Peroxidase Activity of Cytochrome c Generated by Phenylboronic Acid Modification

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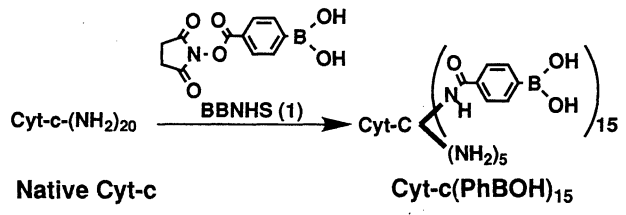
Reactive amino residues of horse heart cytochrome c were successfully modified with 4-boronobenzoic acid N-hydroxysuccinimide ester **1**. The modified cytochrome c shows an enhanced peroxidase activity, which can be regulated by the addition of sugars.

We recently reported that incorporation of phenylboronic acid groups into native myoglobin, a dioxygen storage hemoprotein, can be effective for the control of the myoglobin activity by sugar derivatives.[1,2] These results suggest that phenylboronic acid is one of the intriguing nonnatural functional molecules for modulating the activities of naturally occurring proteins and enzymes.[3] In order to explore the veiled functions of phenylboronic acids, we are now applying this modification method to other proteins. Here we describe a novel peroxidase-activity of cytochrome c(Cyt-c) which is not the original function of native Cyt-c and that the activity is further accelerated by added sugars.

Cytochrome c, a peripheral, electron transport protein, has 20 reactive amino groups (19 Lys and a terminal amine). We modified native Cyt-c (from horse heart, Sigma Chemical Co.,) with 4-boronobenzoic acid N-hydroxysuccinimide ester (BBNHS, **1**) as shown in Scheme 1. To the aqueous



Scheme 1.

solution of Cyt-c (10 mg/ 2 ml, 50 mM (1 mM = 1 mmol dm^{-3}) sodium carbonate buffer, pH 9.5) were added 20 equivalents of **1** dissolved in dioxane (4.3 mg/ 300 μl) at 4 °C. The mixture was incubated for 12 h at 4°C and purified through gel column chromatography (Biogel P-6, eluent 50 mM phosphate buffer, pH 7.5). The averaged modification number of the obtained Cyt-c was estimated by spectroscopic titration of remaining free amino groups with sodium trinitorobenzenesulfonate.[4] By the use of 20 equivalents of **1**, 15 amino groups of Cyt-c are acylated (Cyt-c $(PhBOH)_{15}$).

We evaluated the peroxidase activity of modified Cyt-c using o-methoxyphenol (*o*-MP) as a substrate and hydrogen peroxide (H_2O_2) as an oxidant. In this typical peroxidation reaction, *o*-MP is oxidized to its tetramer (so-called tetraguaiacol), the production of which can be followed spectroscopically by monitoring the absorbance of the product at 470 nm.[5] Figure 1 shows time courses of the absorbance change of this reaction catalyzed by native and modified Cyt-c. The initial rate (V) of the reaction by native Cyt-c is negligible. Apparently, the peroxidation reaction is much accelerated by Cyt-c $(PhBOH)_{15}$ (V = 21.0 μM/min), compared to native Cyt-c. Denaturation and degradation of Cyt-c $(PhBOH)_{15}$ were not observed in UV-visible spectrum after the reaction.

As shown in Figure 1d, the initial rate of the reaction by Cyt-c (PhBOH) 15 in the presence of D-fructose was two-fold more accelerated relative to that in the absence of D-fructose. Such a sugar-response of the activity never occurred in native Cyt-c (Figure 1b)[6]. The sugar-enhanced peroxidase activity of Cyt-c $(PhBOH)_{15}$ is dependent on the added sugar structure. The order of the sugar effect (fructose > arabinose > galactose > mannose > glucose) corresponds with that of the binding

