Design and Synthesis of Sugar-Responsive Semiartificial Myoglobin Triggered by Modulation of Apoprotein–Cofactor Interactions

Itaru Hamachi,* Yusuke Tajiri, Tsuyoshi Nagase, and Seiji Shinkai

Abstract: Phenylboronic acid groups as sugar recognition sites were successfully introduced into native myoglobin by a cofactor-reconstitution method. Spectrophotometric pH titration demonstrated the sugar-induced pK_a shift of the H_2O coordinated to the heme center of the semisynthetic myoglobin bearing phenylboronic acids (met-Mb(PhBOH)$_2$). By means of circular dichroism (CD) and paramagnetic ^{1}H NMR spectroscopies, it was proven that sugars that were bound to phenylboronic acid sites induced the rearrangement of the heme crevice to reinforce the heme cofactor–apoprotein interactions. The structural changes that were induced by the binding of sugars subsequently enhanced the dioxygen storage activity of Mb(PhBOH)$_2$. Such sugar-induced structural and functional changes did not occur for other modified Mbs that had no sugar-recognition units. Interestingly, a randomly modified Mb with phenylboronic acid units did not show any sugar response. In Mb(PhBOH)$_2$, the information from the sugar-binding event was efficiently transmitted to the active center, so that the activity was efficiently altered upon sugar binding. In conclusion, the active site specific incorporation of molecular recognition units as nonnatural functional molecules can provide a novel strategy for the design of stimuli-responsive semisynthetic proteins.

Keywords
boronic acids · heme proteins · molecular recognition · myoglobin · saccharides

Introduction

In the research field of bio-related materials science, proteins and enzymes are considered to be attractive molecular materials bearing individual functions within the nanometer size range. When naturally occurring proteins are hybridized with artificial molecules, the molecular interface capable of introducing sophisticated functions to the hybrid proteins is significant. In recent years, artificial molecules and/or molecular assemblies that are able to regulate enzyme alignments, orientations, and reactivities have been exploited as promising interfaces, instead of passive matrices that simply immobilize the enzymes.[1]

The interface molecules can be introduced into native proteins in a covalent or noncovalent manner. However, it is generally difficult to incorporate nonnatural molecules into specific positions of proteins and enzymes. Solid-phase peptide synthesis and protein semisynthesis have often been used to prepare novel proteins containing nonnatural amino acids.[2] A more general biosynthetic method using aminoacylated suppressor t-RNA has been successfully developed in recent years.[3] Thus, it is now desirable to establish what categories of nonnatural molecules can actively operate in natural proteins at a molecular level. We recently showed that the reconstitution of chemically modified cofactors with apoproteins or apoenzymes can be used to modify active sites with nonnatural molecules (Scheme 1).[4] Our group and others have used this procedure to synthesize a number of unusual hemoproteins containing, for example, a photosensitizer, a redox-active moiety, a molecular recognition site, or a hydrophobic alkyl chain.[4, 5] We previously communicated a preliminary report on a sugar-responsive myoglobin, which was prepared by the reconstitution of a synthetic heme bearing phenylboronic acid pendants with apo-Mb.[6] In this article, we clearly demonstrated that a sugar-induced dynamic change of the cofactor–apomyoglobin interactions is essential to the sugar-responsive activity of the semisynthetic myoglobin, by providing detailed information on the structure and function of various myoglobin derivatives.

[*] I. Hamachi, Y. Tajiri, T. Nagase, S. Shinkai
Department of Chemical Science & Technology
Faculty of Engineering, Kyushu University, Fukuoka, 812 (Japan)
e-mail: itarutcm@mbox.nc.kyushu-u.ac.jp

Results and Discussions

Design and Synthesis of Chemically Modified Heme Derivatives: Phenylboronic acid is known to act as a Lewis acid in forming covalently bonded stable complexes with 1,2- or 1,3-diols in an aqueous medium.[7] In the resulting phenylboronate ester, the acidity of the boronic acid is enhanced to generate a tetrahedral boronate anion. These properties suggested to us that phenylboronic acid might be used to bind sugar derivatives to a protein molecule. We therefore set out to incorporate the former into myoglobin (Mb), a structurally well-defined protein, by the re-

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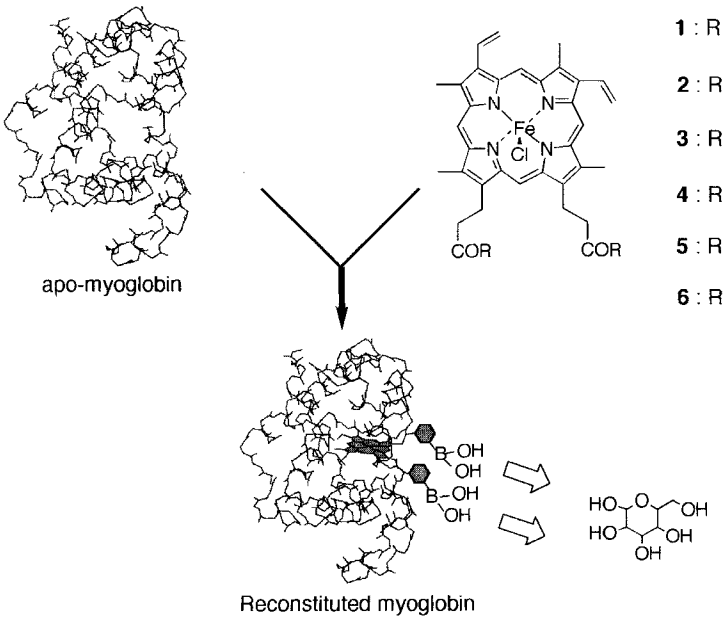


Scheme 1. Reconstitution of myoglobin with synthetic hemes.

constitution of apomyoglobin with a heme having *m*-aminophenylboronic acids attached to two propionic acid terminal groups (**1**). In order to elucidate in detail the effect of the sugar on myoglobin, protoheme derivatives bearing anionic carboxyphenyl groups (**2**), neutral but isoelectronic nitrophenyl groups (**3**), neutral carboethoxylphenyl groups (**4**), and simple phenyl groups (**5**) were synthesized and reconstituted with apo-Mb. These hemes were prepared by Schotten–Baumann condensation of protoporphyrin bis(acid chloride) with corresponding *meta*-substituted anilines, followed by iron insertion under anaerobic conditions (Scheme 2). Protoheme diethyl ester **6** was also synthesized and reconstituted with apo-Mb.

