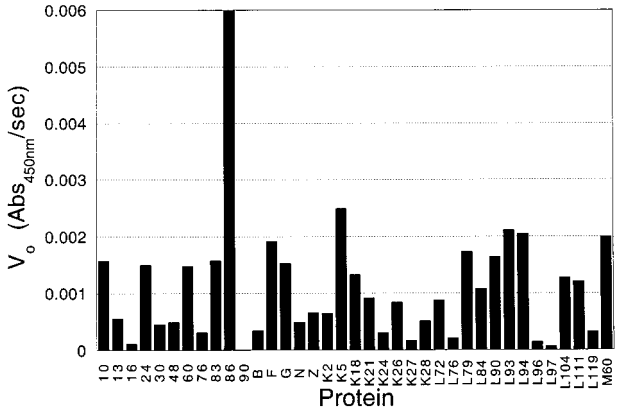

Figure 1. Screen for peroxidase activity. Proteins were expressed in *Escherichia coli* as described previously[1]. All samples displayed a dark band in Coomassie-stained SDS-PAGE. No attempt was made to normalize concentrations. Samples were prepared using a freeze−thaw protcol.[4,5a] Solutions were dialyzed into 50 mM Tris HCl, 50 mM NaCl (pH 8.0) for >4 days to remove endogenous reducing agents. Reaction mixtures contained >50 μM protein extract, 4 μM hemin chloride, 10 μM TMB, and 0.00075% H_2O_2. Absorbance at 450 nm was recorded using a HP8452A diode array spectrophotometer and a 1-cm cuvette. Controls: sequence 48 has a stop in the third codon and does not express protein; protein B is a full-length sequence that does not bind heme.

On the basis of this screen, we chose four proteins for detailed analysis.[9] Proteins were purified, reconstituted with heme, and analyzed for peroxidase activity using ABTS as the reducing agent. Experimental data for protein 86 are shown in Figure 2. Neither the apoprotein nor the heme alone[10] were active, thereby demonstrating the heme−protein complex is the active species.

Maximal velocities (v_{max}) were determined by plotting the data according to the Michaelis−Menten model (Figure 3). Turnover numbers (k_{cat}) were calculated by dividing v_{max} by the concentration of heme protein. More detailed kinetic constants (k_1 and k_3) were determined using peroxidase ping pong kinetics[3] (Table 1 and supporting info.) The k_1 rate constants for our proteins are significantly lower than that for HRP and are more similar to that for myoglobin. This is not surprising, since HRP evolved to bind and transform peroxide, whereas the de novo proteins (and myoglobin) were neither designed nor selected to bind peroxide.

The k_3 values for all four of the purified proteins are 50-fold faster than HRP (Table 1). This second-order rate constant (eq 3) describes the rate at which ABTS reduces compound II back to the resting state of the enzyme. That this final step occurs faster in our de novo proteins than in natural peroxidases suggests an active site that allows ABTS rapid access to the oxidized heme. This is consistent with the kinetics of carbon monoxide binding

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(6) Proteins 10−Z (in Figure 1) have been described[1]. Proteins K2−L119 were taken from a larger collection of several hundred sequences, which was produced as in ref 1, but has not been published.[7] Protein M60 has been described.[8]

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(9) Sequences of 86, G, and M60 are published.[1,8] The sequence of K5 is MGELQHFLEELQKLLQGPDSGHFDNFINHLKKILKGPSGGQLEEMIK-QVEDFLQGPRSGHLKNIIKDLEELLKR.

(10) To minimize precipitation, heme was dissolved in a buffer containing Triton X-100. The turnover number for heme in Triton shown in Table 1 is similar to that reported previously.[16]

10.1021/ja001198q CCC: $19.00 © 2000 American Chemical Society
Published on Web 07/21/2000

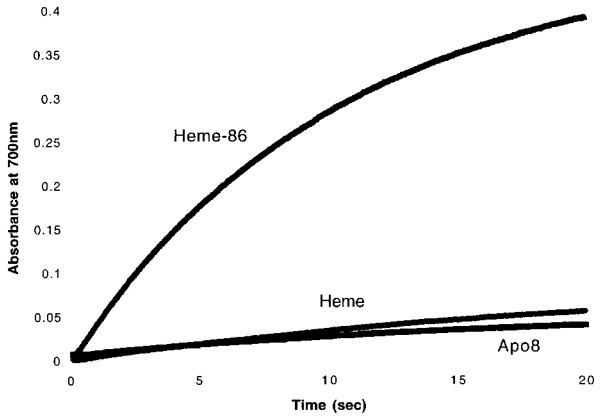

Figure 2. Peroxidase activity of the heme complex of protein 86 compared to heme[10] or apoprotein. Absorbance at 700 nm monitors oxidation of ABTS. (414 nm is less reliable due to interference by the Soret peak and by substrate ABTS.) Proteins were purified as described previously.[1] Protein was concentrated, and buffer was exchanged to 50 mM Tris HCl, 50 mM NaCl, pH 8.0 using a Centricon Plus-20 (Millipore). Purified protein was incubated at room temperature with heme (prepared fresh in 0.1 M NaOH). ABTS was then dissolved in the heme protein solution. Solutions of H_2O_2 were standardized using K_2MnO_4. Final concentrations were 0.5 μM heme, 2.5 mM ABTS, and >5 μM protein mixed with 20 mM H_2O_2. (Concentration of heme protein is limited by the amount of heme added. Excess apo-protein is inactive.) Solutions containing heme protein and ABTS were rapidly mixed with the H_2O_2 solutions in a Hi-Tech Scientific SF-61 DX2 stopped flow with a Hg^+ arc lamp and Brandenburg 4479 photomultiplier module.

