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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 248–251

Non-covalent modification of the heme-pocket of apomyoglobin by a 1,10-phenanthroline derivative

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Received 29 July 2005; revised 4 October 2005; accepted 6 October 2005
Available online 24 October 2005

Abstract—To expand the repertoire of artificial enzymes that are constructed by replacing the natural prosthetic group of hemoproteins with non-natural cofactors, we examined incorporation of a non-porphyrinic ligand (**1**) into the heme-pocket of apomyoglobin in a non-covalent fashion. Ligand **1** is a highly conjugated 1,10-phenanthroline derivative, which shares some structural features with protoporphyrin IX; for example, molecular size and arrangement of hydrophobic and anionic parts. Addition of apomyoglobin to a solution of **1** induces clear changes in the absorption spectrum of **1**, suggesting one-to-one incorporation of **1** into the heme cavity of apomyoglobin with an affinity of $6.3 \times 10^6\ M^{-1}$. We found that the hydrolytic activity of apomyoglobin toward *p*-nitrophenyl hexanoate was greatly suppressed because of the incorporation of **1** into the heme-pocket.

Introduction of non-natural metal complexes into protein cavity has been recognized as a powerful method to create new artificial metalloproteins with desirable functions.[1] The molecules incorporated inside protein cavity often exhibit significant stability and unique reactivities such as enantio- or substrate-selective reactions. For example, Distefano and co-workers have demonstrated that a Cu(II)-1,10-phenanthroline complex that is covalently attached to a cysteine in the protein cavity of an adipocyte lipid-binding protein catalyzes highly enantioselective hydrolysis with up to 86% ee.[1d,2] In addition to the covalent approach,[3] non-covalent incorporation of metal complexes into protein cavity also has been carried out to create artificial metalloproteins with unique functions.[4–6] For example, Hayashi's and Casella's groups have reported that replacement of heme in myoglobin with protoporphyrin IX modified at the propionate group(s) can convert an O_2-storage protein to a peroxidase or a peroxygenase.[4,5] Since this approach takes advantage of strong interactions between protoheme and heme-binding site, incorporation of any other non-natural metal complex that bear much less structural similarity to the native prosthetic group has been considered to be unpromising, although it has been reported that aromatic molecules such as 8-anilino-1-naphthalene sulfonate bind to the heme-pocket of apomyoglobin.[7] Recently, however, Watanabe and co-workers successfully demonstrated that Schiff base complexes can be stably incorporated into the heme cavity of myoglobin with assistance of protein modification by amino acid mutation.[8] Thus, expanding the range of metal complexes that can be incorporated into the heme cavity would enable us to design artificial metalloproteins with functions beyond those of heme proteins (Scheme 1).

Recently, we synthesized a highly conjugated phenanthroline ligand (**1**), 2,9-bis(3,5-dicarboxylphenylethynyl)phenanthroline, as a new water-soluble fluorescent probe.[9] As depicted in Figure 1, ligand **1** shares structural similarity with protoporphyrin IX; that is, both the molecules have a similar molecular size of ca. 1 nm square, an aromatic plane at the upper side, and carboxylate groups at the bottom side. It has well known that protoheme is tightly incorporated into the heme cavity through the ligation of the proximal histidine to the heme iron as well as through multiple non-covalent interactions between the protoheme and amino acid residues, which include hydrophobic interactions between the porphyrin plane and non-polar amino acid side chains as well as polar interactions between the propionate side chains and polar side chains, for example,

