ELSEVIER

Biophysical Chemistry 105 (2003) 263–268

Biophysical Chemistry

www.elsevier.com/locate/bpc

Effect of redox potential of the heme on the peroxidase activity of cytochrome *b*562

Shyamalava Mazumdar[a,b], Stacy L. Springs[a], George L. McLendon[a,*]

[a]*Department of Chemistry, Princeton University, Princeton, NJ 08544, USA*
[b]*Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400005, India*

Received 9 October 2002; accepted 1 November 2002

Abstract

Measurements of peroxidase activities of two site-specific mutants and wild type cytochrome *b*562 suggest that the enzymatic activity correlates with the redox potential of the metal center. A lower value of the Fe^{3+}/Fe^{2+} redox potential seems to be important for promoting peroxidase activity of the hemeprotein possibly by stabilization of the high-valent redox intermediate involved in the catalytic function. The results provide an approach towards rational tuning of enzyme function when 'grafted' into a new protein environment.

Keywords: Cytochrome *b*562; Hemeprotein; Fe^{3+}; Fe^{2+}

1. Introduction

Understanding of structure function relationships lies at the heart of biophysics. The chemistry of redox enzymes offers an attractive class of systems for such investigations. For example, much effort has been devoted to understanding how active site redox potentials vary in response to protein environments [1,2]. With such data in hand, more complex functional processes may be approached. For example, reactions of hemeproteins with peroxides often involve formation of high-valent redox species containing a ferryl heme $[Fe{=}O]^{2+}$ center [3]. The formation and stabilization of such ferryl heme species depend on the nature of the amino acid environment around the heme [4]. The natural peroxidase enzymes, such as horseradish peroxidase (HRP) have been shown to stabilize the high-valent intermediate and catalyze oxidation of reducing substrates by H_2O_2 at a very high rate [3,4]. There have been extensive studies [5–7] on the peroxidase reaction in recent years. Several hemeproteins and their mutants were studied to delineate the specific roles of the surrounding amino acids on the promotion of peroxidase-type activity in hemeproteins. Stabilization of the intermediate by hydrogen bonding involving the distal histidine and arginine residues was shown [4,8–10] to play a key role on the catalytic activity of peroxidases like HRP. Hemeproteins devoid of such suitable hydrogen bonding residues, e.g. cytochromes, often show little or no peroxidase activity [5–7,11,12]. However, the enzymatic activity of these heme proteins can be enhanced by suitable

*Corresponding author.
E-mail address: glm@princeton.edu (G.L. McLendon).

0301-4622/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0301-4622(03)00075-9

mutations at the active site [5,13,14]. Horse heart myoglobin showed [6] enhanced peroxidase activity on random mutagenesis of amino acids lying at the periphery and distal side of the heme center, which are not directly involved in coordination or other interactions with the prosthetic group.

The interaction of H_2O_2 with the heme forming the high-valent redox intermediate is primarily a redox coupled atom transfer reaction where H_2O_2 acts as a two-electron acceptor and oxygen atom donor [3]. The reaction of the heme with H_2O_2 can be schematically written as:

$$Fe^{3+} + H_2O_2 \rightleftharpoons [Fe{=}O]^{3+} + H_2O \quad (1a)$$

$$[Fe{=}O]^{3+} + e^- \rightleftharpoons [Fe{=}O]^{2+} \quad (1b)$$

$$[Fe{=}O]^{2+} + e^- \rightleftharpoons Fe^{3+} + O^{2-} \quad (1c)$$

The formation of the intermediates, compound I: $[Fe{=}O]^{3+}$ a radical cation, compound II $[Fe{=}O]^{2+}$ and the catalytic oxidation of the reducing substrates (e^- donor) by the hemeproteins would thus also depend on the redox potential of the metal center. There has, however, yet been no report on study of the effect of redox potential on the peroxidase activity of hemeproteins.

The effect of redox potential on the peroxidase catalytic activity of the hemeprotein becomes difficult to investigate, as variation of redox potential often is associated with significant structural changes such as change of ligation of the metal center in the hemeprotein. One way of identifying any effect of redox potential is to compare the peroxidase activity of structurally homologous variants of a hemeprotein with varying redox potentials where the peroxidase intermediate is not stabilized by hydrogen bonding or any other interactions.

The small four-helix bundle protein, cytochrome *b*562 provides an attractive laboratory for 'grafting' peroxidase activity into a stable and well-characterized hemeprotein. Cytochrome *b*562 does not possess any known peroxidase activity in the native form indicating [12] that the heme cavity does not significantly stabilize the peroxidase intermediate in cytochrome *b*562. Furthermore, systematic in vitro evolution of cytochrome *b*562 has been carried out to reveal a wide range of functional variety in redox potential, whilst maintaining the basic structure intact [1,2]. Thus, cytochrome *b*562 provides an attractive system to study how peroxidase activity might be introduced into a protein, and systematically tuned by variation of the active site.

