Sugar-enhanced aniline hydroxylase activity of semisynthetic myoglobins possessing *m*-dihydroxyborylphenylalanine

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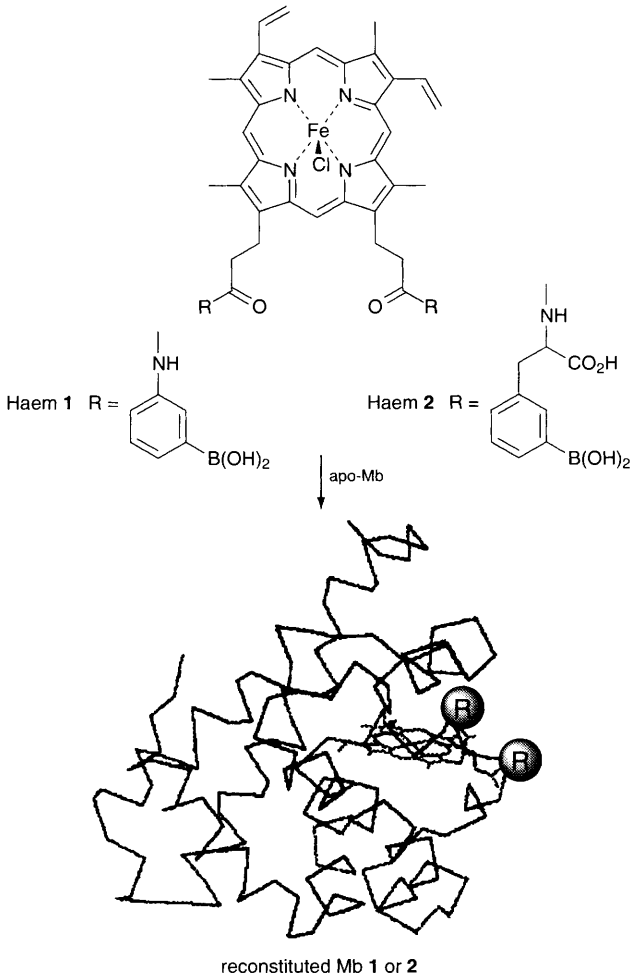
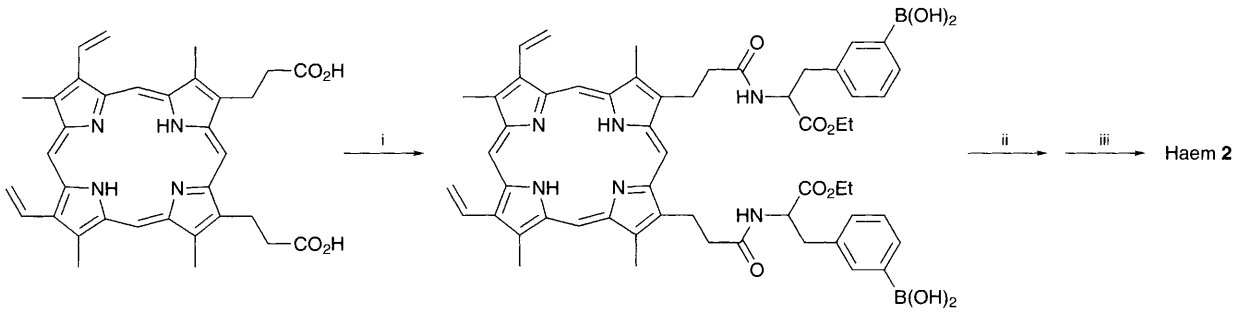
Semisynthetic myoglobin bearing *meta*-dihydroxyborylphenylalanine groups as sugar binding sites displays sugar-responsive aniline hydroxylase activity.

Incorporation of molecular recognition sites into naturally occurring enzymes and proteins is gradually becoming one of the most important methods for modulating or switching enzyme activities. As pioneering works, Schultz and co-workers demonstrated successful conversion of a nonspecific phosphodiesterase to a site-selective nuclease by the conjugation of an oligonucleotide as a recognition site.[1] We recently reported that the dioxygen storage capability of myoglobin (Mb) bearing unnatural phenylboronic acids is enhanced by monosaccharides such as D-fructose and D-glucose.[2] We describe herein that the enzymatic activity of phenylboronic acid-appended Mb derivatives can be facilitated by the binding of sugars. Structure stabilization effected by added sugars shows good agreement with the enhanced hydroxylase activity of our engineered Mbs.