Keywords: Apomyoglobin; Heme-pocket; Phenanthroline; Reconstitution.
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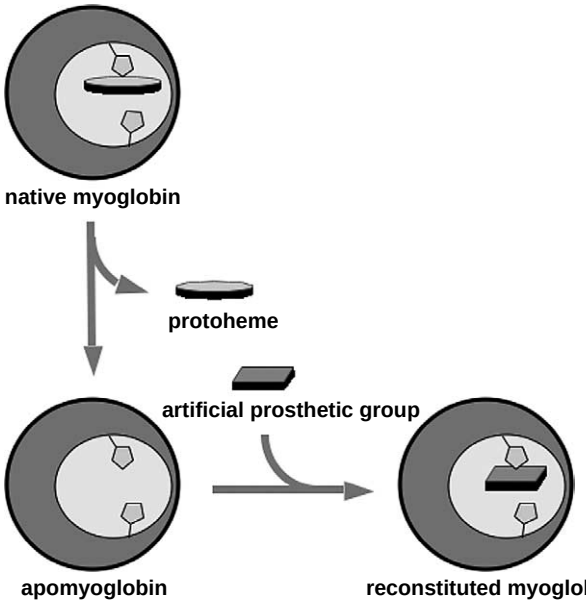
doi:10.1016/j.bmcl.2005.10.016



Scheme 1.

Ser92 and Arg45 in sperm whale myoglobin.[10] Therefore, the above-mentioned structural similarity between the phenanthroline **1** and protoheme suggests the possibility that ligand **1** binds into the heme cavity of apomyoglobin in the same fashion to protoheme. Here, we have investigated the above possibility using sperm whale apomyoglobin[11] as a host protein.

First, UV titration studies were carried out by addition of sperm whale apomyoglobin to a HEPES-buffered solution (pH 8.1) of **1**. The absorption spectral changes (Fig. 2A) exhibit two isosbestic points at 305 and 348 nm, and an increase at around 280 nm, which is caused by tryptophan residues of added apomyoglobin. The molar ratio graph at 317 and 370 nm is shown in the inset of Figure 2A. The absorption at 317 nm decreases and the absorption at 370 nm increases almost linearly until a ligand-to-apomyoglobin ratio of 1:1 is reached, and further addition of apomyoglobin did not affect the absorption spectrum, except for the absorption region increased by added apomyoglobin (250–300 nm). The spectral change between 250 and 400 nm was analyzed using a non-linear least-squares fit algorithm based on singular value decomposition to determine the binding constant to be log $K = 6.8 \pm 0.3$ (SPECFIT software[12]). The binding constant of **1** for apomyoglobin is rather smaller than that of the native prosthetic group hemin ($K_d \sim 10^{-12}$–10^{-15} M).[10a] The lower affinity of **1** may be caused by lacking the metal ion that can coordinate to the proximal histidine.

On the other hand, addition of holomyoglobin to **1** did not alter the spectrum of **1**. To confirm the incorporation of **1** in the heme-pocket, we prepared tetrazole-myoglobin, whose distal histidine residue is modified to bear a tetrazole ring according to the reported procedure,[13] and added its apoprotein to a solution of **1**. The resulting spectral changes were very much similar to those for the unmodified apomyoglobin (Fig. 3). However, the estimated binding constant shows a decrease by ca. 10-fold (log $K = 5.7 \pm 0.2$). Thus, these results clearly suggest that **1** is tightly incorporated into the heme cavity of apomyoglobin due to its structural homology with the native prosthetic group.

On the basis of the spectral analysis, spectrum for **1** inside heme-pocket can be calculated and is shown in Figure 2B. The spectrum shows red-shifted absorption bands compared with that for **1** in the absence of apomyoglobin.