Effects of the Sugar on Heme Reconstitution with Apomyoglobin: The reconstitution of heme **1b** with apo-Mb was conducted by the addition of **1b** dissolved in a small amount of pyridine to apo-Mb in aqueous solution (isolated yield = 20%). The reconstituted Mb (met-Mb(PhBOH)$_2$) was found to be sufficiently pure, since the ratio of the absorbance at 408 nm (Soret band of heme) to that at 280 nm (aromatic side chain of apo-Mb) was 4.4, which is comparable to the value of 4.8 for native Mb and is greater than those of other Mbs (3.4 for Mb(PhCO$_2^-$)$_2$, 4.2 for Mb(PhH)$_2$, and 3.8 for Mb(OEt)$_2$[8]). The ligand exchange, redox, and dioxygen-binding reactions, in addition to various spectroscopic measurements by UV/Vis, circular dichroism (CD), electron paramagnetic resonance (EPR), and nuclear magnetic resonance (NMR), confirmed that the heme **1** was satisfactorily fixed in the native heme pocket of myoglobin.

When we used dimethyl sulfoxide (DMSO) as a solvent for **1b** instead of pyridine, the reconstitution failed (yield = 0%). However, the reconstitution was found to proceed smoothly in the presence of D-fructose (yield = 32%). Under these conditions, the change in the absorption spectrum of **1b** with the addition of apo-Mb gave a good titration curve, which was saturated at a 1/1 ratio of apo-Mb to **1b** (see Figure 1). The ability of monosaccharides to facilitate the reconstitution depends on their structure. The strength

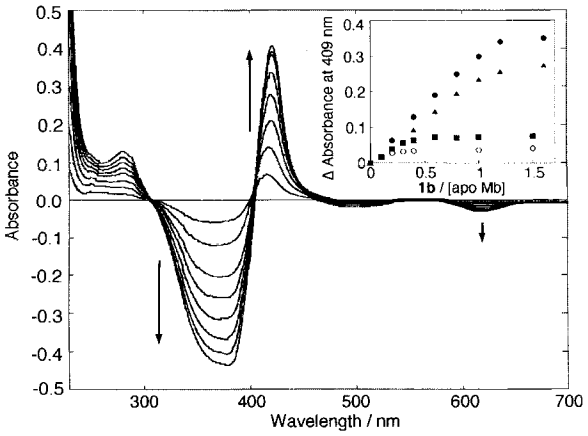
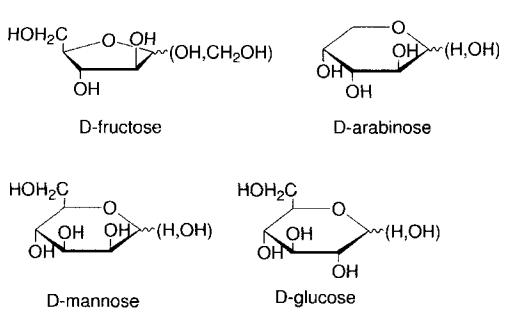


Figure 1. Changes in the absorption spectrum of heme **1b** (10 μM in carbonate buffer, 50 mM, pH 10.5, 25 °C) with addition of apo-Mb in the presence of D-fructose (0.1 M). Inset: titration curves of **1b** with apo-Mb in the presence of various sugars; D-fructose (●), D-arabinose (▲), D-mannose (■), D-glucose (○).

of this effect increases in the same order as their binding affinities to phenylboronic acid (D-fructose > D-arabinose > D-mannose > D-glucose).[9] 1,3-propanediol is less effective in promoting the Mb reconstitution than sugars. Sugars do not assist the insertion of any of the other hemes (**2b**–**6b**) into apo-Mb. These results indicate that the hydrophilicity of **1b** is increased



Abstract in Japanese:

糖類に対するレセプターとなるフェニルホウ酸を修飾したヘム補因子が、アポミオグロビンに再構成された。得られた再構成ミオグロビンは、単糖の結合によってホウ酸アニオンが生じ、それに伴って活性中心に存在する配位水のpKaが塩基性側にシフトした。円二色性あるいは核磁気共鳴スペクトルから見るとフェニルホウ酸ペンダントミオグロビンの二次構造は糖類を結合しても余り変わらなかった。しかし活性中心近傍は糖類の結合によって効果的に安定化を受けることが示された。さらにこの人工タンパク質の酸素貯蔵能は、糖類の添加に応答して大きく増幅されることが明らかになった。再構成法を用いた人工レセプター分子の天然タンパク質への導入は、タンパク質の人工機能化のための有力な方法論となるであろう。

