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A simple method to engineer a protein-derived redox cofactor for catalysis

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ABSTRACT

The 6×-Histidine tag which is commonly used for purification of recombinant proteins was converted to a catalytic redox-active center by incorporation of Co^{2+}. Two examples of the biological activity of this engineered protein-derived cofactor are presented. After inactivation of the natural diheme cofactor of MauG, it was shown that the Co^{2+}-loaded 6×His-tag could substitute for the hemes in the H_2O_2-driven catalysis of tryptophan tryptophylquinone biosynthesis. To further demonstrate that the Co^{2+}-loaded 6×His-tag could mediate long range electron transfer, it was shown that addition of H_2O_2 to the Co^{2+}-loaded 6×His-tagged Cu^{1+} amicyanin oxidizes the copper site which is 20 Å away. These results provide proof of principle for this simple method by which to introduce a catalytic redox-active site into proteins for potential applications in research and biotechnology.

1. Introduction

This paper describes a simple procedure for the introduction of a highly-potential oxidizing species into a specific location on a protein using standard molecular biology techniques. This is done by adding cobalt to a polyhistidine tag that is commonly used for protein purification. We show that this engineered cofactor is competent in both oxidative catalysis and electron transfer. Two examples that demonstrate the biological activity of this engineered protein-derived cofactor are presented. It was shown that the Co^{2+}-loaded 6×His-tag could substitute for the natural diheme cofactor of MauG in H_2O_2-driven tryptophan tryptophylquinone (TTQ) biosynthesis after inactivation of the native hemes. It was also shown that the Co^{2+}-loaded 6×His-tag could mediate long range electron transfer when attached to the cupredoxin amicyanin.

MauG is a *c*-type diheme enzyme [1] which catalyzes posttranslational modifications of methylamine dehydrogenase (MADH) [2] that complete the formation of the protein-derived TTQ [3] cofactor (Fig. 1). This catalytic reaction requires a 6-electron oxidation of two specific tryptophan residues of the protein substrate [4]. These oxidation reactions proceed via a bis-Fe^{IV} redox form of the two hemes [5] of MauG in which one heme is Fe^{IV}=O with a His axial ligand and the other is Fe^{IV} with axial heme ligands provided by His and Tyr side chains [6]. Catalysis requires long range electron transfer from the preTTQ substrate to the Fe^{IV} hemes of MauG (Fig. 1) which was shown to occur via a hole hopping mechanism [7,8] of electron transfer in which Trp residues of MauG are reversibly oxidized [9,10]. A tightly bound Ca^{2+} is present in MauG which is positioned in the vicinity of the two hemes and which is connected to each heme via H-bonding networks that include bound waters [6]. Removal of this Ca^{2+} by chelators yields a Ca^{2+}-depleted MauG which has no TTQ biosynthesis activity [11]. This is due to changes in the diheme site which prevent formation of the bis-Fe^{IV} state [12]. Whereas addition of H_2O_2 to native MauG generates the bis-Fe^{IV} state, the hemes of Ca^{2+}-depleted MauG are not reactive towards H_2O_2. The recombinant MauG used in these previous studies possesses a 6×His-tag at the C-terminal end of the protein which was added to facilitate purification. It is located ~35 Å from the preTTQ site of preMADH and 27 Å from Trp199 which mediates the hole hopping that is required for catalysis [9] (Fig. 1). This study investigated the possibility that Co^{2+} could be incorporated into the 6×His-tag and converted by H_2O_2 to a potent oxidant that could substitute for the inactive hemes in Ca^{2+}-depleted MauG in catalyzing TTQ-biosynthesis. The results describe the functionality of the Co^{2+}-loaded 6×His-tag in the catalytic reaction of MauG-dependent TTQ biosynthesis.

For additional proof of principle of this approach, the ability of a Co^{2+}-loaded 6×His-tag to participate in another long range electron transfer reaction was also demonstrated. This study used a type I copper

Abbreviations: 6×His-tag, 6x-Histidine; TTQ, tryptophan tryptophylquinone; MADH, methylamine dehydrogenase; preMADH, the biosynthetic precursor protein of MADH with incompletely synthesized TTQ; ET, electron transfer; *bis*-Fe^{IV} MauG, redox state of MauG with one heme as Fe^{IV}=O and the other as Fe^{IV}

