Sugar-Responsive Semisynthetic Myoglobin Bearing Phenylboronic Acid Groups as Recognition Sites

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There are a number of naturally occurring proteins such as hemoglobin and G-proteins, the activities of which are regulated by association and/or dissociation of a small molecule at a specific position.[1] Perturbation given by a small molecule induces a 3D structural change of a protein so as to switch the net reactivity. Development of a general strategy for the effector-mediated control of a protein activity can facilitate the design and synthesis of stimuli-responsive artificial proteins that are among the most important targets in recent protein engineering.[2]

We describe herein the sugar-mediated regulation of the activity of a semisynthetic myoglobin. In a reconstituted myoglobin with a heme cofactor bearing two phenylboronic acid groups at the proximity of its active site, the interaction between the heme cofactor and the apoprotein is suitably perturbed by binding an additive sugar, and, as a result, the dioxygen storage activity of the semisynthetic myoglobin, suppressed in the absence of the sugar, is recovered.

The phenylboronic-acid-appended myoglobin (Mb(PhB-$(OH)_2)_2$) was prepared from a synthetic heme **1**[3] and horse heart apomyoglobin according to the standard reconstitution method.[4] The purified $Mb(PhB(OH)_2)_2$ was nearly as α-helical as native Mb, as monitored by circular dichroism (CD) spectroscopy,[5] and had a typical high-spin iron(III) ($g_x = g_y = 5.9$, $g_z = 2.0$) in its active center of heme, as determined by electron paramagnetic resonance (EPR) spectroscopy at pH 6.0.[6] The absorption spectra of $Mb(PhB(OH)_2)_2$ derivatives obtained by ligand-exchange reactions from the sixth axial H_2O to other anions (fluoride, azide, and cyanide) and those obtained by the reduction and oxygenation reactions were almost identical with those of native Mb.[7] These results show that the semisynthetic $Mb(PhB(OH)_2)_2$ was successfully reconstituted in its structure similar to native Mb.

We observed a slight change in the UV–visible spectrum of the oxidized form of $Mb(PhB(OH)_2)_2$ (met-form) when D-fructose, a monosaccharide, was added to the aqueous solution (pH 7.5)

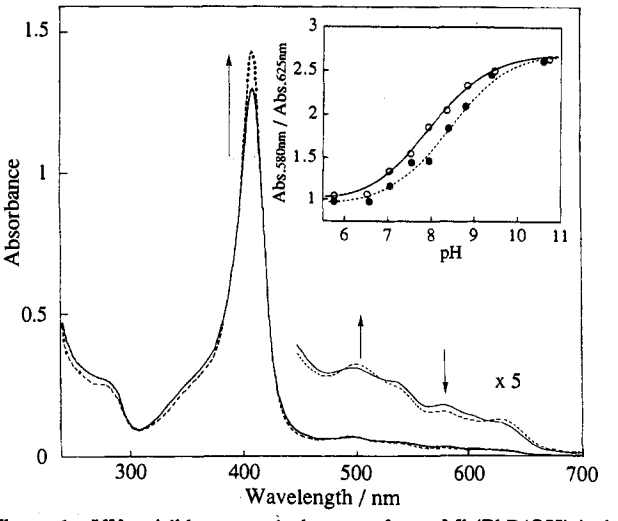
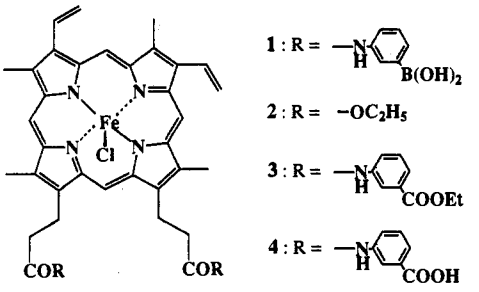


Figure 1. UV—visible spectral changes of met-$Mb(PhB(OH)_2)_2$ by addition of D-fructose (0 mM (—), 100 mM (- - -)) to met-$Mb(PhB(OH)_2)_2$ 10 μM, 50 mM phosphate buffer, pH 7.5 at 25 °C. Inset: pH titration curve of met-$Mb(PhB(OH)_2)_2$ in the absence (○) and the presence (●), of D-fructose (0.1 M) at 25 °C.