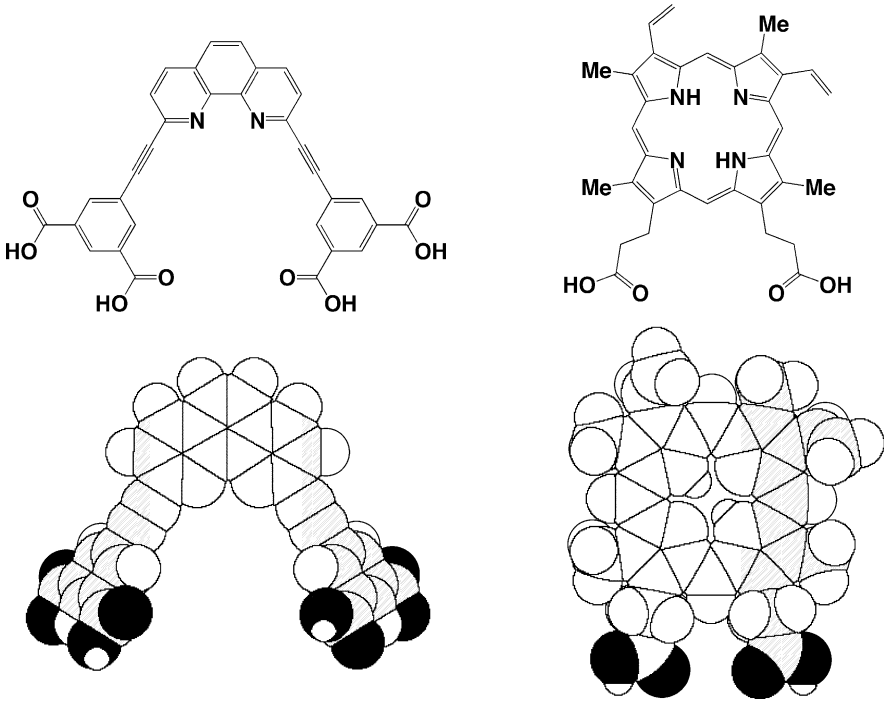


Figure 1. Structures and CPK models of **1** and protoporphyrin IX.

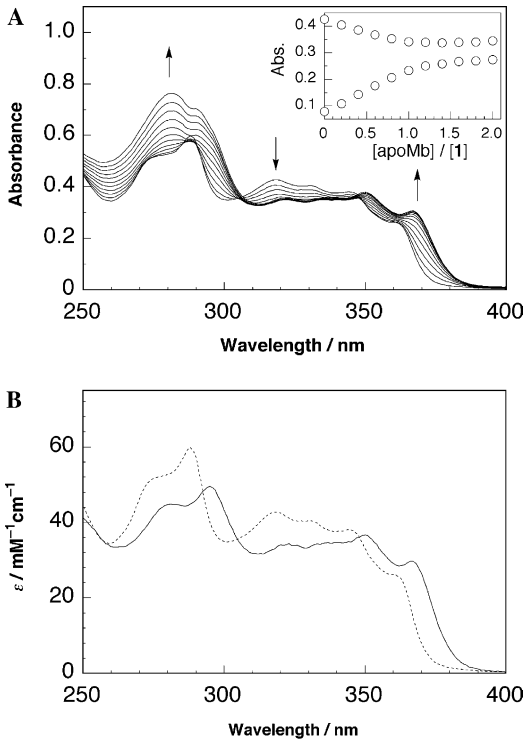


Figure 2. (A) Absorption spectral changes of **1** upon addition of apomyoglobin. Conditions: 10 mM HEPES buffer, pH 8.1, 25 °C. [**1**] = 10 μM. [apomyoglobin] = 0–20 μM. Inset: absorption as a function of added apomyoglobin monitored at 317 and 370 nm (molar ratio plot). (B) Calculated spectrum of **1** inside apomyoglobin (solid line), together with that of **1** (dotted line).

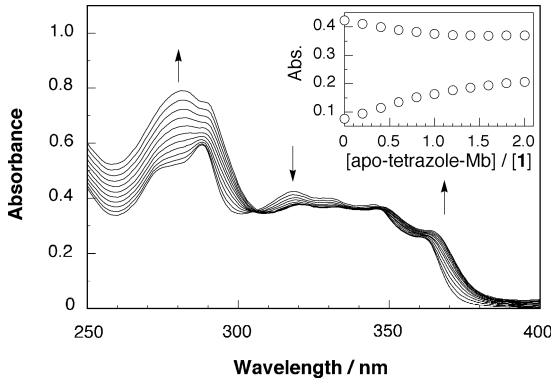


Figure 3. Absorption spectral changes of **1** upon addition of apo-tetrazole-myoglobin. Conditions: 10 mM HEPES buffer, pH 8.1, 25 °C. [**1**] = 10 μM. [apo-tetrazole-myoglobin] = 0–20 μM. Inset: absorption as a function of added apomyoglobin monitored at 317 and 370 nm (molar ratio plot).

