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Transition-Metals Facilitated Electron Transfer of Semisynthetic Myoglobin Bearing Bis(iminodiacetic Acid) Moiety

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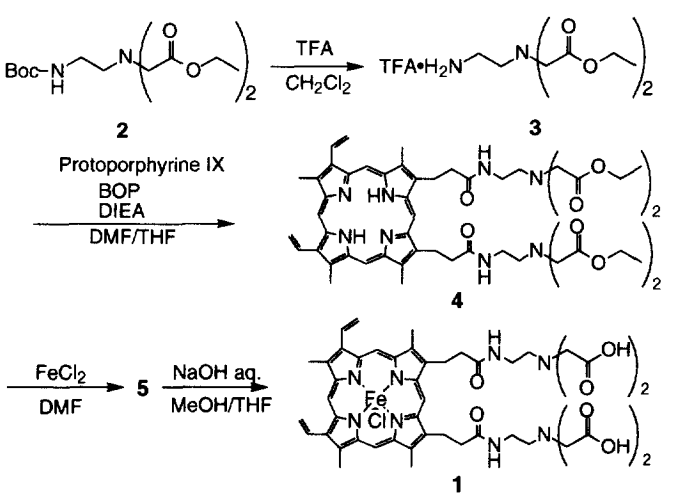
Abstract: A novel bis(iminodiacetic acid)-appended myoglobin is conveniently synthesized by a cofactor reconstitution method. Electron transfer from ascorbate to the engineered myoglobin is facilitated by the transition-metal binding. Copyright © 1996 Elsevier Science Ltd

Well-characterized proteins provide valuable starting frameworks for the construction of novel biomacromolecules.[1] In order to confer a sophisticated function on these proteins, new chemical strategies for the artificial modification and regulation of naturally occurring proteins are desired. We recently reported, for example, that site-specific incorporation of phenylboronic acid into native myoglobin (Mb) can give a novel sugar-responsive property.[2] This finding suggests to us that rationally designed introduction of a molecular recognition site is one of the promising approaches for modulating a protein activity. It is now important to expand a variety of artificial receptors which are able to operate on native proteins in active manners. Here we describe that introduced iminodiacetic acids can tightly bind transition metal cations, so as to facilitate an electron transfer from ascorbate to the heme center of the engineered Mb.

Scheme 1



Bis(iminodiacetic acid)-appended heme **1** was synthesized as shown in Scheme 1. Protoporphyrin IX was condensed with *N*,*N*-di(ethoxycarbonylmethyl)ethylenediamine in the presence of BOP and DIEA[3], followed by iron insertion to the porphyrin center, and by hydrolysis of four ester bonds to afford **1**.[4] The heme **1** was incorporated into apo-Mb according to the conventional cofactor reconstitution method.[5] UV-visible, circular dichroism (CD) and NMR spectroscpoies demonstrated that the purified bis(iminodiacetic acid)-appended Mb (IDA_2-Mb, yield for the reconstitution = 95 %) was almost identical to native Mb in its structure.[6]

Figure 1 displays UV-visible spectral changes of IDA_2-Mb by addition of Co(II). With an increasing amount of $CoCl_2$, Soret band at 409 nm and two Q-bands at 505 and 630 nm are lessened, whereas Q-bands at 540 and 580 nm are intensified. These changes are clearly saturated at a 1 : 1 ratio of Co^{2+} to IDA_2-Mb (See Figure 2), indicating that a 1 : 1 complex between IDA_2-Mb and Co^{2+} is quantitatively formed in the present conditions. The spectral changes by addition of Cu(II), on the other hand, are saturated at a 2 : 1 ratio (Cu^{2+}: IDA_2-Mb). Probably, two iminodiacetic acid groups act cooperatively to form a stable hexagonal complex for Co^{2+} (i. e., $Co(IDA)_2$), while the two groups independently bind Cu^{2+} to form two square planner complexes for Cu(II) (i. e., $2Cu(IDA)_1$). No spectral changes take place for Mg^{2+} addition because of its low stability constant.[7]

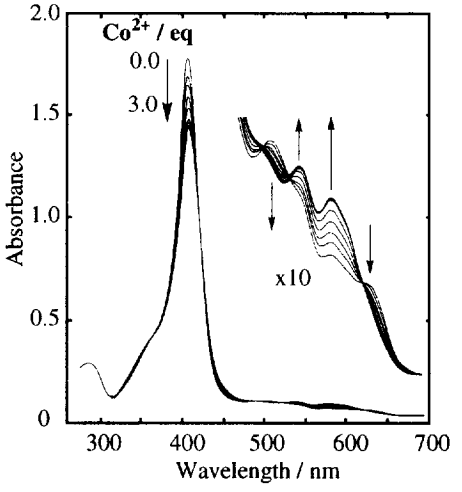


Figure 1. UV-vis spectral change of IDA_2-Mb by addition of Co^{2+}. [IDA_2-Mb]=13.7μM, pH 8.0, 50mM KH_2PO_4/NaOH buffer, 25°C.