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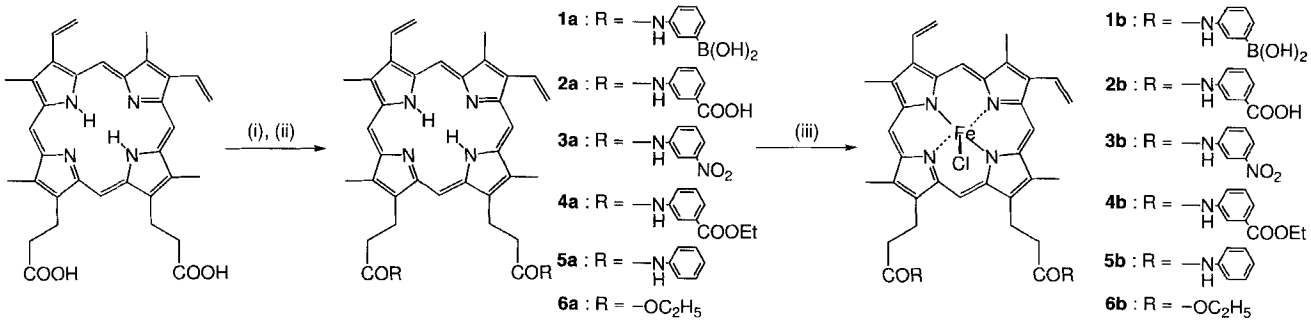

Scheme 2. Synthetic route to the hemes examined in this paper. i) $(COCl)_2$/dry CH_2Cl_2, ii) H_2NR or HOEt, iii) $FeCl_2$/dry DMF.

through complexation of sugars at the boronic acid sites, because of the generation of a boronate anion and the binding of the sugar molecules. The sugar-associated heme **1b** can then be incorporated more readily because of its increased amphiphilicity, in the same way as propionate terminal groups in hemin give better results than propionic acids in the conventional reconstitution.[10] In addition, saccharide–protein interactions in the proximity of the heme crevice may play some role in the stabilization of the obtained holoprotein.

Sugar-Induced pK_a Shift of H_2O Coordinated to the Heme Iron(III) Center: Substituents incorporated into the heme are known to change the pK_a of the axial H_2O molecule coordinated to the iron(III) center of heme in myoglobin. This pK_a shift can be used as an index to evaluate the interactions in the microenvironment of the heme crevice. Table 1 summarizes the pK_a values of the reconstituted Mbs with **1b**–**6b** in the presence and in the absence of D-fructose (0.1 M). The pK_a values of all the Mb derivatives are lowered compared to that for the unmodified Mb. It is widely accepted that propionate anions of native protoheme form a tight ion pair with Lys CD3 and a hydrogen bond with Ser F7, both of which greatly contribute to the stable fixation of heme cofactor in apo-Mb.[11] The loss of these stabilization factors by chemical modification causes the lowering of the pK_a. Turning to the Mbs with appended phenyl derivatives, we observe that the pK_a for $Mb(PhBOH)_2$ is lower than that for $Mb(PhH)_2$. This may be due to the fact that the bulkier $-B(OH)_2$ substituents disturb the three-dimensional structure of the apoprotein to a greater extent than do the smaller -H groups. The instability of $Mb(PhCO_2Et)_2$ and $Mb(PhNO_2)_2$, which prevents us from determining their precise pK_a values, supports this explanation of the effect of bulky substituents on the holoprotein structures. In contrast, the pK_a of $Mb(PhCO_2^-)_2$ is close to that of native Mb. This suggests that the incorporation of the negatively charged benzoate groups considerably stabilizes the holoprotein complex, even though the negative charges are slightly further from the protein skeleton than the native propionates. The pK_a for $Mb(OEt)_2$ is rather low, in spite of its small substituents. The less polar ester bond might have less effect on the heme–apoprotein stabilization than the more polar amide bond.

Table 1. pK_a values of coordinated H_2O molecules in various Mb derivatives in the presence and in the absence of D-fructose (0.1 M).

Myoglobin	Heme	pK_a	pK_a(D-fru)
$Mb(PhBOH)_2$	**1b**	8.0	8.5
$Mb(PhCO_2H)_2$	**2b**	8.5	8.5
$Mb(PhNO_2)_2$	**3b**	– [a]	– [a]
$Mb(PhCO_2Et)_2$	**4b**	– [a]	– [a]
$Mb(PhH)_2$	**5b**	8.3	8.3
$Mb(OEt)_2$	**6b**	8.0	8.0
native Mb	protoheme IX	9.1	9.1

[a] These proteins are not stable enough to allow determination of their pKa. However, no spectral changes were observed on addtion of D-fructose (0.1 M).

A more important observation is that the pK_a of $Mb(PhBOH)_2$ changes from 8.0 to 8.5 on addition of D-fructose. It is conceivable that the newly generated boronate anions formed upon sugar complexation facilitate a tight fixation of the heme cofactor in the pocket of apo-Mb, so that the pK_a in the presence of D-fructose is increased to a value comparable to that of $Mb(PhCO_2^-)_2$. A sugar-induced pK_a shift is not observed for any of the other chemically modified Mb derivatives.

