Hemoprotein Engineering

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Direct *meso*-Alkynylation of Metalloporphyrins Through Gold Catalysis for Hemoprotein Engineering**

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Abstract: *A method was developed for the direct functionalization of metalloporphyrins at the methine protons (meso positions) to yield asymmetric alkynylated derivatives by using gold catalysis and hypervalent iodine reagents. This single-step procedure was applied to b-type heme and the product was incorporated into a gas-sensor heme protein. The terminal alkyne allows fluorophore labeling through copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC). Hemoproteins with this type of engineered cofactor have several potential applications in labeling and imaging technologies. Additionally, the alkyne provides a handle for modulating porphyrin electron density, which affects cofactor redox potential and ligand affinity. This method will be helpful for investigating the chemistry of natural heme proteins and for designing artificial variants with altered properties and reactivities.*

Heme proteins perform a remarkable array of functions, which encompass oxygen transport and storage (globins), cellular respiration and energy production (cytochromes), catalysis and oxidation chemistry (P450 enzymes), and signal transduction (gas-sensor proteins).[1] The key component underlying this broad chemical versatility is the common heme cofactor (iron protoporphyrin IX, FePPIX, *b*-type heme), one of the most abundant metalloporphyrins in nature. Accordingly, the replacement of the heme prosthetic group with artificial derivatives is a very successful strategy for engineering altered or enhanced chemical functionality in hemoproteins.[2]

Progress in this area of research depends not only on tailored cofactor surrogates compatible with proper protein binding but also on the synthetic accessibility of these molecules. Heme proteins recognize and bind their cofactor through apolar contacts, metal coordination, and ionic interactions with the carboxylic acid moieties (propionates). Some heme proteins also form covalent thioether bonds between the porphyrin vinyl side chains and cysteine residues.[3] Given that these components of the heme topology, as well as steric considerations, dictate protein compatibility,[2,3] we considered the methine protons (*meso* positions) to be promising sites for functionalization.[4] Furthermore, we reasoned that an acetylenic moiety at the *meso* position of FePPIX would serve as a handle to modulate the electron density of the porphyrin core. A terminal alkyne would also provide an attachment site for chemical tags or markers for chemical biology experiments through copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC, click chemistry)[5] or the Sonogashira reaction.[6]

To explore this variant of cofactor labeling, we chose the well-studied heme nitric oxide and/or oxygen (H-NOX) gas-sensor protein from the thermophilic bacterium *Thermoanaerobacter tengcongensis* (*Tt*) as a model.[7] Several features make this member of the H-NOX protein family an ideal candidate for this purpose. The replacement of the natural heme cofactor has been facilitated by a robust, expression-based method.[8] Importantly, the crystal structure of native *Tt* H-NOX reveals a solvent accessible opening at the 20-*meso* position of FePPIX that could accommodate an alkyne functional group (Figure 1 a).[9] The result of these considerations was the design of target molecule **1** (Figure 1 b). However, the synthesis of *meso*-alkynyl FePPIX **1** has not been demonstrated and synthetic approaches based upon existing reports require multiple steps.[10] Consequently, we considered unconventional alternatives for direct alkynylation and ultimately chose gold-catalyzed C–C coupling methods.

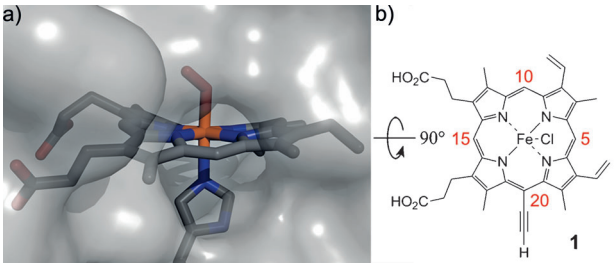

Figure 1. a) The *Tt* H-NOX protein crystal structure (Fe^{II}–O_2 complex), which highlights a natural opening that exposes the 20-*meso* position of the heme cofactor.[9] b) Bioinspired target molecule **1**, which is based on the iron protoporphyrin IX parent structure modified at one *meso* position with a terminal alkyne.[11]