In order to overcome the low yield and low stability of Mb **1** due to the loss of two carboxylic acids, a new haeme derivative **2** was synthesized as shown in Scheme 1.[3] The haeme **2** containing two carboxylic acids was successfully reconstituted to afford the dihydroxyborylphenyl alanine-appended Mb **2** in almost quantitative yield (98%) (Scheme 2).[4] The stability of Mb **2** was examined by urea-denaturation experiments. An increasing amount of a denaturant (urea) destroys the 3D-structure of Mb, so as to release the haeme cofactor from the native haeme crevice. This denaturation process can be spectrophotometrically monitored by the broadening of the Soret band of the haeme. Fig. 1 displays typical denaturation curves, plotting the absorbance of the Soret band against the urea concentration. It is apparent that Mb **2** is more resistant to denaturation, relative to Mb **1**. Free energy changes ($\triangle G°$) for this process were evaluated by the linear extrapolation method as follows: $\triangle G°$ = 8.0 kcal mol^{-1} (1 cal = 4.184 J) for Mb **2** and 6.3 kcal mol^{-1} for Mb **1**.[5] Introduction of two carboxylic acid groups into the haeme **2** stabilized Mb **2** by about 2 kcal mol^{-1}, compared to Mb **1**. More interestingly, Mb **2** is further stabilized in the presence of D-fructose ($\triangle G°$ = 10.1 kcal mol^{-1}) by 2 kcal mol^{-1}, relative to Mb **2** in the absence of D-fructose. Similar stabilization by D-fructose was also observed for Mb **1** ($\triangle G°$ = 8.3 kcal mol^{-1} in the presence of D-fructose). Such a sugar contribution to the structural stabiliza-



Scheme 2 Reconstitution of synthetic haems **1** and **2** to apo-Mb



Scheme 1 *Reagents and conditions*: i, $(COCl)_2$, dry CH_2Cl_2; then *m*-dihydroxyborylphenylalanine ethyl ester hydrochloride, pyridine, dry CH_2Cl_2, room temp.; ii, $FeCl_2$, dry DMF, 65 °C; iii, 1 M NaOH, THF–MeOH, room temp.

tion has not been previously observed for other Mb derivatives lacking the phenylboronic acid group ($\Delta G°$ for native Mb: 14.3 kcal mol^{-1} and 13.6 kcal mol^{-1} without and with D-fructose, respectively).

The structural stabilization by D-fructose was directly reflected in the definitive alteration of the active site environment. A sugar-responsive change of the active site was detected in the UV–VIS spectra of met-Mb **2**, which was attributed to protonation or deprotonation of the axial water. The pK_a of the H_2O coordinated to the haem iron centre in Mb **2** was determined to be 8.5 by spectrophotometric pH titration. With addition of D-fructose (0.1 M), the pK_a value clearly shifted to 9.0, which is almost identical to that of native Mb.[6] As reported previously, the pK_a of Mb **1** was shifted from 8.0 to 8.5 by D-fructose.[2] These results imply that the microenvironment of the active site of Mb **2** is modulated from a partially disturbed state to an almost native one by the binding of D-fructose in the vicinity of the haem crevice as well as by the incorporation of two carboxylic acid moieties. It is worthwhile to emphasize that the sugar-induced pK_a shift does not take place in other Mbs.

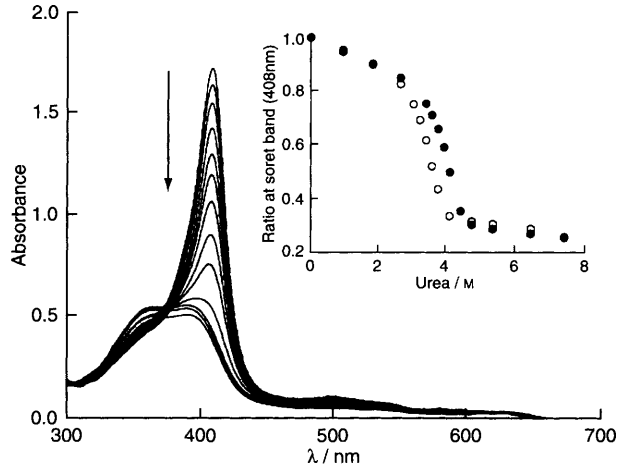


Fig. 1 Typical UV–VIS spectral change of met-Mb **2** by addition of urea: 8 μM met-Mb **2** in phosphate buffer (50 mM, pH 7.5) at 25 °C. Inset: denaturation curve of met-Mb **2** in the absence (○) and the presence of 0.1 M D-fructose (●).