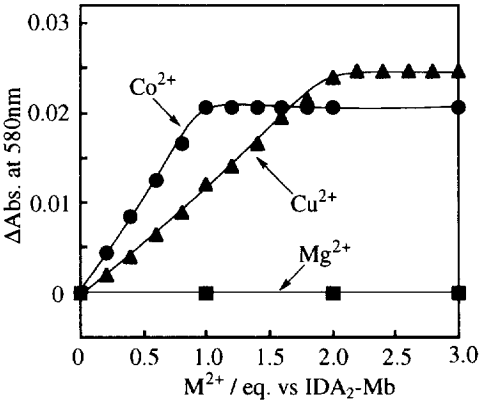


Figure 2. Titration curve of IDA_2-Mb by metal cations. Experimental conditions are identical with Fig. 1.

The above-mentioned spectral changes induced by transition metals are ascribed to the pK_a shift of the H_2O coordinated to iron(III) heme. Detailed pH titration in the presence and the absence of metal cations gave corresponding pK_a values summarized in Table 1. In the absence of metals, the pK_a value of IDA_2-Mb is almost same as that of native Mb, implying that the microenvironment of the heme crevice is similar to the native one. An acidic pK_a shift occurs by about one order magnitude when one equivalent of Co^{2+} is added to IDA_2-Mb. Addition of two equivalent of Cu^{2+} induces similar pK_a shift, but one equivalent of Cu^{2+} is less sufficient.

The pK_a is not affected by Mg^{2+}. It is clear that transition metals bound to iminodiacetic acid moieties efficiently modulate the microenvironment of the active site in IDA_2-Mb.[8]

Table 1. pK_a values of IDA_2-Mb in the presence of metal cations.

metal cation	no metal	Co^{2+}(1eq)	Cu^{2+}(1eq)	Cu^{2+}(2eq)	Mg^{2+}(5eq)
pK_a	8.9	8.2	8.5	8.0	8.9

Experimental conditions : [IDA_2-Mb]=13.7μM, 50mM KH_2PO_4/NaOH buffer, 25°C. pH of the solution was controlled by the addition of 0.1N NaOH aq.

Such considerable changes in the active site microenvironment influence its electron transfer capability. Reduction of met-IDA_2-Mb (Fe(III) state) by ascorbate gives oxy-IDA_2-Mb (dioxygen complex of Mb) under aerobic conditions. By monitoring the time course of the absorbance change of oxy-IDA_2-Mb, we evaluate apparent electron transfer rates (v_{init}). As shown in Figure 3, the initial rate is enhanced by about 8 fold in the presence of $CoCl_2$ (1eq.). In the case of Cu(II), the rate for 2 eq. of Cu^{2+} is greater than that for 1 eq. of Cu^{2+}, like the pK_a shift behavior. Cu(II) addition (even in 2 eq.) is less effective for the rate enhancement rather than Co(II) addition (1eq.). Such a transition-metal-accelerated reduction is never observed for native Mb.

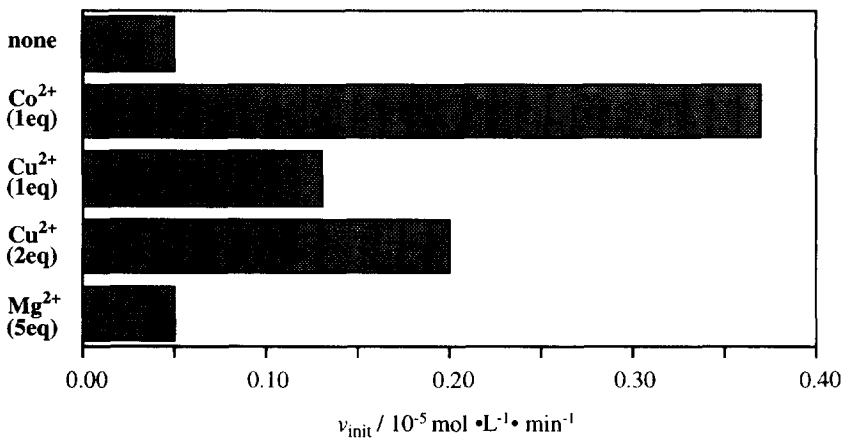


Figure 3. Initial rates of the reduction of met-IDA_2-Mb by ascorbic acid.
[IDA2-Mb]=1.37x10^{-5}M, [ascorbic acid]=2.0x10^{-4}M, pH 7.0, 50mM KH_2PO_4/NaOH buffer, 25°C