The sugar-induced spectral changes of $Mb(PhBOH)_2$—sharpening of the Soret band (408 nm) and increase in the intensities of Q-bands due to aqua-met-Mb (503 and 630 nm) with simultaneous decrease in the intensities of the Q-bands due to hydroxide-Mb (540 and 580 nm)[12]—show a typical saturation behavior with respect to the D-fructose concentration (Figure 2). We can estimate the binding constant of $Mb(PhBOH)_2$ to D-fructose from this saturation curve. In the presence of excess D-fructose, the Benesi–Hildebrand plot gives a good linear relationship for the reciprocal square of the D-fructose concentration. This indicates that a 2:1 complex of D-fructose to

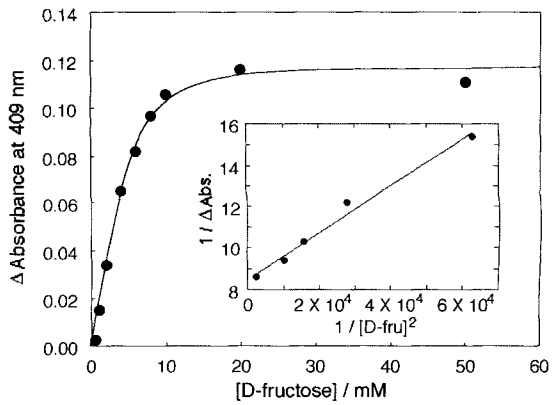

Figure 2. Spectrophotometric titration curve of met-$Mb(PhBOH)_2$ (10 μM in phosphate buffer, 50 mM, pH 7.5, 25 °C) with D-fructose. Inset: Benesi–Hildebrand plot obtained from the titration curve.

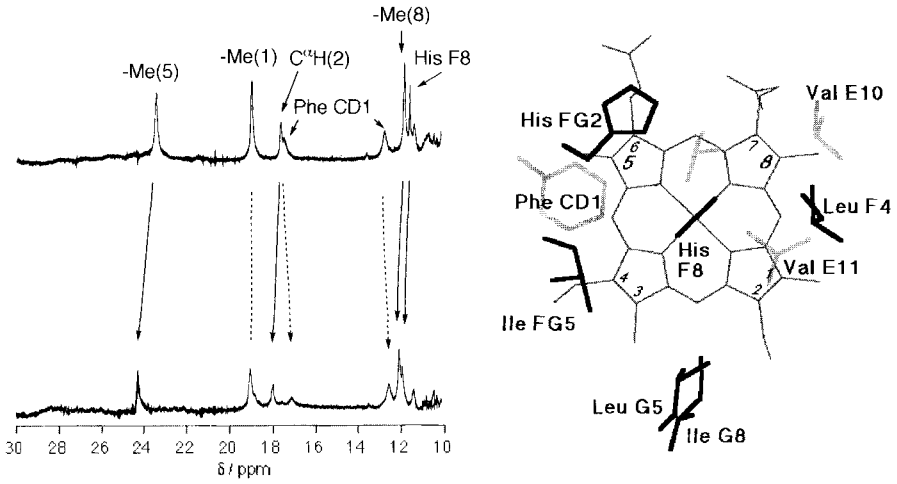

Figure 3. ^{1}H NMR spectra of met-Mb(PhBOH)$_2$ in the absence (top) and in the presence (bottom) of D-fructose (met-Mb(PhBOH)$_2$ 2.8 mM, D-fructose 50 mM, phosphate buffer 50 mM, pD 7.5) containing KCN (10 mM) at 25 °C.

Mb(PhBOH)$_2$ is predominantly formed under the present conditions with an association constant of 8×10^4 M^{-2}. Based on this value, it is clear that more than 99% of Mb(PhBOH)$_2$ binds D-fructose under our standard conditions (10 μM of Mb, 0.1 M of sugars).

Structural Changes in Mb(PhBOH)$_2$ on Binding with Sugar Derivatives: Direct evidence for the sugar-induced structural changes of Mb(PhBOH)$_2$ was obtained by CD spectroscopy. In these experiments, we used the low-spin form of met-Mbs in which a cyanide anion was bound as an axial ligand, because the sugar-induced ligand exchange from H_2O to hydroxide can be neglected in cyanide complexes.[13] A positive peak at 408 nm, characteristic of the induced CD of the heme moiety, clearly increased in intensity to 120% on addition of racemic fructose. In the UV region, on the other hand, two negative peaks (225 and 208 nm) and a positive peak (190 nm), which are characteristic of α-helix structure, scarcely changed. It is evident that the heme–apomyoglobin interactions are efficiently reinforced upon fructose binding.

We next conducted ^{1}H NMR measurements on Mb(PhBOH)$_2$ with the aim of investigating the detailed structural perturbations in proximity to the active site. Figure 3 shows the ^{1}H NMR spectra between $\delta = 10$–30 in the presence and in the absence of D-fructose. Some of the proton signals due to the heme and amino acid residues of Mb changed on addition of D-fructose. The methyl proton at C_5 of the heme shows a significant downfield shift from $\delta = 23.3$ to 24.2. Similar downfield shifts are observed for the methyl protons at C_8 ($\delta = 11.7$ to 11.9) and vinyl protons of the heme C_2 ($\delta = 17.6$ to 17.9). In the amino acid residues of the Mb polypeptide skeleton, upfield shifts of *para* and *meta* protons of Phe CD1 are detected ($\delta = 17.5$ to 17.0 and $\delta = 12.7$ to 12.5, respectively). On the other hand, there is no change in the C_1 methyl protons of the heme and in the methyl protons of Ile FG5.[14] In general, it is clear that the D-fructose-induced changes in the chemical shifts are greatest for the protons of the amino acid residues and of the heme side chains, which are located at the entrance of the heme crevice. This is consistent with the results obtained from the CD spectra. These results demonstrate that the sugars bound at the two phenylboronate sites can efficiently rearrange the heme–apoprotein interactions through the steric and electrostatic perturbations around their vicinal sites. No changes in the CD and NMR spectra were observed for the other Mb derivatives bearing no boronic acid groups.