As for most hemeproteins, the heme is localized within a hydrophobic cavity in the protein interior, but with some residual solvent exposure. Variation of surrounding residues, especially the aromatic groups F61 and F65, leads to increased contact between the heme and solvent water (Fig. 1). The redox potential for the Fe^{2+}/Fe^{3+} couple for the heme in cytochrome *b*562 is decreased in the first-generation mutants [1] containing variations at the F61 and F65 residues of the proteins. The potential was shown to further decrease in a second-generation of mutants [2] containing variations on the surface exposed Arg106 or Arg98 residues. The mutants F61A (+151 mV) and F61I/F65Y/R106L (+9 mV) show very drastic variation in the Fe^{2+}/Fe^{3+} redox potential compared to the wild type protein (+167 mM) with very small effect on the structure of the heme cavity [1,2]. For ferryl proteins the situation is less obvious: the heme 'Fe(IV)O' unit is formally neutral, but may be significantly dipolar. If so, the same variations which stabilize the Fe^{3+} heme might also stabilize the ferryl intermediate resulting in enhanced peroxidase activity. We have investigated oxidation of guaiacol by H_2O_2 catalyzed by these two structurally characterized variants of cytochrome *b*562 and the wild type protein and evaluated their peroxidase activity to investigate how the redox potential of the metal center affects the enzymatic activity.

2. Materials and methods

Mutants of cytochrome *b*562 were prepared by PCR based mutagenesis as described before [1]. Wild type and mutant cytochrome *b*562 were expressed in *E. coli* using plasmid pRW2 as described earlier [1]. Isolation of the protein from the bacterial culture was carried out by repeated freeze-thaw lysis to remove most of the insoluble

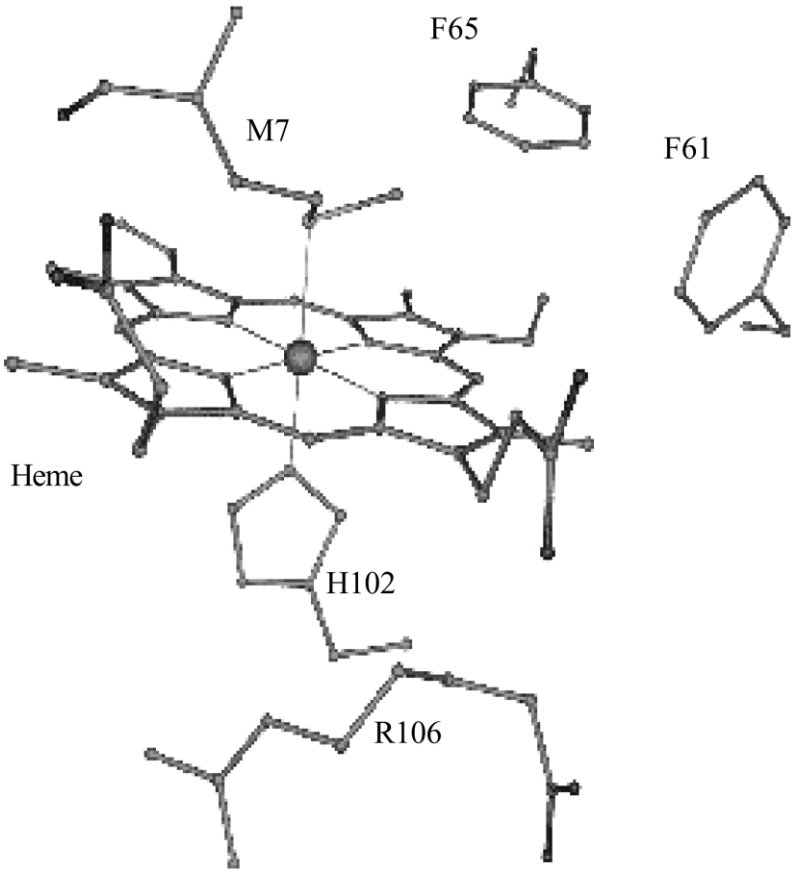

Fig. 1. Schematic structure of the heme coordination geometry and the F61, F65 and R106 residues of cytochrome *b*562.

proteins and membrane components. The cytochrome *b*562 collected in the soluble fraction was purified by size exclusion chromatography on a Superdex 30 column equilibrated at pH 7.4 by 20 mM potassium phosphate buffer. The protein was further purified on a SP-Sepharose FF column using a gradient 0–0.5 M NaCl at pH 4.2 prior to experiment. The purity of the protein checked by the ratio of A418/A280 > 5.7, was more than 90%.

