Thermostable Synthetic Hemoproteins: Thermophilic Xylanases Hybridized with Dioxygen-Carrying *meso*-Tetrakis(*o*-pivalamidophenyl)porphinatoiron(II) Derivative

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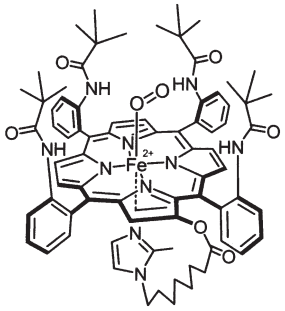
meso-Tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphinatoiron(II) derivative is incorporated into thermophilic xylanases, giving novel thermostable synthetic hemoproteins, which can form a dioxygen adduct in aqueous media (pH 7.3) at 90 °C.

It is of current interest to construct artificial hemoprotein architectures by replacing the prosthetic heme groups with synthetic metalloporphyrinoids, which often yields unique functions never seen in nature.[1,2] However, the porphyrin moiety of the guest molecule is structurally restricted, because (i) it should be planar to enter the geometric hemepocket, and (ii) its binding forces to the apoprotein host are usually vectorial bindings, for example, the central metal coordination, and hydrogen bonding to the nearest amino acid residues. Human serum albumin (HSA), the most abundant plasma protein in our blood stream, also incorporate tetraphenylporphinatoiron(II) [Fe(II)TPP] derivative, and the obtained albumin-heme hybrid reversibly binds dioxygen (O_2) under physiological conditions in the same manner as hemoglobin (Hb) and myoglobin.[3] In this case, the heme inclusion is only due to hydrophobic interaction, therefore, varieties of modified hemes (Mw 680–1940) are nonspecificcally incorporated into the apolar domains of HSA, and the O_2-binding properties of the composites largely depend on their guest structures.[4] If this simple hybridization strategy can be universally applied to the other proteins having hydrophobic cavity and totally different characteristic, this chemistry will lead to construct a new class of artificial hemoproteins. Xylanases catalyze the hydolysis of β-1,4-D-xylans, the major hemicellulose component, to xylooligosaccharaides and xylose. We have recently found that thermophilic xylanases incorporate 2-[8-(2-methylimidazolyl)octanoyloxymethyl]-5,10,15,20-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl) porphinatoiron(II) (FeP) into their hydrophobic pockets, and the obtained xylanase-FeP hybrids can bind and release O_2 at the wide range of temperature up to 90 °C. This paper reports for the first time the O_2-binding ability of the novel thermostable synthetic hemoproteins.



The catalytic domain regions of thermophilic xylanase B from *Dictyoglomus thermophilum* (XynBD: Mw 23 kD) and xylanase B from *Thermotoga maritima* (XynBT: Mw 39 kD) were expressed in *Escherichia coli*, and partially purified.[5] An EtOH solution of carbonyl FeP (80 μM, 2.5 mL) was slowly injected into the phosphate buffer solution (25 mM, pH 7.3, 7.5 mL) containing XynBD or XynBT (26.7 μM) under a carbon monoxide (CO) atmosphere. The mixture was dialyzed with cellulose membrane against phosphate buffer (pH 7.3) at 4 °C to remove excess EtOH. Finally, the total volume was adjusted to 10 mL, giving carbonyl xylanase-FeP solution (FeP/xylanase = 1 (mol/mol), [FeP] = 20 μM), which was stable for more than six months at room temperature without precipitation.

The UV-vis. absorption spectrum of the aqueous XynBD-FeP(CO) showed the formation of the typical CO-coordinated low-spin Fe(II)TPP derivative (λ_{max}: 427, 536 nm) (Figure 1).[6] Preliminary titration experiments suggested that there were several binding sites for FeP in a xylanase, that involve one major site with high binding constant. In order to simplify the system, we prepared the equivalent composite in this experimental setup. From the quantitative analyses of the absorption intensity for the Soret band, the binding number of FeP to a xylanase was determined to be one.

In CD spectrum of XynBT, negative and positive peaks appeared at 226 and 268 nm, respectively. The intensity ratio of these two peaks was unchanged after incorporation of FeP, suggesting that the heme inclusion does not induce any two-dimentional structure changes of XynBT host. Moreover, isoelectric points of XynBT-FeP was found to be ca. 7.0; this value is exactly the same as that of XynBT itself. The FeP interacts XynBT with hydrophobic interaction, hence its surface net charges are always identical.

Light irradiation using a 500 W halogen lamp to the aqueous XynBD-FeP(CO) solution under an O_2 atmosphere led to CO dissociation, affording the O_2-adduct complex (λ_{max}: 426, 550 nm).[3,6] Upon exposure of N_2, the spectral pattern immediately changed to that of the five-N-coordinated high-spin iron(II) with an intramolecularly coordinated axial imidazole (λ_{max}: 441, 535, 562 nm), which indicates that no amino acid residue binds to the central iron(II) of FeP in the XynBD structure. This dioxygenation was reversibly observed depending on the O_2-partial pressure and quite stable at 25 °C [half-lifetime ($\tau_{1/2}$: 5 h)]. Identical O_2-coordination was also observed in the XynBT-FeP as well.