Armaroli and co-workers reported the redshift of the absorption bands of 1,10-phenanthroline derivatives upon protonation of the phenanthroline nitrogens due to stabilization of the lowest $^1\pi\pi^*$ level.[14] Thus, it is suggested that nitrogen atoms of the phenanthroline moiety of **1** become protonated upon the incorporation into the heme-pocket. The heme-pocket environments surrounding **1** might affect the equilibrium between **1** and its protonated form. There are two histidine residues in the heme-pocket of myoglobin; that is, the proximal histidine that coordinates to the heme iron and the distal histidine in the vicinity of the heme iron. Taking into account the structural homology of **1** with protoheme, these histidine residues might be located close to nitrogen atoms of the phenanthroline moiety of **1**. Thus, the spectral change of **1** suggests a possible interaction between nitrogen atoms of the phenanthroline moiety of **1** and the proximal and/or distal histidine residue(s).

Circular dichroism (CD) spectra measurements provided another evidence to support the localization of **1** inside the heme-pocket. Figure 1 shows the CD spectra of apomyoglobin in the absence and presence of **1**. A very small amount of alpha-helix induction was observed in the presence of **1** (Fig. 4A). This result shows that **1** does not work as a denaturing reagent but does stabilize apomyoglobin to a small extent, which might correlate to the smaller binding affinity of **1** with apomyoglobin compared to that of the native prosthetic group, hemin. More interestingly, the **1**–apomyoglobin complex exhibited a positive induced CD signal in the absorption region of **1** (Fig. 4B), which clearly supports the location of **1** in chiral environments. Thus, the CD experiments clearly indicate the localization of **1** inside the heme-pocket of apomyoglobin.

In 1987, Zemel reported the hydrolytic activity of apomyoglobin.[15] The reaction proceeds more efficiently as the substrate becomes more hydrophobic; that is, *p*-nitrophenyl acetate < *p*-nitrophenyl hexanoate (PNH) < *p*-nitrophenyl nonate. Thus, the hydrolytic reaction is promoted by histidine residues in the hydrophobic heme-pocket, which is also supported by no hydrolytic activity of holomyoglobin. Therefore, it is expected that the incorporation of **1** in the heme-pocket would effect this hydrolytic reaction. In this study, we examined the catalytic hydrolytic activity of sperm whale apomyoglobin by using PNH as a hydrophobic substrate in the presence and absence of **1** in 20 mM HEPES buffer

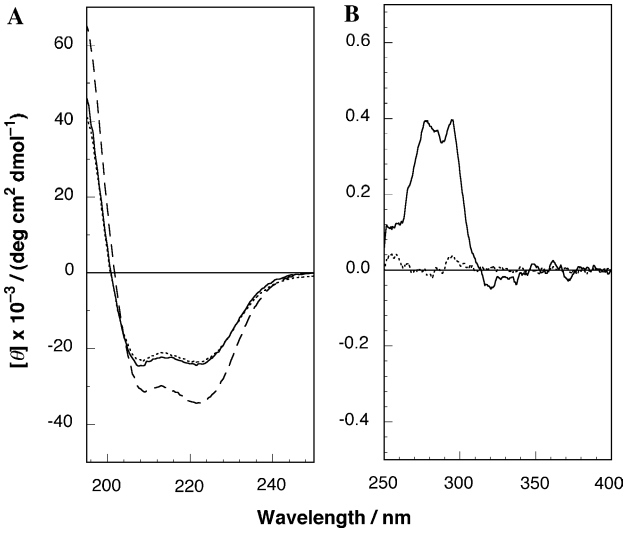


Figure 4. CD spectra of apomyoglobin in the absence and presence of **1**. Conditions: 20 mM phosphate buffer, pH 6.9, 10 °C. [apomyoglobin] = 10 μM, [**1**] = 11 μM (solid line); [apomyoglobin] = 10 μM (dotted line); [native myoglobin] = 10 μM (dashed line).