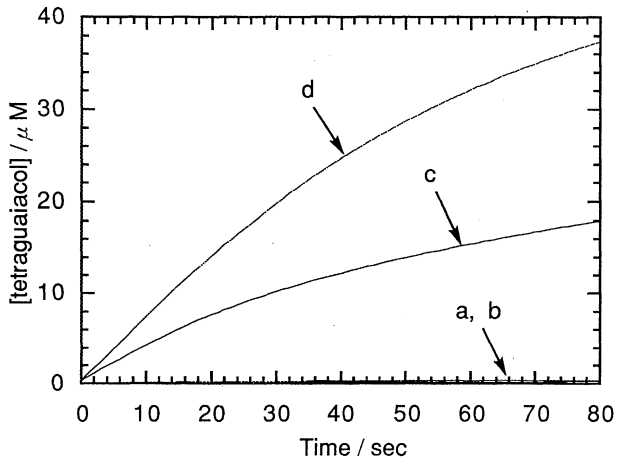


Figure 1. Initial rates of peroxidation reaction by Cytochrome c
a) native Cyt-c without D-fructose, b) native Cyt-c with D-fructose, c) Cyt-c $(PhBOH)_{15}$ without D-fructose, d) Cyt-c $(PhBOH)_{15}$ with D-fructose; [Cyt-c $(PhBOH)_n$] = 5.0 x 10^{-2} mM (n=0,15), [D-fructose] = 0, 100 mM, [*o* -MP] = 10 mM, [H_2O_2] = 3 mM; pH 9.5 50 mM carbonate buffer.

constants of phenylboronic acid with various sugars.[7] These results suggest that the sugar response in Cyt-c $(PhBOH)_{15}$ can be attributed to the binding of sugars with phenylboronic acids locating on the Cyt-c surface.

The overall peroxidation reaction can be approximated by the following two steps: the reaction of Cyt-c with H_2O_2 to generate its active species and the subsequent oxidation of *o*-MP.[8] The initial rate does not change with the *o*-MP concentration, while the rate depends on the H_2O_2 concentration with saturation kinetics. These reveal that the rate-determining step of the overall reaction is the formation process of an active intermediate of Cyt-c by H_2O_2 rather than the oxidation of *o*-MP, both in the presence and the absence of sugars. It is clear that the enhanced peroxidase activity of Cyt-c with sugars is due to increased reactivity of Cyt-c to H_2O_2.

Figure 2 shows circular dichroism (CD) spectra of Cyt-c $(PhBOH)_{15}$ with and without D-fructose. Split Cotton effect due to a Soret band of the heme active center (420 nm (negative), 405nm (positive)) and two negative bands due to protein backbone (221nm and 208 nm) appear, that are practically identical to native Cyt-c. Both Cotton effects are weakened to about 80% in their intensity (81% in ICD of Soret band and 77% in protein band) by D-fructose, indicating the decrease of α-helix content by added D-fructose. Conceivably, the binding of D-fructose to phenylborobic acids induces the perturbation of 3D-structure of Cyt-c backbone, probably due to

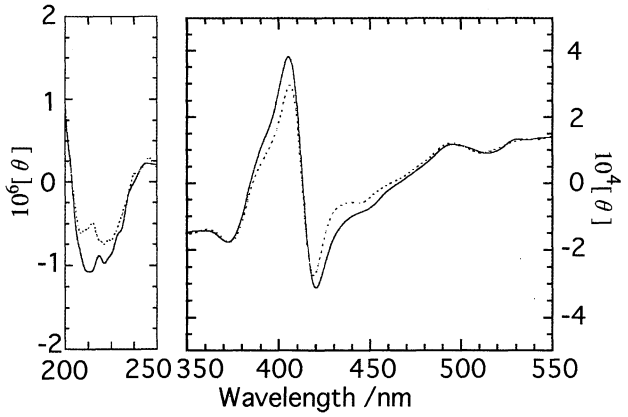


Figure 2. CD spectra of Cyt-c$(PhBOH)_{15}$ without (——) and with (- - - -) D-fructose; [Cyt-c$(PhBOH)_{15}$] = 1.0 x 10^{-2} mM, [D-fructose] = 0.100 mM pH 9.5 50 mM carbonate buffer.

the electrostatic repulsion between newly generated borate anions and carboxylate anions of Glu and Asp.[9] Such a structural disorder on the protein surface leads to the change of the heme active site, so as to enhance the peroxidase activity.[10]

In conclusion, a sugar responsive Cyt-c is synthesized by attachment of phyenylboronic acids to the protein surface. The idea on sugar-sensitive enzyme with peroxidase activity can be applied to a novel biosensor. This new enzyme may also be useful to the boron-neutron therapy because of its high density of boron atom within a globular enzyme.

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References and Notes

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