Hydrogen peroxide solutions were prepared from a 3% stock solution by diluting with appropriate buffer. The concentration of H_2O_2 was determined from absorbance using $\varepsilon_{240} = 39.4$ M^{-1} [11]. Guaiacol and other chemicals were obtained from Sigma Chemicals Co.

Peroxidase activity of the wild type and mutants of cytochrome *b*562 was determined from oxidation of guaiacol by H_2O_2 in presence of the protein. The kinetics of oxidation of guaiacol [4,11] were measured spectrophotometrically from the formation of tetraguaiacol (at 470 nm) using $\varepsilon_{470} = 26\,600$ $M^{-1}\,cm^{-1}$ with a HiTech Sf61 DX2 stopped-flow spectrometer. The rate of reaction of H_2O_2 with guaiacol was determined at different concentrations of guaiacol to determine Michaelis–Menten constants for guaiacol binding to the protein. Similarly, rates were also determined at a constant guaiacol concentration with varying H_2O_2 to evaluate the binding constant of H_2O_2 to the protein. Rates were determined both by exponential analysis as well as by initial rate method and the results were the same.

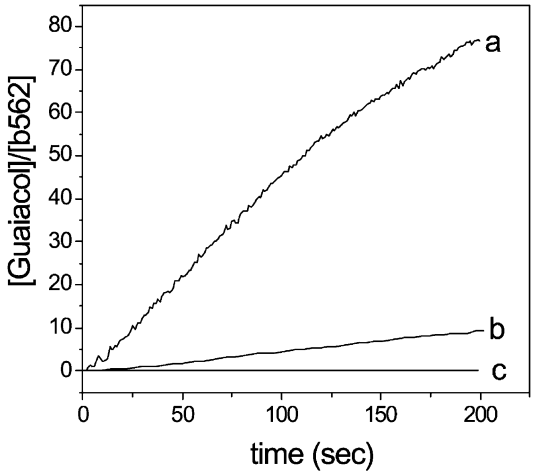

Fig. 2. Formation of tetraguaiacol (detected from absorbance at 470 nm) during reaction of guaiacol with H_2O_2 at pH 6 catalyzed by (a) F61I/F65Y/R106L; (b) F61A and (c) wild type cytochrome *b*562.

3. Results and discussion

The rate of oxidation of guaiacol with H_2O_2 was found to depend on the concentrations of guaiacol, H_2O_2 and cytochrome *b*562. A slow phase was observed at very low (<5 mM) H_2O_2 and also at high guaiacol (>50 mM) concentrations. This indicated that the reaction of the protein with H_2O_2 forms the slower step and excess guaiacol tends to inhibit the reaction. The initial rate (v) was determined from the slope of the linear region of the time evolution of the oxidized product (Fig. 2).

Unlike in the case of cytochrome *c* [11], no induction step was observed in the catalysis by cytochrome *b*562 in the range of 20–200 mM H_2O_2 and up to 30 mM guaiacol concentrations, when the concentrations of cytochrome *b*562 and its mutants were 0.2 to $\sim$20 μM. The methionine residue bound at the distal side of the heme probably is modified by H_2O_2 and initial binding of H_2O_2 to the metal center does not affect the experimental reaction rate observed in the stopped-flow time-scale. Analogous results [11] were also reported for the peroxidase reaction of cytochrome *c*550. The initial rate of oxidation of guaiacol was found to show saturation behavior with the substrate. Michaelis–Menten analysis [7,9,12] of the initial rate at different guaiacol concentrations, shown in Fig. 3 gave the values of k_{cat} and K_m for the reaction.

The results shown in Fig. 3 show peroxidase activity increase in the F61I/F65Y/R106L mutant of cytochrome *b*562. The activity of the wild type protein is extremely small and that of the F61A mutant was well above wild type but smaller than the triple mutant.