The O_2-binding affinities ($P_{1/2}$) of xylanase-FeP series were carefully determined on the basis of the UV-vis. absorption spectral changes during the O_2 titration,[3] and their O_2-association and -dissociation rate constants (k_{on}, k_{off}) were also obtained by

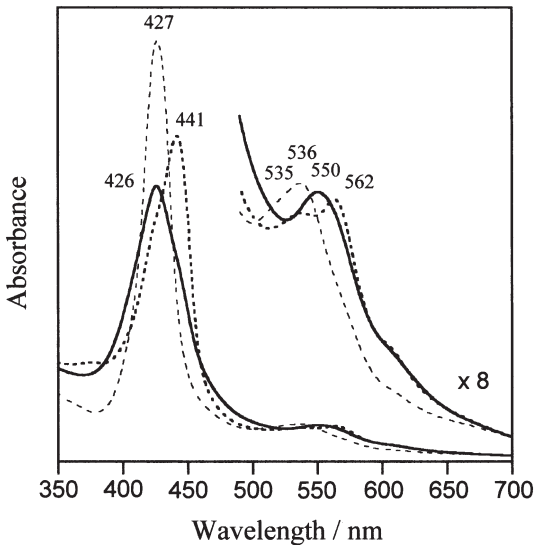


Figure 1. UV-vis. absorption spectral changes of XynBD-FeP hybrid in phosphate buffer solution (pH 7.3, 25 mM) at 25 °C. - - - - -: under N_2, ——: under O_2, – – – –: under CO.

laser-flash photolysis experiments (Table 1).[7] The O_2-binding affinities were relatively low (high $P_{1/2}$) compared to that of HSA-FeP, which were kinetically caused by the high k_{off} values. It has been known that the O_2 recombination to HSA-FeP was influenced by the molecular environments around the each heme as same as to the synthetic porphyrinatoiron(II)s in organic solutions.[7,8] Most probably, FeP is incorporated into the hydrophobic substrate-binding pockets of xylanases, and the low polarity around the O_2-binding site could enhance the O_2-dissociation.

Table 1. O_2-Binding parameters of xylanase-FeP hybrids in phosphate buffer solution (pH 7.3, 25 mM) at 25 °C

System	$P_{1/2}$/Torr	k_{on}/$M^{-1}s^{-1}$	k_{off}/s^{-1}
XynBD-FeP	23	3.2×10^7	1.2×10^3
XynBT-FeP	27	3.2×10^7	1.4×10^3
HSA-FeP[a]	13	3.2×10^7	7.2×10^2

[a]Ref. 7.

Accompanying the autooxidation of the central iron(II), the absorption band (λ_{max}: 550 nm) slowly decreased, leading to the formation of the inactive iron(III) porphyrinate. It is rather remarkable that XynBD-FeP exhibited great heat resistance up to 90 °C. Although the absorption pattern of the O_2-adduct complex at high temperature slightly involved the ingredient of the deoxy form, the half-lifetime ($\tau_{1/2}$) of the oxy and deoxy species was 5 min at 90 °C and 10 min at 75 °C (Figure 2). This is quite contrast to the result of Hb, which was immediately denatured and its protohemes were oxidized within one minute under the same conditions. As a result, the dioxygenated XynBD-FeP showed an unusually tough thermostability; the $\tau_{1/2}$ is over 10-fold longer than that of Hb.

In conclusion, thermophilic xylanases successfully incorporate Fe(II)TPP complex with an intramolecularly coordinated proximal base, giving stable xylanase-FeP hybrids, which can form stable O_2 adduct at great wide range of temperature up to

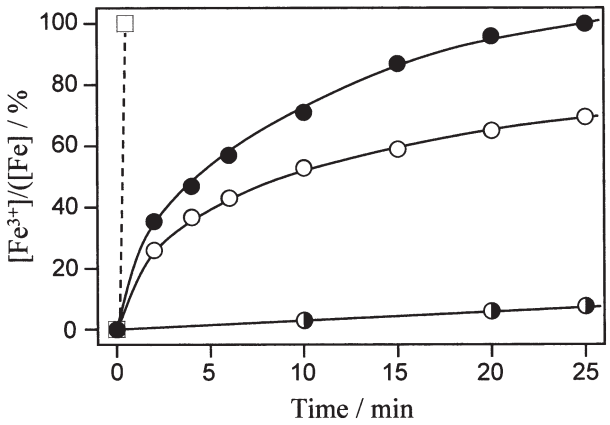


Figure 2. Time course of Fe^{3+} complex formation in XynBD-FeP hybrid determined by the UV-vis. absorption decay at 550 nm (Abs. of dioxygenated Fe^{2+}P) in phosphate buffer solution (pH 7.3, 25 mM) at high temperatures ([O_2]=760 Torr). ●: at 90 °C ($\tau_{1/2}$: 5 min), ○: at 75 °C ($\tau_{1/2}$: 10 min), ◑: at 25 °C ($\tau_{1/2}$: 5 h), □: Hb at 90 °C.

90 °C. These are the first examples of the heat-resistant synthetic O_2-carrying hemoproteins. This simple preparation method using nonspecific hydrophobic interaction will be applied to the other host–guest combinations, which could afford us a totally new class of artificial hemoprotein architectures. Studies on novel metalloprotein-clusters including synthetic porphyrins are now underway.

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