The Effect of Sugars on the Dioxygen Storage Activity of Modified Mbs: Structural perturbation in a variety of semisynthetic myoglobins directly influences their activity (i.e., dioxygen storage capability). The dioxygen complex of Mb (oxy-Mb) gradually decomposes to the ferric Mb (met-Mb). This process can be monitored spectrophotometrically (Figure 4). Table 2 shows the autooxidation rate constants (k_{ox}) of oxy-Mb. These rates are good parameters for measuring the lifetimes of the active states. The oxy-Mb states of all the chemically modified hemes are less stable than that of native Mb. The autooxidation rates of oxy-Mb(PhNO$_2$)$_2$ and oxy-Mb(PhCO$_2$Et)$_2$, both of which bear noncharged and sterically bulky substituents, were espe-

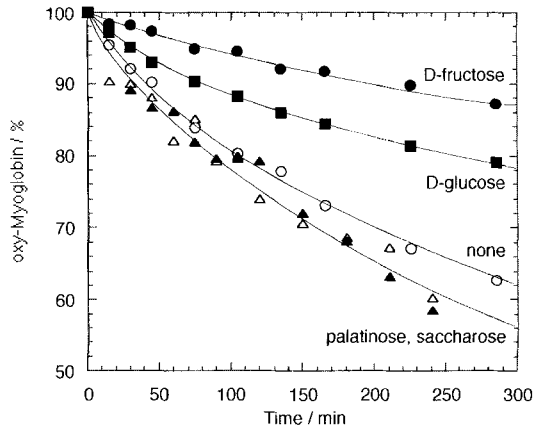

Figure 4. Time courses of the autooxidation of oxy-Mb(PhBOH)$_2$ in the absence (○) and in the presence of D-fructose (●), D-glucose (■), palatinose (▲), and saccharose (△).

Table 2. Autooxidation rate constants (k_{ox}) of the dioxygen complexes of semisynthetic Mbs in the absence and the presence of D-fructose (0.1 M) [a].

Myoglobin	Heme	k_{ox}/h^{-1}	k_{ox}(D-fru)/h^{-1}
Mb(PhBOH)$_2$	**1b**	0.11	0.03
Mb(PhCO$_2$H)$_2$	**2b**	0.12	0.18
Mb(PhNO$_2$)$_2$	**3b**	>0.42 [b]	[b]
Mb(PhCO$_2$Et)$_2$	**4b**	>10 [c]	>10 [c]
Mb(PhH)$_2$	**5b**	0.16	0.16
Mb(OEt)$_2$	**6b**	0.13	0.13
native Mb	protoheme IX	0.02	0.02
randomly modified Mb	protoheme IX	0.08	0.08

[a] Experimental conditions: [Mb] = 10 μM, 50 mM phosphate buffer, pH 7.5 at 25 °C. [b] Dioxygen complex of Mb(PhNO$_2$)$_2$ was not stable enough to allow accurate evaluation of k_{ox}. [c] Typical absorption spectrum of dioxygen complexes was not observed, but autooxidation rate was roughly estimated from the observed disordered spectrum.

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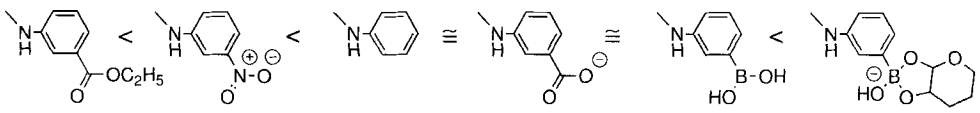


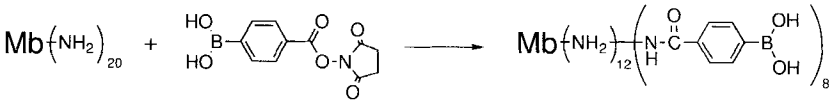
Scheme 3. The effect of the phenyl substituents on oxy-Mb stabilization.

cially accelerated. $Mb(OEt)_2$ and $Mb(PhH)_2$ bearing noncharged but less hindered groups formed considerably more stable dioxygen complexes. Oxy-$Mb(PhCO_2^-)_2$ is more stable than oxy-$Mb(PhNO_2)_2$ having a pseudo-isoelectronic but noncharged substituent. This indicates that the introduction of negative charge is important for the stabilization of oxy-Mb, as is the minimization of steric hindrance (Scheme 3). Oxy-$Mb(PhCO_2Et)_2$ was remarkably destabilized by a factor of 100 relative to oxy-$Mb(PhCO_2^-)_2$. This also supports the significance of anionic charges. The lifetime of oxy-$Mb(PhBOH)_2$ is in the same range as those of $Mb(PhH)_2$ and $Mb(PhCO_2^-)_2$.

Most significantly, the stability of oxy-$Mb(PhBOH)_2$ ($1/k_{ox}$) was enhanced by D-fructose. The lifetimes of the other oxy-Mbs did not change with the addition of D-fructose. At pH 7.5 or 8.5, the dioxygen complex of $Mb(PhBOH)_2$ is four times more stable in the presence of D-fructose (Table 3); however, at lower pH (pH 6.5), this is no longer the case. The pH dependence of k_{ox} correlates well with the fact that phenylboronic acid cannot bind sugar derivatives in acidic solution. In detailed experiments, k_{ox} showed saturation kinetics with regard to the D-fructose concentration (0–100 mM). Based on these results combined with the preceding structural data, sugar-mediated stabilization of oxy-$Mb(PhBOH)_2$ can be ascribed to the modulation of the heme cofactor–apomyoglobin interactions induced upon complexation of D-fructose in the vicinity of the heme pocket.

The lifetime of oxy-$Mb(PhBOH)_2$ also depends on the identity of the added saccharide (see Table 3). D-Glucose, a monosaccharide, is less effective than D-fructose, as expected from its lower association constant. Palatinose and saccharose, disaccharides bearing the D-fructose unit at their C_1 end, do not have any distinct effect on the stabilization of the oxy complex. This might be due to the higher molecular volume of disaccharides cancelling out the sugar-induced stabilization factor.