Table 1 shows the values of K_m and k_{cat} obtained by non-linear least squares fit of the specific activity to the Michaelis–Menten equation:

$$v/[E]\ \ (\mathrm{s}^{-1}) = \frac{k_{cat}\ \ (\mathrm{s}^{-1}) \times [\mathrm{Guaiacol}]\ \ (\mathrm{M})}{K_m\ \ (\mathrm{M}) + [\mathrm{Guaiacol}]\ \ (\mathrm{M})} \qquad (2)$$

where K_m refers to the Michaelis–Menten constant for the protein for binding of guaiacol, $[E]$ is the concentration of the protein, v is the rate of the reaction and k_{cat} is the effective catalytic rate constant for oxidation of guaiacol. The values of catalytic constants were independent of H_2O_2 in $\sim$20–200 mM concentration range. At lower concentrations of H_2O_2 the rates of the reaction were too slow to determine accurately while high con-

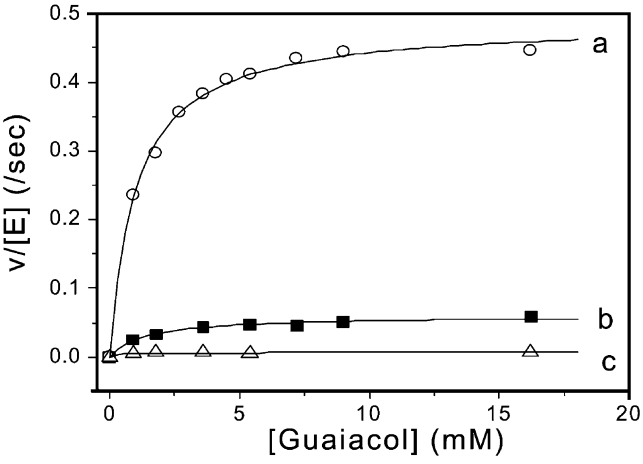

Fig. 3. Variation of specific rate of oxidation of guaiacol by H_2O_2 in presence of (a) F61I/F65Y/R106L; (b) F61A and (c) wild type cytochromes *b*562. The Michaelis–Menten kinetics was followed in presence of 100 mM H_2O_2.

Table 1
Catalytic constants for oxidation of guaiacol by H_2O_2 catalyzed by various hemeproteins

Enzyme	K_m (M)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	E^0 (mV)	Reference
HRP	5.8×10^{-3}	2.5×10^{4}	4.3×10^{6}	−266	[4]
Myoglobin	32×10^{-3}	21.6	1.2×10^{3}	~0	[14]
H42A HRP	4×10^{-6}	0.9	24×10^{4}	–	[9]
F61I/F65Y/R106L cytochrome *b*562	1.0×10^{-3}	29.2	2.9×10^{4}	9	This work
F61A cytochrome *b*562	1.4×10^{-3}	3.6	2.5×10^{3}	151	This work
M7A cytochrome *b*562	31×10^{-6}	2.7	9.7×10^{4}	–	[12]
Wt cytochrome *b*562	1.2×10^{-3}	0.5	0.4×10^{3}	167	This work

centrations of H_2O_2 gave rise to rapid decomposition of the protein.

The reaction rates were also determined at different concentrations of H_2O_2 keeping the guaiacol concentration constant at ~9 mM and results were analysed using Michaelis–Menten equation Eq. (2) with $[H_2O_2]$ as the independent variable. The values of k_{cat} for the cytochrome *b*562 mutants were found to be almost the same as those obtained from the guaiacol dependence of the rate of reaction. However, the value of K_m^{Per} was found to be ~100 mM for H_2O_2 as a substrate.

The Michaelis constant K_m (Table 1) obtained from variation of the reaction rate with guaiacol corresponds to binding of the reducing substrate to the protein in the intermediate state. Most peroxidases are relatively non-selective for the reducing substrate indicating that there is no specific recognition site for the substrate at the heme active site. Observation of very similar values of K_m for HRP and the cytochrome *b*562 shown in the Table 1 thus support non-specific interaction between the reducing substrate and the proteins. The value of k_{cat} corresponds to the enzymatic turnover number. The turnover number of F61A mutant was significantly larger than the wild type protein, which has very small enzymatic turnover. The F61I/F65Y/R106L cytochrome *b*562 shows almost a tenfold increase in the value of k_{cat} compared to the F61A mutant of the protein, indicating significant enhancement of the catalytic efficiency on mutation. The value of k_{cat} of cytochrome *b*562 mutants studied by us was found to increase with decreasing redox potential of the protein. The low redox potential of the Fe^{3+}/Fe^{2+} couple for native HRP [10] of −266 mV coupled with specific recognition of H_2O_2 at the heme cavity leads to a very high catalytic efficiency of HRP. The magnitude of the K_m^{Per} corresponding to the binding of H_2O_2 to cytochrome *b*562 was the same for both the mutants (100 mM) indicating weak non-selective interaction of the peroxide with the heme. This value can be compared to K_m^{Per} for cytochrome c_{550} [11] to be ~240 mM, which supports absence of specific recognition site for interaction of the peroxide with these hemeproteins where the peroxidase activity has been grafted into a native protein by active site mutations.