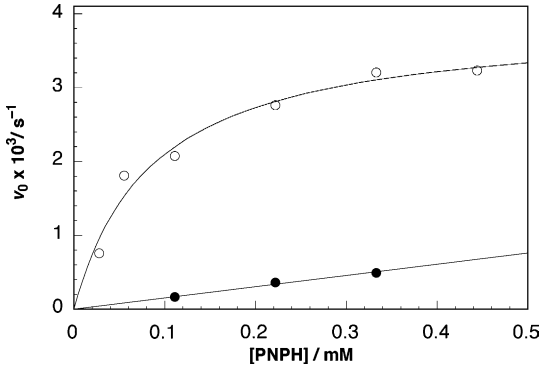


Figure 5. Initial rate of the PNH hydrolysis as the PNH concentration. Conditions: 10 mM HEPES buffer, pH 8.1, 10 °C. [apomyoglobin] = 10 μM without (filled circle) **1** and with **1** (11 μM) (open circle).

(pH 8.1, $I = 0.014$) containing 2% CH_3CN at 10 °C. Figure 5 shows a plot of the initial rate constant as a function of the PNH concentration, which shows saturation behavior in the absence of **1**, where the intrinsic hydrolysis rate of PNH ($k = 1.0 \times 10^{-6}$ s^{-1}) is subtracted from the observed reaction rate constants following the reported procedure.[16] The Michaelis–Menten parameters were estimated as follows: $K_m = 0.086 \pm 0.017$ mM and $k_{cat} = (3.9 \pm 0.2) \times 10^{-3}$ s^{-1}. These parameters are in agreement with those reported by Zemel; $K_m = 0.074$ mM and $k_{cat} = 48 \times 10^{-3}$ s^{-1} (pH 8.0, 25 °C).[15] In the presence of **1**, on the other hand, the initial reaction rate does not show saturation behaviors but increases almost linearly with substrate concentrations, which leads to the second-order rate constant of $k_2 = 1.5 \pm 0.1$ M^{-1} s^{-1}. Thus, the incorporation of **1** inside the heme cavity inhibits the hydrolysis of PNH by the histidines in the heme-pocket.

The result presented here shows that the water-soluble highly conjugated phenanthroline ligand **1** is readily incorporated into the hydrophobic heme-pocket of sperm whale apomyoglobin probably due to the structural similarity to protoheme. The incorporation of **1** into the heme-pocket inhibits the hydrolytic activity of apomyoglobin toward activated esters. Thus, the present study demonstrates that even non-porphyrinic molecules can be smoothly inserted into the heme cavity of apomyoglobin through proper molecular design. The resulting **1**–apomyoglobin complex could provide a novel metal-binding site in the heme cavity. The construction and characterization of artificial metalloproteins based on the **1**–apomyoglobin complex are currently undergoing in our laboratory.

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9. Characterization of **1**: ^{1}H NMR (DMSO, 400 MHz): δ 8.61 (d, $J = 8.4$ Hz, 2H), 8.52 (t, $J = 1.8$ Hz, 2H), 8.45 (d, $J = 1.2$ Hz, 4H), 8.14 (d, $J = 7.6$ Hz, 2H), 8.08 (s, 2H), ^{13}C NMR (DMSO, 100 MHz): δ 165.6, 144.9, 141.8, 137.0, 136.0, 132.1, 130.5, 128.2, 127.3, 127.1, 122.4, 90.9, 87.6; FAB MS (DEA/DMSO): *m/z* 556 [M$^-$]; HRMS (FAB, DEA/DMSO, [M$^-$−H]) Calcd for $C_{32}H_{15}O_8N_2$ 555.0828. Found 555.0840.
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