In order to clarify whether the site-specific incorporation of the nonnatural phenylboronic acid molecules is a crucial factor, we used a randomly modified Mb as a control protein. It was prepared by the condensation reaction of ε-amino groups of lysine residues of the native Mb with ((succinimidyloxy)carbonyl)phenylboronic acid (Scheme 4, an average of 8 groups were incorporated per Mb).[15] As shown in Table 2, no activity enhancement by D-fructose was observed for the randomly modified Mb. The information ensuing from the binding of the sugar molecules to the phenylboronic acid groups is therefore much more efficiently transmitted to the active center in $Mb(PhBOH)_2$ than in randomly modified Mb. This result clearly demonstrates the value of our active site directed modification based on the cofactor reconstitution method.

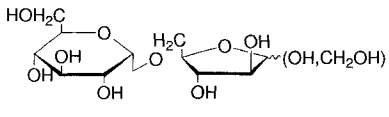


Scheme 4. Synthesis of a randomly modified Mb.

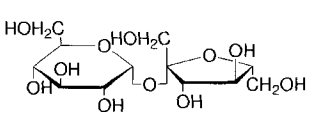
Table 3. Autooxidation rate constants for dioxygen complexes of $Mb(PhBOH)_2$ under various conditions [a].

Conditions		Autooxidation rate constants (k_{ox})/h^{-1}			
pH	[sugar]/mM	D-fructose	D-glucose	palatinose	saccharose
6.0	0	0.36	0.36	0.36	0.36
	100	0.59		–	–
7.5	0	0.11	0.11	0.11	0.11
	5	0.10	–	–	–
	20	0.06	–	–	–
	50	0.04	–	0.15	0.11
	100	0.03	0.06		
8.5	0	0.13	0.13	0.13	0.13
	100	0.03	–	–	–

[a] Experimental condition; [Mb] = 10 μM, 50 mM phosphate buffer at 25 °C.



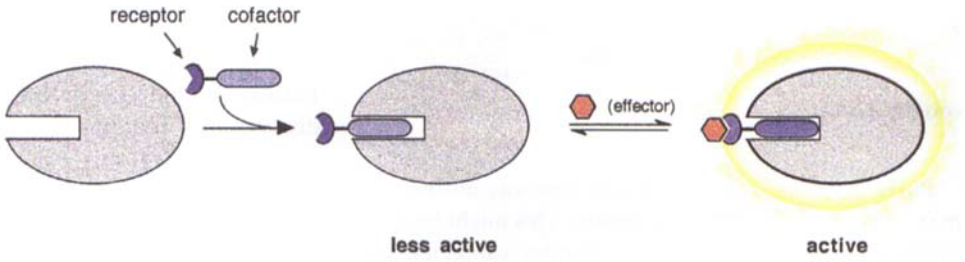
Palatinose



Saccharose

Conclusion

A variety of functions of hemoproteins and enzymes, such as dioxygen transport and storage, electron transport, oxidation and oxygenation of substrates, are generally considered to be regulated by the apoprotein–cofactor (heme) interactions.[16] Two major controlling factors for the interactions are 1) the nature of the axial ligands and 2) the microenvironment in the heme pocket. In most recent examples hemoproteins have been modified by the *static* replacement of an axial ligand or of amino acids located in the heme pocket.[17] In sharp contrast, the present phenylboronic acid appended myoglobin is quite novel in its *dynamic* response to the sugars: the introduced recognition site acts as a "reporter" group. The subtle change in the heme–apoprotein interactions upon sugar binding results in the regulation of *net* protein activity. Thus, it is established that our methodology can confer an allosteric property upon a protein with a specific effector modifying the molecular recognition site (Scheme 5). We expect that artificial interface molecules having molecular recognition functions, such as phenylboronic acid, will significantly expand the possibilities for the design and synthesis of stimuli-responsive semiartificial proteins for use in protein-based science and technology.[18]



Scheme 5. Illustration of basic concept of stimuli-responsive semisynthetic proteins.

Experimental Section

Materials: Protoporphyrin IX was purchased from Aldrich. Myoglobin (horse heart) was purchased from Sigma. All chemicals were used without further purification.

General Procedures: Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silica gel 60 (Merk 5554). Column chromatography was performed on silica 60 (Merk 9385, 230–400 mesh). Melting points were determined on a micro melting point apparatus Yanaco MP-500D and are uncorrected. UV/Vis spectra were recorded on a Shimazu UV-3000 spectrometer. ^{1}H NMR spectra were recorded on either a Bruker AC-250P (250 MHz) or a JEOL GSX-400 (400 MHz) spectrometer. IR spectra were recorded on a Jasco A-100 spectrophotometer. CD spectra were recorded on a Jasco J-720 spectrometer.

Synthesis of Heme Derivatives:

2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-boronophenyl)carbamoyl)-ethyl)porphyrin (1a) was prepared according to literature procedure.[19]

[2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-boronophenyl)carbamoyl)-ethyl)porphyrinate]iron(III) Chloride (1b): Under N_2 atmosphere, **1a** (50 mg, 0.06 mmol) and $FeCl_2 \cdot nH_2O$ (80 mg, 0.63 mmol) were suspended in dry DMF (15 mL) and stirred at 50 °C for 12 h in the dark. The reaction was followed by absorption spectroscopy and TLC (SiO_2; $CHCl_3$–MeOH, 10/1 (*v*/*v*)). The mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The residue was washed with water containing one drop of 1 N HCl. The precipitation was separated by filtration and dried to give **1b** as a dark brown powder: 83% (46 mg) yield; Anal. calcd for $C_{46}H_{44}N_6O_6B_2FeCl$: C, 62.09; H, 4.98; N, 9.44. Found: C, 62.35; H, 5.00; N, 9.34.