The results thus suggest that the Fe^{3+}/Fe^{2+} redox potential and peroxidase activity of the heme protein are correlated, and proteins with lower potentials, which stabilize the higher oxidation state of heme, would provide better enzymatic activity. The redox reaction between the protein and H_2O_2 leads to oxidation of the ferric heme forming ferryl heme radical ($[Fe{=}O]^{3+}$) and ferryl heme ($[Fe{=}O]^{2+}$) intermediates (Eqs. (1a), (1b) and (1c)). The heme group having lower redox potential for Fe^{3+}/Fe^{2+} couple would be a better electron donor to H_2O_2 and would thus favor formation of the high-oxidation state intermediates than the one with higher redox potential. This observation provides a rational approach towards evolution of a novel activity within all existing hemeproteins of synthetic active site. The variation of (hydrophobic) character by site directed mutagenesis could scarcely have been seen by Kauz-

mann [15] when he published his prescient review of the hydrophobic effect. However, the implications of the hydrophobic effect for enzyme activity indeed were well anticipated.

References

[1] S.L. Springs, S.E. Bass, G.L. McLendon, Cytochrome *b*562 variants: a library for examining redox potential evolution, Biochemistry 39 (2000) 6075–6082.

[2] S.L. Springs, S.E. Bass, G. Bowman, I. Nodelman, C.E. Schutt, G.L. McLendon, A multigeneration analysis of cytochrome *b*562 redox variants: evolutionary strategies for modulating redox potential revealed using a library approach, Biochemistry 41 (2002) 4321–4328.

[3] D.B. Dunford, Peroxidases in chemistry and biology, in: J. Everse, K.E. Everse, M.B. Grishman (Eds.), Horseradish Peroxidase: Structure and Kinetic Properties, vol. 2, CRC Press, Boca Raton, FL, 1991, pp. 1–24.

[4] M.I. Savenkova, J.M. Kuo, P.R. Ortiz de Montellano, Improvement of peroxygenase activity by relocation of a catalytic histidine within the active site of horseradish peroxidase, Biochemistry 37 (1998) 10828–10836.

[5] S.-I. Ozaki, S. Matsui, M.P. Roach, Y. Watanabe, Rational molecular design of a catalytic site: engineering of catalytic functions to the myoglobin active site framework, Coord. Chem. Rev. 198 (2000) 39–59.

[6] L. Wan, M.B. Twitchett, L.D. Eltis, A.G. Mauk, M. Smith, In vitro evolution of horse heart myoglobin to increase peroxidase activity, Proc. Natl. Acad. Sci. USA 95 (1998) 12825–12831.

[7] R.E.M. Diederix, M. Ubbink, G.W. Canters, Effect of the protein matrix of cytochrome *c* in suppressing the inherent peroxidase activity of its heme prosthetic group, Chembiochem (2002) 110–112.

[8] T.L. Poulos, J. Kraut, The stereochemistry of peroxidase catalysis, J. Biol. Chem. 255 (1980) 8199–8205.

[9] M.I. Savenkova, P.R. Ortiz de Montellano, Horseradish peroxidase: partial rescue of the His-42→Ala mutant by a concurrent Asn-70→Asp mutation, Arch. Biochem. Biophys. 351 (1998) 286–293.

[10] M. Tanaka, S. Nagano, K. Ishimori, I. Morishima, Hydrogen bond network in the distal site of peroxidases: spectroscopic properties of Asn70→Asp horseradish peroxidase mutant, Biochemistry 36 (1997) 9791–9798.

[11] R.E.M. Diederix, M. Ubbink, G.W. Canters, The peroxidase activity of cytochrome *c*-550 from *Paracoccus versutus*, Eur. J. Biochem. 268 (2001) 4207–4216.

[12] N. Kamiya, Y. Okimoto, Z. Ding, et al., How does heme axial ligand deletion affect the structure and the function of cytochrome *b*562?, Protein Eng. 14 (2001) 415–419.

[13] T. Matsui, S.-I. Ozaki, E. Liong, G.N. Phillips Jr., Y. Watanabe, Effects of the location of distal histidine in the reaction of myoglobin with hydrogen peroxide, J. Biol. Chem. 274 (1999) 2838–2844.

[14] T. Hayashi, Y. Hitomi, T. Ando, et al., Peroxidase activity of myoglobin is enhanced by chemical mutation of heme-propionates, J. Am. Chem. Soc. 121 (1999) 7747–7750.

[15] W. Kauzmann, Some factors in the interpretation of protein denaturation, Adv. Protein Chem. 14 (1959) 1–63.