Chart 1



(Figure 1). By analogous UV–visible spectra of native Mb in acidic pH, this spectral change is reasonably ascribed to the protonation of the axial OH^- on iron(III).[8] The detailed pH titration of met-$Mb(PhB(OH)_2)_2$ both in the absence and in the presence of D-fructose (0.1 M) clearly shows that the pK_a of the coordinated H_2O shifted from 8.0 to 8.5 by addition of D-fructose (inset of Figure 1). In the other reconstituted Mb with a heme **2** ($Mb(OEt)_2$)[9] which has no phenylboronic acid group, the pK_a (8.0) did not change, irrespective of D-fructose. Also, no influence of the additive D-fructose on the pK_a (9.0) of native Mb was observed.[10] It is well-known that a neutral boronic acid group changes to a negatively charged borate anion even in the neutral pH by complexation with sugars.[11] The newly generated negative charges in the side chains of the heme **1** may induce the tight ion-pair formation between cofactor and apo-Mb, as in the case of native Mb assisted by two propionate anions of protoheme.[12] Such a rearrangement of heme in holoprotein partially explains the result that the acidity of the axial H_2O in met-$Mb(PhB(OH)_2)_2$

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(2) For recent examples: (a) Willner, I.; Willner, B. In *Bioorganic Photochemistry-Biological Application of Photochemical Switches*; Morrison, H., Ed.; Wiley: 1993; Vol. 2, p 1 and references therein. (b) Westmark, P.; Kelly, J. P.; Smith, B. D. *J. Am. Chem. Soc.* **1993**, *115*, 3416. (c) Porter, N. A.; Bruhnke, J. D. *Photochem. Photobiol.* **1990**, *51*, 37. (d) Mendel, D.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2758. (e) Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128. (f) Hamachi, I.; Tanaka, S.; Shinkai, S. *J. Am. Chem. Soc.* **1993**, *115*, 10458.

(3) Synthesis of the heme **1** was briefly described elsewhere: (a) Hamachi, I.; Tajiri, Y.; Murakami, H.; Shinkai, S. *Chem. Lett.* **1994**, 575. (b) Murakami, H.; Nagasaki, T.; Hamachi, I.; Shinkai, S. *Tetrahedron Lett.* **1993**, *34*, 6273.

(4) The heme **1** dissolved in a minimum amount of pyridine (1.2–2.0 equiv to apo-Mb) was added dropwise to an aqueous solution of apo-Mb (0.1 mM, at 4 °C). (a) Axup, A. W.; Albin, M.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435. (b) Hamachi, I.; Nakamura, K.; Fujita, A.; Kunitake, T. *J. Am. Chem. Soc.* **1993**, *115*, 4966. (c) Asakura, T.; In *Methods in Enzymology*; Fleiser, S., Packer, L., Eds.; Academic Press: New York, 1978; Part C, p 446.

(5) CD spectra were obtained at 20 °C (1.3 μM in 10 mM phosphate buffer, pH 6; Jasco-700 spectrometer).

(6) EPR spectra of the lyophilized sample were measured at 4 K on a JEOL JES-2X X-band spectrometer equipped with a liquid helium cryostat (Oxford).

(7) Tamura, M.; Asakura, T.; Yonetani, T. *Biochim. Biophys. Acta* **1973**, *295*, 467.

(8) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands*; North-Holland: Amsterdam, the Netherlands 1971.

(9) The heme **2** was synthesized from the diacid chloride of protoporphyrin IX and ethanol, followed by iron complexation. The heme **3** was obtained by condensation of the acid chloride with ethyl 3-aminobenzoate and iron insertion. The heme **3** was hydrolyzed to afford **4**. Experimental details will be described elsewhere.

(10) This value is almost identical to that of sperm whale Mb ($pK_a = 8.95$). Brunori, M.; Amiconi, G.; Antonini, E.; Wyman, J.; Zito, R.; Rossi Fanelli, A. *Biochim. Biophys. Acta* **1968**, *154*, 315.

(11) (a) Lorand, J. P.; Edwards, J. O. *J. Org. Chem.* **1954**, *24*, 769. (b) Tsukagoshi, K.; Shinkai, S. *J. Org. Chem.* **1991**, *56*, 4089.