2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-carboxyphenyl)carbamoyl)-ethyl)porphyrin (2a): Under N_2 atmosphere, protoporphyrin IX (100 mg, 0.18 mmol) was suspended in dry CH_2Cl_2 (10 mL) and treated with oxalyl chloride (1 mL, excess). The mixture was stirred in the dark for 2 h. After removal of the solvent, the residue yield the protoporphyrin bis(acid chloride) as a dark green solid, which was used immediately without further purification. Under N_2 atmosphere, the bis(acid chloride) in the dry CH_2Cl_2 (15 mL) was added dropwise to a solution of dry DMF (80 mL) and dry pyridine(20 mL) containing 3-aminobenzoic acid (1.22 g, 8.18 mmol). The mixture was stirred in the dark overnight. The solvent was removed under reduced pressure, and the residue dissolved in THF. The organic layer was washed with brine and dried over sodium sulfate, and the solvent was removed under reduced pressure to give **2a** as a black powder: 55% (78 mg) yield; m.p. > 300 °C (decomp.); IR (KBr): $\tilde{\nu}$ = 1720–1650 (C=O) cm^{-1}; ^{1}H NMR ([D$_6$]DMSO, 250 MHz): δ = −4.04 (2H, s, NH), 3.35, 4.45 (4H each, t each, 13^1, 17^1, 13^2, 17^2, CH_2), 3.62–3.70 (12H, br, 2, 7, 12, 18, CH_3), 6.19, 6.41 (2H each, d each, 3, 8, $=CH_2$), 7.29, 7.52, 7.75, 8.18 (2H each, t, d, d, and s, respectively, Ar-H), 8.45 (2H, m, =CH-), 10.16–10.31 (4H, brs, methine-H).

Other derivatives were synthesized according to the same methods as described above.

[2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-carboxyphenyl)carbamoyl)-ethyl)porphyrinate]iron(III) Chloride (2b): Dark brown powder: 36% (33 mg) yield; Anal. calcd for $C_{48}H_{42}N_6O_6FeCl \cdot 1.5H_2O$: C, 62.86; H, 4.95; N, 9.16. Found: C, 62.68; H, 4.71; N, 9.07.

2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-nitrophenyl)carbamoyl)ethyl)-porphyrin (3a): Black powder: 70% (0.10 g) yield; m.p. > 300 °C (decomp.); IR (KBr): $\tilde{\nu}$ = 1680 (C=O), 1530, 1350 (N=O) cm^{-1}; ^{1}H NMR ([D$_6$]DMSO, 250 MHz): δ = 3.34, 4.46 (4H each, t each, 13^1, 17^1, 13^2, 17^2, CH_2), 3.62–3.70 (12H, br, 2, 7, 12, 18, CH_3), 6.22, 6.40 (2H each, d each, 3, 8, $=CH_2$), 7.79, 8.37, (4H each, d and m, respectively, Ar-H), 8.56 (2H, m, =CH-), 10.09–10.24 (4H, s, methine-H). Anal. calcd for $C_{46}H_{42}N_8O_6 \cdot 2.5H_2O$: C, 65.16; H, 5.59; N, 13.22. Found: C, 64.98; H, 5.21; N, 12.98.

[2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-nitrophenyl)carbamoyl)ethyl)-porphyrinate]iron(III) Chloride (3b): Dark brown powder: 14% (14 mg) yield; Anal. calcd for $C_{46}H_{40}N_8O_6FeCl \cdot 0.5H_2O$: C, 61.31; H, 4.59; N, 12.43. Found: C, 61.24; H, 4.86; N, 12.50.

2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-ethoxycarbonylphenyl)-carbamoyl)ethyl)porphyrin (4a): Black powder: 79% (0.12 g) yield; m.p. 243–245 °C; IR (KBr): $\tilde{\nu}$ = 3300 (NH), 1720 (ester C=O), 1660 (amide C=O) cm^{-1}; ^{1}H NMR ([D$_7$]DMF/[D$_8$]THF, 250 MHz): δ = −3.80 (2H, s, NH), 1.23 (6H, t, ethyl-CH_3), 3.45, 4.56 (4H each, t each, 13^1, 17^1, 13^2, 17^2, CH2), 3.58, 3.75 (6H each, s each, 2, 7, 12, 18, CH_3), 4.23 (4H, q, ethyl-CH_2-), 6.20, 6.45 (2H each, d each, 3, 8, $=CH_2$), 7.31, 7.57, 7.83, 8.28 (2H each, t, d, d, and s, respectively, Ar-H), 8.52 (2H, m, =CH-), 10.25–10.45 (4H, s, methine-H). Anal. calcd for $C_{52}H_{52}N_6O_6 \cdot CH_3OH$: C, 72.60; H, 6.35; N, 9.45. Found: C, 72.50; H, 6.20; N, 9.63.

[2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(((3-ethoxycabonylphenyl)-carbamoyl)ethyl)porphyrinate]iron(III) Chloride (4b): Dark brown powder: 73% (41 mg) yield; Anal. calcd for $C_{52}H_{50}N_6O_6FeCl \cdot CH_3OH \cdot CHCl_3$: C, 59.09; H, 5.50; N, 7.66. Found: C, 59.29 H, 5.34; N, 7.27.