It is known that the modification of the heme propionate decreases the pK_a value of the coordinated water in Mb. Tsukahara and coworkers pointed out that decrease of the pK_a is roughly correlated with the facilitated Mb reduction by ascorbate.[9] In our IDA_2-Mb, the metal-induced pK_a shift shows considerable correspondence with the accelerated electron transfer rate. Incorporation of iminodiacetic acid groups does not cause strong structural disorder based on its pK_a value. The transition-metal binding, however, may induce breakage of electrostatic and hydrogen bonding interactions between the IDA ends of the heme and Lys 45 and His 97 of Mb skeleton to expand the heme crevice.[10] These dynamic changes reasonably explain the origin of the transition-metal response of IDA_2-Mb in terms of both the pK_a and the enhanced Mb reduction rate. It is thus established

that iminodiacetic acid which can dynamically alter its structure and charge upon transition-metals binding is one of the promising artificial receptor molecules for protein engineering.

References

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2. Hamachi, I.; Tajiri, Y.; Shinkai, S. *J. Am. Chem. Soc.*, **1994**, 116, 7437.
3. BOP: Benzotriazole-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate
   DIEA: Diisopropyl ethylamine
4. All new compounds have been characterized by ^{1}H-NMR, IR and elemental analysis. Protoporphyrin derivative **4**, ^{1}H-NMR ($CDCl_3$, 250 MHz) : δ 0.97 (12H, t, -CH_2CH_3), 2.40 (4H, m, -CH_2NCH_2CO-), 3.02 (8H, m, -$CH_2CONHCH_2$-), 3.14 (8H, s, -NCH_2CO-), 3.56-3.68 (12H, m, Ar-CH_3), 3.82 (6H, q, -CH_2CH_3), 4.30 (4H, m, Ar-CH_2-), 6.18-6.45 (4H, dd, Ar-CH=CH_2), 7.79 (2H, s, -CONH-), 8.38-8.50 (2H, m, Ar-CH=CH_2), 10.10-10.17 (4H, m, meso-H). IR (KBr, cm^{-1}) : 3300 (ν_{NH}), 1730(ν_{CO}). Analytical Found: C, 65.28, H, 7.06, N, 11.17%. Calcd for $C_{54}H_{70}N_8O_{10}$: C, 65.44, H, 7.12, N, 11.30%. Heme **1**, analytical Found: C, 56.67, H, 5.46, N, 11.49%. Calcd for $C_{46}H_{52}N_8O_{10}ClFe$ $0.4H_2O$: C, 56.64, H, 5.46, N, 11.49%
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6. The spectrophotometric titration of **1** with apo-Mb confirms the quantitative formation of a 1:1 complex. The ratio of the absorbance at 409 nm to that at 280 nm is 5.1, which is greater than that of native Mb. This indicates that the IDA_2-Mb is pure enough for the future studies. Absorption maxima for the ligand-exchanged IDA_2-Mb are following : 419, 542 and 575 nm for N_3^- form, 406 and 606 nm for F^- form. Absorption maxima for met-IDA_2-Mb are 408, 502 and 630 nm, for deoxy-IDA_2-Mb are 432 and 557 nm, and for oxy-IDA_2-Mb are 413, 542 and 579 nm. These values are identical to those for the corresponding forms of native Mb.
7. A. E. Martel, R. M. Smith, " Crystal Stability Constants Vol. 1", Plenum Press; New York and London, (1989), p. 116-119: log*K* = 6.94 (Co^{2+}), 10.57 (Cu^{2+}), 2.98 (Mg^{2+}).
8. This is supported by the results of the CD spectral measurement. A CD peak of the heme crevice (a positive cotton effect at 408nm) is clearly lessened up to about 90% in its intensity by addition of Co^{2+}, whereas the α-helix region (two negative peaks at 220 and 208 nm and a positive peak at 190 nm) scarcely changes. Such a transition-metal effect was not observed for the reconstituted Mb with heme **5**.
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