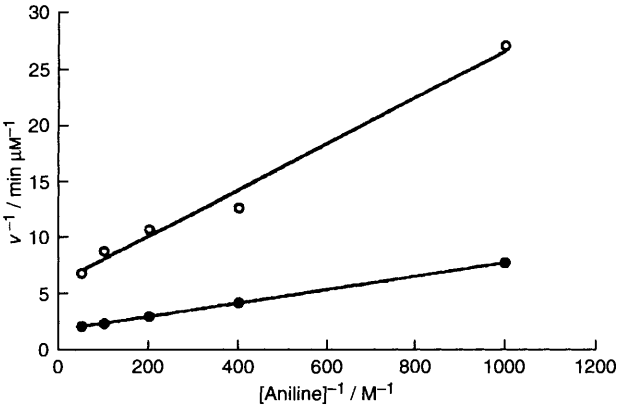


Fig. 2 Double reciprocal plots in the initial rates of the aniline hydroxylation reaction against aniline concentration in the absence (○) and the presence of (●) of 0.1 M D-fructose. Each point represents the average of three experiments: met-Mb **2** 8 μM, FMN 50 μM, NADH 1 mM in phosphate buffer (50 mM, pH 7.5) at 37 °C.

Based on the structural results, we next conducted the aniline hydroxylation reaction catalysed by Mb **2** in the presence and the absence of sugars. Following the reported procedure, dihydronicotinamide adeninedinucleotide (NADH) as an electron donor and flavin mononucleotide (FMN) as an electron mediator were employed under aerobic conditions in this reaction.[7] The initial rates show saturation kinetics with respect to the aniline concentration. As shown in Fig. 2, double reciprocal plots of the initial rates against the aniline concentrations gave good linear relationships, yielding the Michaelis–Menten parameters as follows: k_{cat} 2.1 × 10^{-2} (min^{-1}) and K_m 3.5 × 10^{-3} (M) in the absence of D-fructose, k_{cat} 7.0 × 10^{-2} (min^{-1}) and K_m 3.4 × 10^{-3} (M) in the presence of D-fructose. It is clear that the net hydroxylation (k_{cat}/K_m) by Mb **2** is enhanced 3.5 fold by D-fructose. The binding affinity for aniline ($1/K_m$) does not significantly change with the addition of D-fructose, while the catalytic efficiency, k_{cat}, on the other hand, increases by a factor of 3.3. Conceivably, the sugar-facilitated hydroxylase activity could be mainly ascribed to the accelerated k_{cat}, but not to K_m. The sugar-effect was saturated with respect to the D-fructose concentration both in the case of Mb **2** and Mb **1** (data not shown). As control experiments, we also confirmed that no enhancement by D-fructose occurs in native Mb [saturation kinetics are also observed for native Mb: k_{cat} 5.6 × 10^{-2} (min^{-1}) and K_m 8.8 × 10^{-3} (M) without D-fructose, k_{cat} 4.8 × 10^{-2} (min^{-1}) and K_m 9.6 × 10^{-3} (M) with D-fructose]. These results indicate that D-fructose operates as an active effector molecule to regulate the enzymatic activity of Mb through the binding to phenylboronic acid moieties appended to the proximity of the active site.

In the diverse field of bio-science and technology, many antibodies conjugated with reactive enzymes have been used to detect substances of biological importance. We envisage that small, functional molecules may replace huge antibodies due to their rationally-designed hybridization with native enzymes. The present study facilitates such a promising approach.

References

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3 *meta*-Dihydroxyborylphenylalanine was synthesized by the method reported for the *para*-derivative; H. R. Snyder, A. J. Reedy and W. J. Lennarz, *J. Am. Chem. Soc.*, 1958, **80**, 835. All new compounds were characterized by various spectrometric methods and elemental analysis. Haem **2** (racemic mixture) was characterized by IR spectroscopy [(KBr): ν_{OH} 3300 cm^{-1}, ν_{CO} 1650 cm^{-1}] and elemental analysis (Calc.: C, 60. 41; H, 5.07; N, 8.13. Found: C, 60.63; H, 5.11; N, 7.98%).

4 The reconstituted Mb **2** was spectrophotometrically characterized by ligand exchange reactions (aqua-met Mb **2**: λ_{max}, 408, 502 and 630 nm, azide-met Mb **2**: 419, 540 and 573 nm, fluoro-met Mb **2**: 406 and 603 nm) and redox reactions [deoxy (Fe^{II})-Mb **2**: 430 and 559 nm, oxy-(O_2-complex)-Mb **2**: 415, 541 and 579 nm]. The spectral data were almost identical to those of native Mb.

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