2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis((phenylcarbamoyl)ethyl)porphyrin (5a): Black powder: 55% (70 mg) yield; m.p. > 300 °C (decomp.); IR (KBr): $\tilde{\nu}$ = 3300 (NH), 1650 (C=O) cm^{-1}; ^{1}H NMR ([D$_7$]DMF, 250 MHz): δ = −3.59 (2H, s, NH), 3.46, 4.57 (4H each, t each, 13^1, 17^1, 13^2, 17^2, CH_2), 3.70, 3.72, 3.80, 3.81 (3H each, s each, 2, 7, 12, 18, CH_3), 6.23, 6.95 (2H each, d each, 3, 8, $=CH_2$), 6.94, 7.17, 7.60 (2H, 2H and 4H, respectively, t, t, and d, respectively, Ar-H), 8.58 (2H, m, =CH-), 10.11, 10.38, 10.46, 10.52 (1H each, s each, methine-H). Anal. calcd for $C_{46}H_{44}N_6O_2 \cdot CH_3OH$: C, 75.78; H, 6.49; N, 11.72. Found: C, 75.98; H, 6.23; N, 11.56.

[2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis((phenylcarbamoyl)ethyl)-porphyrinate]iron(III) Chloride (5b): Dark brown powder: 38% (24 mg) yield; Anal. calcd for $C_{46}H_{42}N_6O_2FeCl \cdot H_2O$: C, 67.36; H, 5.41; N, 10.25. Found: C, 67.56; H, 5.35; N, 10.06.

2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(ethoxyethyl)porphyrin (6a): This was prepared according to the literature procedure.[20]

[2,7,12,18-Tetramethyl-3,8-divinyl-13,17-bis(ethoxyethyl)porphyrinate]iron(III) Chloride (6b): Dark brown powder: 51% (70 mg) yield; Anal. calcd for $C_{38}H_{40}N_4O_4FeCl$: C, 64.46; H, 5.69; N, 7.91. Found: C, 64.86; H, 5.76; N, 7.47.

Reconstitution of apomyoglobin with various protoheme derivatives: Apomyoglobin was prepared from horse heart metmyoglobin by the acid–butanone method.[21] Protoporphyrin derivatives were incorporated into the heme pocket of apomyoglobin by slightly modifying a procedure described in the literature.[10, 22] Iron protoporphyrins (**1b, 3b, 4b, 5b, 6b,** 1.5–2.0 equiv) dissolved in pyridine/CH_3OH (3:1 *v*/*v*) (1 mg/0.1 mL) were added dropwise to an aqueous apomyoglobin solution with ice-cooling. The mixture was diluted with 10 mM phosphate buffer (pH 7.0) to keep the concentration of the organic solvent below 5–10% (*v*/*v*). The resulting mixtures were incubated at 4 °C for 3–5 h and centrifuged at 4 °C (10000 rpm, 15 min). The supernatant was dialyzed against 10 mM phosphate buffer (pH 7.5) and purified by gel column chromatography (Sephadex G-25). The reconstituted myoglobins were concentrated by ultrafiltration, if necessary. Iron porphyrin **2b** was dissolved in pyridine and aqueous NaOH (0.1 mol dm^{-3}, 1:1 (v:v), 1 mg/0.1 mL) as a solvent. The solution was then diluted 10 times with H_2O and added to an apomyoglobin solution. The purification was conducted by the same method described above. Molar extinction coefficients of the Soret band

0947-6539/97/0307-1030 $ 17.50+.50/0 Chem. Eur. J. 1997, 3, No. 7

for the reconstituted Mbs were determined by the conventional pyridine–hemochromogen method[23] (ε_{409} (Mb(PhBOH)$_2$): 148 mM^{-1}cm^{-1}, ε_{409} (Mb(PhCO$_2$H)$_2$): 207 mM^{-1}cm^{-1}, ε_{409} (Mb(PhH)$_2$): 138 mM^{-1}cm^{-1}, ε_{407} (Mb(OEt)$_2$):[8] 145 mM^{-1}cm^{-1}, ε_{409} (native Mb):[8] 188 mM^{-1}cm^{-1}). Based on the corresponding values, Mbs concentrations were spectrophotometrically determined.

Reconstitution of apomyoglobin with 1b in the presence of sugars: Compound **1b** (6 mM) in DMSO solution (2.5 μL) was added to 50 mM carbonate buffer (pH 10.5, 3 mL) containing 0.1 M monosaccharide. To the solution of **1b**, an appropriate amount of 0.25 mM apo-Mb solution was added dropwise and then UV/Vis spectral changes were measured by using a differential spectrum technique at 25 °C.

Assay of dioxygen storage activity of modified Mbs in the presence of sugars: To the Mb solution containing 0.1 M sugar in 50 mM phosphate buffer (pH 7.5), 5 mg of sodium dithionite was added. Dioxygen gas was carefully bubbled through the solution for 1 min. The stabilities of dioxygen-binding Mbs (oxy-Mbs) were monitored by means of their UV/Vis spectra, through the Q-bands (absorption maximum at 540 and 580 nm), which are characteristic of typical dioxygen complex of Mb(oxy-Mb).

^{1}H NMR spectra of cyano-Mb(PhBOH)$_2$:[14] The cyano-Mb(PhBOH)$_2$ sample was concentrated, and the solvent replaced by 50 mM phosphate buffer of D_2O (pD 7.5) containing 2 mM KCN and 0.1 M (or 0 M) D-fructose by using centricon 30 (Amicon). ^{1}H NMR spectra of the cyano-Mb(PhBOH)$_2$ samples were recorded at 25 °C on a JEOL GSX-400 NMR spectrometer and required 30000 transients collected over a 24 KHz bandwidth with a 5 μs, 45° pulse. Peak positions were referenced against internal DSS.

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