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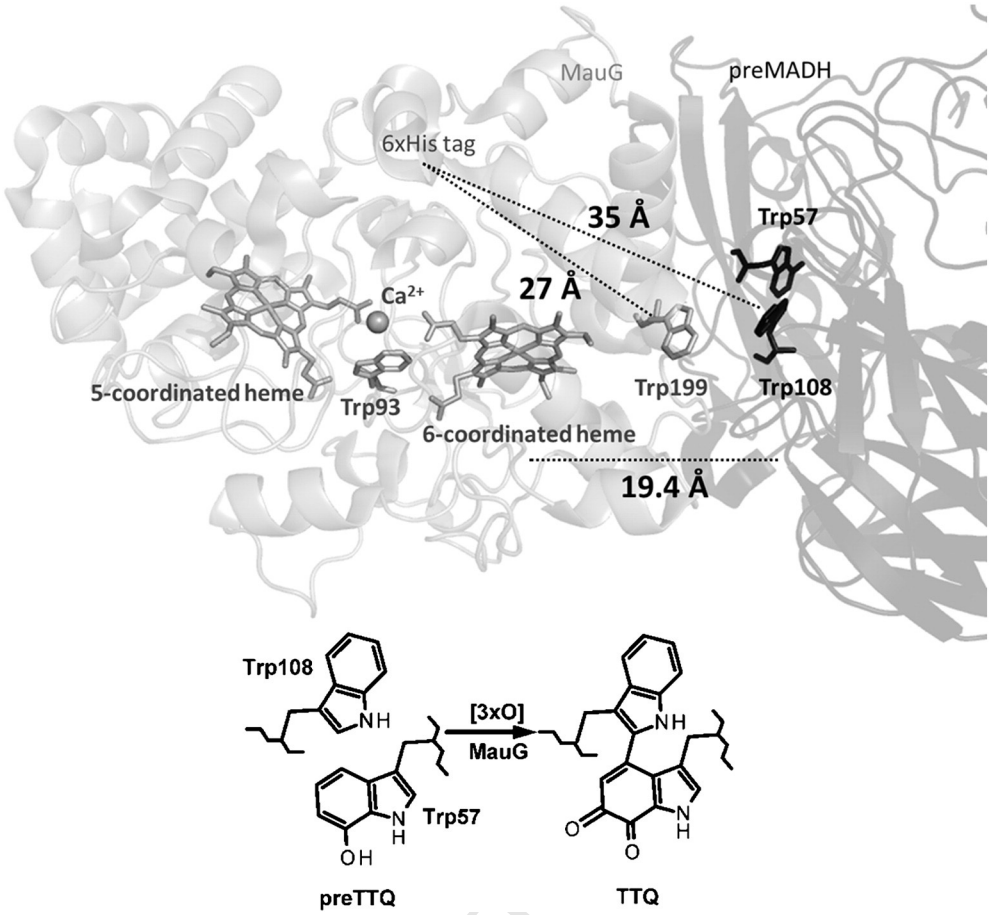

Fig. 1. The MauG-preMADH complex and the reaction that it catalyzes. The structure of the MauG-preMADH complex from *P. denitrificans* (PDB ID: 3L4M) is displayed with the hemes, Ca^{2+} and residues of interest indicated. The distances from the 6×His-tag site at the C-terminus of MauG to the preTTQ site, and from the nearest heme of MauG to the preTTQ site are indicated. The reaction catalyzed by MauG is shown below the structure. [O] represents oxidizing equivalents which may be provided by H_2O_2.

protein, amicyanin from *Paracoccus denitrificans* [13,14] with a 6×His-tag added to the N-terminus of the protein. Type 1 copper sites are found in a wide range of redox proteins in bacteria, plants and animals, and function as electron transfer mediators [15,16]. In the type 1 site a single copper is coordinated by three equatorial ligands that are provided by a Cys and two His residues, and by a fourth weak axial ligand, usually provided by a Met and they are characterized by an intense blue color and absorption centered near 600 nm that result from a $S(Cys)\pi \rightarrow Cu(II)d_{x2-y2}$ ligand-to-metal charge transfer transition [17]. It was shown that the 6×His-tag-bound Co^{2+} can be oxidized by H_2O_2 and subsequently oxidize the Cu^{1+} of reduced amicyanin via intraprotein electron transfer over a distance of over 20 Å. This system was also used to characterize some of the properties of the Co^{2+}-loaded 6×His-tag site. These studies illustrate the utility of a relatively simple and inexpensive method for the introduction of a potent oxidizing species into a specific site on a protein for potential use as a catalyst or electron transfer mediator.

2. Materials and methods

2.1. Protein expression and preparation

Recombinant MauG is produced in a homologous expression system using *P. denitrificans* [1]. The *mauG* gene was fused with the promoter region of the *cycA* (cytochrome *c*-550) gene [18] of *P. denitrificans* that was cloned into the pBluescript II KS(+) vector. A 6×His-tag was inserted by site-directed mutagenesis at the C-terminal of *mauG*. The cycA promoter-*mauG*-6×His segment was excised and ligated into pRK415-1, a broad-host-range vector. This plasmid was introduced into *P. denitrificans* by conjugation with the mobilizing *Escherichia coli* strain S17-1. As the N-terminal signal sequence of *mauG* was retained, the 6×His tagged MauG protein was isolated directly from the periplasmic fraction using Ni-NTA Superflow resin. It was eluted from the Ni-NTA resin in 70 mM imidazole. Ca^{2+}-depleted MauG was prepared by incubation of native MauG with 0.01 M EDTA disodium salt [11]. Methods for the expression and purification of recombinant preMADH, the substrate for MauG, from a *Rhodobacter sphaeroides* expression system were as described previously [19].

Amicyanin is encoded by the *mauC* gene of *P. denitrificans* [20]. The *mauC* gene was cloned into a pUC19 vector and a 6×His-tag was inserted by site-directed mutagenesis between the codon of the N-terminal amino acid and the native signal sequence of the gene which directs expression of the mature protein into the periplasmic space. This plasmid was introduced into *E. coli* strain BL-21(DE3) to express the 6×His-tagged amicyanin. The recombinant protein was purified from the periplasmic fraction of the harvested cells which was prepared by treatment with lysozyme followed by a mild osmotic shock [21]. This fraction was subjected to chromatography using a Ni-NTA Superflow resin and the 6×His-tagged amicyanin was eluted from the resin with 70 mM imidazole. MADH [22] and cytochrome *c*-551i [23] were purified from *P. denitrificans* as described previously.

2.2. Mechanistic studies

The steady-state spectrophotometric assay of MauG-dependent TTQ biosynthesis using preMADH as the substrate was performed using H_2O_2 as the source of oxidizing equivalents as was previously described [24]. The reaction was performed in a 0.05 M Tris–HCl buffer, pH 7.5.

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