(12) X-ray structural analysis showed that two propionate groups of protoheme strongly interacted with 45-Arg, 97-His, and 128-Gln in native Mb (sperm whale). 45-Arg is replaced by Lys in horse Mb. Takano, T. *J. Mol. Biol.* **1977**, *110*, 537.

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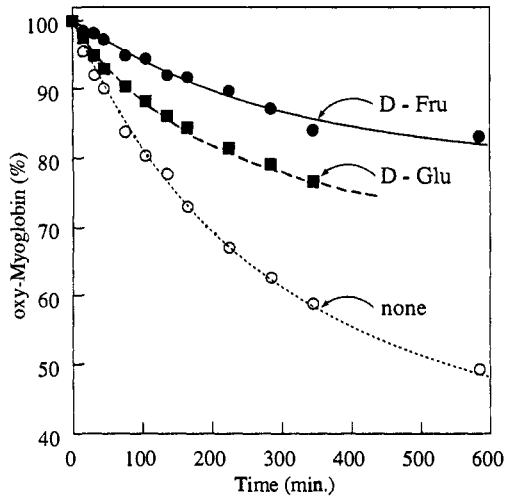

Figure 2. Lifetimes of oxy-Mb(PhB(OH)$_2$)$_2$ in the absence of sugars (○) and in the presence of 0.1 M D-fructose (●) and of 0.1 M D-glucose (■). The oxy-Mb(PhB(OH)$_2$)$_2$ was prepared by $Na_2S_2O_4$ reduction of met-Mb(PhB(OH)$_2$)$_2$ and subsequent O_2 gas introduction. Time courses of reaction of oxy-Mb(PhB(OH)$_2$)$_2$ were monitored by the decrease of the absorbance at 580 nm, characteristic of the oxy complex: Mb(PhB-(OH)$_2$)$_2$ 10 μM, 50 mM phosphate buffer, pH 7.5 at 25 °C.

shifts from the value identical with that of met-Mb(OEt)$_2$ to that close to the value of the native upon addition of D-fructose.

Figure 2 compares the relative stability of the dioxygen complex of Mb(PhB(OH)$_2$)$_2$ (oxy-Mb(PhB(OH)$_2$)$_2$) with and without sugars. In the absence of sugars, the oxy form is gradually autoxidized to the met form with a first-order rate constant (k_{ox}) of 0.11 h^{-1}. Compared to native Mb (k_{ox} = 0.02 h^{-1}), the stability of the dioxygen complex (i.e., active state of Mb) is reduced, probably due to the lack of two propionate anions and the incorporation of bulky phenyl groups of **1**. By addition of D-fructose, oxy-Mb(PhB(OH)$_2$)$_2$ was greatly stabilized (k_{ox} = 0.03 h^{-1}) to recover the dioxygen-storage activity comparable to that of native Mb.[13] D-Glucose, which has a lower affinity with phenylboronic acid than D-fructose,[14] is less effective for the Mb activity (k_{ox} = 0.06 h^{-1}). The sugar-facilitated stabilization of the dioxygen complex occurs in the range of pH 7.5–8.5 (at pH 8.5, k_{ox} was 0.13 and 0.03 h^{-1} in the absence and in the presence of D-fructose, respectively). No effects of sugars were observed in pH 6.0 and 9.6. The pH dependence is consistent with the pK_a shift induced by D-fructose. The lifetimes of the dioxygen complex of native Mb and Mb(OEt)$_2$ (k_{ox} = 0.13 h^{-1}) were not affected by D-fructose. Conceivably, the subtle change in cofactor–apoprotein interaction induced by sugar-binding was directly reflected in the protein activity (see Figure 3).[15]

As one of the important control experiments, we prepared a randomly modified Mb with 4-((succinimidyloxy)carbonyl)-phenylboronic acid (averaged modification number, eight phenylboronic acid groups per Mb).[16] Neither the sugar-induced pK_a shift nor stabilization of the dioxygen complex occurred in the randomly modified Mb (pK_a = 9.6, k_{ox} = 0.10 h^{-1}, both in the presence and in the absence of 0.1 M D-fructose). It is clear that the active-site-directed introduction of nonnatural functional groups is much more efficient for the sophisticated functionalization of native proteins than the random one.