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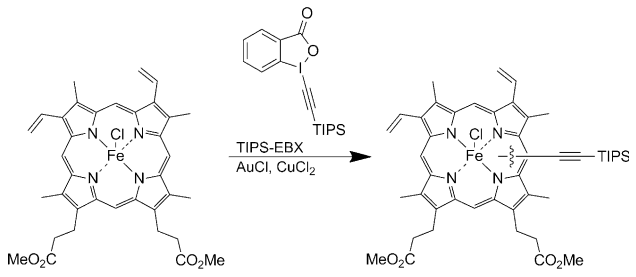
Gold has emerged as a remarkably potent catalyst for numerous reactions in organic synthesis, foremost in C–C cross-coupling and C–H functionalization.[12] Among the notable attributes of gold(I) are a strong π acidity and the ability to coordinate and activate triple bonds. For the installation of an alkyne, the traditional approach is its reaction as a nucleophile (e.g., the Sonogashira reaction), through which acetylides are readily accessible through intermediate deprotonation. The reverse approach with the alkyne acting as an electrophile, however, requires an inversion of reactivity. This umpolung is far less developed for acetylenes.[13] Waser and co-workers used hypervalent iodine reagents to preactivate silane-protected acetylenes for electrophilic aromatic substitutions mediated by gold(I) chloride, first on pyrrole and indole,[14] and then on thiophene and furane heterocycles.[15] We have adapted this acetylene transfer reaction for the derivatization of metalloporphyrins at the *meso* position. Notably, this approach does not require porphyrin metalation/demetalation or other intermediates. With this strategy, FePPIX was *meso*-alkynylated and the product was successfully incorporated into the *Tt* H-NOX protein.

FePPIX was converted into the dimethylester **2** to facilitate solubility in organic solvents. For the conversion of **2** into the alkynylated derivative **3**, 1-[(triisopropylsilyl)-ethynyl]-1,2-benz-iodoxol-3(1*H*)-one (TIPS-EBX) was used as the acetylene transfer reagent in combination with catalytic amounts of AuCl (5 mol%) and several reaction conditions were surveyed to maximize product formation (Table 1).[16] The initial low reaction yields were ascribed to substantial decomposition of the starting material, a common obstacle in synthetic reactions with this porphyrin.[17] We speculated that the presence of colloidal Au^0, which forms through the disproportionation of Au^I into Au^{III} and Au^0, may be the source of the observed degradation. Aside from ensuring anhydrous conditions, the strategy of Graf et al. was adopted, which involves the addition of $CuCl_2$ to reoxidize colloidal Au^0 to Au^I.[18] Eventually, the use of 1 mol% Cu^{II} and 5 mol% Au^I culminated in a 64% yield of isolated alkynylated porphyrin **3**. Subsequent removal of the TIPS group led to the corresponding terminal alkyne (C≡C–H) **4**, which after saponification gave the target compound **1**. The identities of the dimethylesters were confirmed by high-resolution mass spectrometry and NMR analysis.[16] The applicability of the gold-catalyzed reaction was also tested with zinc protoporphyrin IX. HPLC analysis confirmed a 55% yield of the alkyne product after a slight modification of the reaction conditions (Figure S1 in the Supporting Information).[16]

Table 1: Reaction optimization for the direct *meso*-alkynylation of iron protoporphyrin IX dimethylester **2**.



2 **3**

Entry	AuCl [mol%]	$CuCl_2$ [mol%]	Conditions[a]	Yield[b] [%]
1	5	–	14 h, RT[c]	traces
2	5	–	14 h, reflux[c]	<10
3	5	–	3 h, 80°C[d]	15
4	5	–	3 h, 80°C[d,e]	<10
5	5	0.5	14 h, 80°C[d]	27
6	5	1	3 h, 80°C[d]	64
7	5	10	3 h, 80°C[d]	<10

[a] **2** (147 μmol), TIPS-EBX (177 μmol; 1.2 equiv), and trifluoroacetic acid (100 equiv) in THF (30 mM porphyrin); [b] Yields of isolated product after chromatography; [c] Conventional heating; [d] Microwave irradiation; [e] 2.0 equiv pyridine instead of TFA.