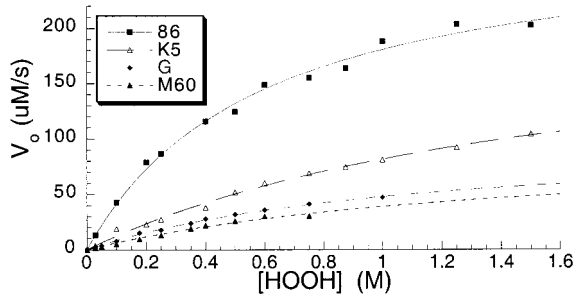

Figure 3. Michaelis−Menten plot. Absorbance at 700 nm was converted to concentration ($\epsilon_{700\ nm} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$).[23] Protein, heme, and ABTS concentrations are the same as in Figure 2.

to the de novo proteins, which indicate that the heme is relatively accessible.[18]

Previous studies of artificial porphyrin-based peroxidases include microperoxidase (a proteolytic product of cytochrome C),[15] a catalytic antibody,[16] heme-binding DNA or RNA aptamers,[17] designed coiled-coil peptides,[19] and several small molecule analogues.[20] We compared the activities of our binary code peroxidases to the heme-based peroxidases containing peptide, DNA, or RNA. As Table 1 shows, protein 86 is 13.5-fold faster than microperoxidase. Moreover, all of the binary code peroxidases were considerably faster than either the catalytic antibody of Cochran and Schultz,[16] the heme-binding DNA aptamer of Sen and co-workers,[17a] or the coiled-coil peptides of Sakamoto et al.[19] When compared to these previous synthetic peroxidases, the turnover number (k_{cat}) for protein 86 (17000 min^{-1}) is >40 times faster.

Table 1. Kinetic Constants of Peroxidases[a]

protein	turnover (min−1)	k_1 ($M^{-1}\ s^{-1}$)	k_3 ($M^{-1}\ s^{-1}$)	reference
86	17000	500	11×10^4	this work
K5	13000	125	9.5×10^4	this work
G	6000	75	11×10^4	this work
M60	5600	55	11×10^4	this work
heme	140			this work, 10
HRP	~60000	9×10^6	0.2×10^4	11, 12
myoglobin	21	540	0.88×10^4	13, 14
μperoxidase	1260	9910		15
antibody	394			16
DNA aptamer	300			17a

[a] Turnover numbers were determined by dividing v_{max} by the concentration of heme protein. k_1 and k_3 were determined by fitting kinetic data to the equation: $2[E_o]/V = 1/k_1[H_2O_2] + 1/k_3[ABTS]^3$. Due to the low k_1, high concentrations of peroxide were necessary to determine the rate constants. Like natural proteins, our de novo proteins are inactivated by high peroxide concentrations. Hence the turnover numbers given are a lower limit of the catalytic potential of these enzymes. Kinetics were measured in 25 mM Tris HCl, 25 mM NaCl, pH 8.0 using 2.5 mM ABTS. The concentration of ABTS had only minor influence on the constants. An alternative mechanism in which ABTS is oxidized by hydroxyl radicals via Fenton chemistry can be ruled out because our heme proteins (i) do not catalyze hydroxylation of *p*-nitrophenol, and (ii) have reduction potentials <+100 mV and thus exist in an Fe^{III} resting state (data not shown).

When comparing the binary code proteins to natural or synthetic peroxidases, it should be emphasized that the binary code peroxidases were *not* subjected to genetic selections for heme binding or peroxidase activity. Moreover, they were *not* explicitly designed to bind heme. They were isolated from a library of sequences designed by binary patterning of polar and nonpolar amino acids to fold into α-helical bundles. Among this unselected collection of binary code proteins (i) all of the purified proteins form α-helical structures,[1,21] (ii) approximately half bind heme,[2] (iii) several function as peroxidases (Table 1), and (iv) at least one protein (86) exhibits a rapid catalytic turnover.

The organization of the genetic code suggests a role for polar/nonpolar patterning in the evolution of protein structure and function. Our previous findings with libraries of de novo sequences showed that binary patterning plays a key role in dictating structure.[1,7,8,21,22] Our current finding that several proteins from a small sampling of a binary code library bind heme and accomplish catalysis suggests that binary patterning coupled with binding to pre-organized activity modules (heme or other cofactors) may have provided a facile route toward the evolution of functional enzymes.

Acknowledgment. We thank John Groves and George McLendon for helpful discussions and use of the stop flow instrument.

Supporting Information Available: Plots of the kinetic data according to the steady-state model[3] and absorption spectra of proteins in the presence and absence of cyanide (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA001198Q

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