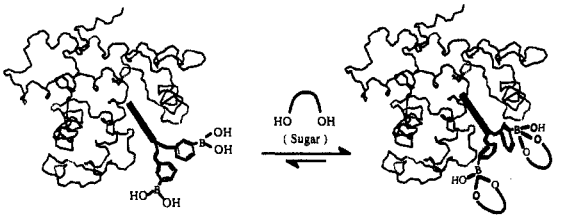

Figure 3. Schematic illustration of the sugar-induced change of the heme–apomyoglobin interaction.

We conclude that a sugar-sensitive, semiartificial myoglobin, which has a potential application to a novel biosensor, is successfully synthesized by a convenient reconstitution method of apo-Mb, with a synthetic heme covalently appending a nonnatural recognition site. The present results also suggest that incorporation of a rationally designed nonnatural group at a suitable site is an essential technique in the rapidly developing field of protein-based materials science.[17] Our methodology would be applicable to other cofactor-depending proteins and enzymes.

Acknowledgment. We are grateful to Dr. Hiroto Murakami for the synthesis of heme **1**. We also thank Dr. Takeshi Nagasaki and Mr. Onari Kimura for the preparation of the randomly modified Mb with phenylboronic acid. This research is supported by Shorai Science and Technology Foundation and the Grant-in Aid for Scientific Research on Priority Areas No. 06240241 from the Ministry of Education, Science and Culture, Japan.

(13) The sugar-enhanced stabilization of oxy-Mb(B(OH)$_2$)$_2$ was found to depend on the concentration of D-fructose: k_{ox} = 0.11, 0.05, and 0.03 h^{-1} at 5, 25, and 100 mM of D-fructose, respectively.

(14) Yoon, J.; Czarnik, A. W. *J. Am. Chem. Soc.* **1992**, *114*, 5874.

(15) In a reconstituted Mb with **4** (Mb(PhCO$_2$H)$_2$), the dioxygen complex became more stable (k_{ox} = 0.20 h^{-1} both in the absence and in the presence of D-fructose) than that of a Mb with **3** (k_{ox} > 10 h^{-1}), indicating that anionic charges are effective for the enhanced stability of the oxy form. However, the autoxidation rate in Mb(PhCO$_2$H)$_2$ is still 7 times faster, relative to that in Mb(PhB(OH)$_2$)$_2$ with D-fructose. These results suggest that the bound sugar may play additional roles to stabilize the dioxygen complex besides the ion-pair formation of borate with apo-Mb. Detailed studies on the sugar effect are now under progress in our laboratory.

(16) The activated ester **5** dissolved in 1,4-dioxane (10 equiv to native Mb) was added to a solution of native Mb (100 mM phosphate buffer, pH 8.0), incubated at 4 °C for 12 h, and then purified by ultrafiltration and gel chromatography (Bio-gel P-6, eluent 50 mM phosphate buffer, pH 7.5). The averaged modification number was determined by titration of free amino groups with sodium trinitrobenzenesulfonate. Details will be reported later: Kimura, O.; Nagasaki, T.; Hamachi, I.; Shinkai, S., manuscript in preparation.

(17) Incorporation of nonnatural amino acids to naturally occurring proteins is now actively developed: (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182. (b) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R.; Diala, E. D. *J. Am. Chem. Soc.* **1989**, *111*, 8013. (c) Chung, H. H.; Benson, D. R.; Schultz, P. G. *Science* **1993**, *259*, 806. (d) Chung, H. H.; Benson, D. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1993**, *115*, 6414. (e) Ellman, J. A.; Mendel, D.; Schultz, P. G. *Science* **1992**, *255*, 197. (f) Bain, J. D.; Switzer, C.; Chamberlin, A. R.; Benner, S. A. *Nature (London)* **1992**, *356*, 537. (g) Wuttke, D. S.; Gray, H. B.; Fisher, S. L.; Imperiali, B. *J. Am. Chem. Soc.* **1993**, *115*, 8455. (h) Wallace, C. J. A.; Clark-Lewis, I. *J. Biol. Chem.* **1992**, *267*, 3852. (i) Milton, R. C. deL.; Milton, S. C. F.; Kent, S. B. H. *Science* **1992**, *256*, 1445.