Next, we determined the *meso* regioselectivity of the gold-catalyzed reaction. FePPIX contains four different methine protons and thus a mixture of alkynylated regioisomers can be expected. HPLC chromatography and mass spectrometry led to the identification of four regioisomers for **4** and the isomer ratio was 1:2:4:3 (Figure S2).[16] NMR analysis of these porphyrins was impeded by strong aggregation as a result of π stacking and solubility issues. Furthermore, Fe^{III} is paramagnetic whereas the diamagnetic Fe^{II} porphyrin rapidly oxidizes with trace amounts of oxygen. These problems were overcome by reducing the Fe^{III} with $SnCl_2$ in the absence of oxygen at dilute concentrations.[19] Rotating-frame nuclear Overhauser effect spectroscopy (ROESY) allowed full assignment of the protons in all four regioisomers of **4** (Figures S3, S4, Table S1).[16] This analysis showed that FePPIX was mainly alkynylated at the 5-*meso* and 10-*meso* positions (together 70% of the total product mixture). The remaining 30% of the product mixture corresponds to alkynylation at the 15-*meso* and 20-*meso* positions. The measured *meso* regioselectively can be attributed to the electron-polarizing effect of the vinyl substituents in combination with steric effects. Indeed, experimental data from a study for two heme-related porphyrins indicate that the substituents have a marked effect on the *meso*-carbon reactivities in electrophilic aromatic substitution reactions.[20] With regard to protein incorporation, it was reasoned that the protein scaffold itself enforces the selection of only structurally compatible *meso* alkynes from this mixture.[11]

The incorporation of **3** into the *Tt* H-NOX protein was carried out during protein expression. Past approaches to cofactor replacement have relied on harsh methods and partial denaturation of the protein scaffold. By contrast, the method used here exploits two characteristics of the *E. coli* RP523 strain, namely porphyrin-permeability of the cell membrane and disrupted heme biosynthesis, to achieve exclusive utilization of the exogenously supplied unnatural porphyrin.[8] The triisopropylsilyl-protected heme was incorporated first to test the limits of steric bulk compatible with membrane permeability and protein incorporation. The protein was expressed in the presence of mixture **3** and purified by using standard procedures (Figure S5).[16] The identity and structural integrity of the metalloporphyrin-incorporated protein was validated by nondenaturing protein mass spectrometry[16] and UV/Vis spectroscopy (Figure 2).

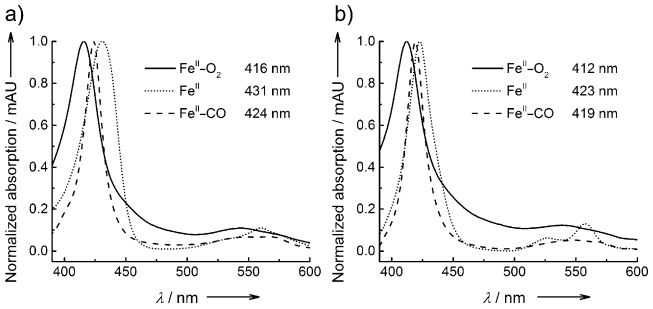


Figure 2. Normalized absorption spectra of *Tt* H-NOX protein. The spectra for unligated and gas-ligand complexes are shown with Soret maxima indicated. The protein was coexpressed with natural heme (a) or the alkynylated FePPIX analogue (b).

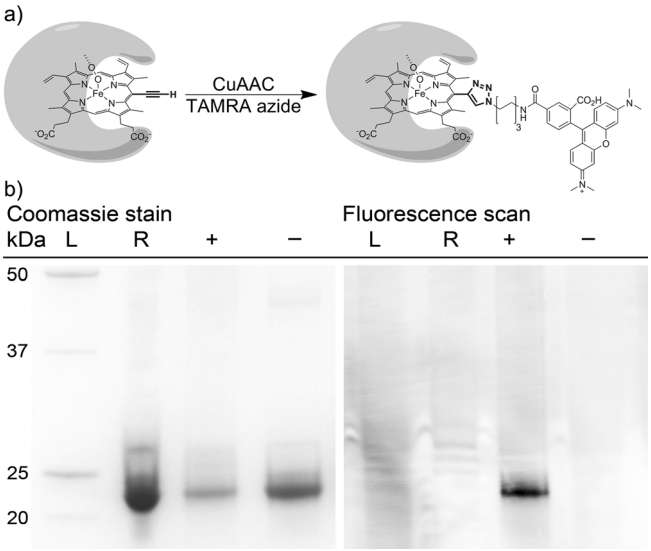


Figure 3. a) Site-specific CuAAC labeling of the *Tt* H-NOX protein bearing an alkynylated heme with a fluorophore (TAMRA azide). b) SDS-PAGE gel stained with Coomassie for protein detection (left) and scanned for fluorescence (right). The lanes correspond to molecular weight ladder (L), unlabeled *Tt* H-NOX protein (R), TAMRA-labeled protein (+), and a negative control with native *Tt* H-NOX that lacks the alkyne subjected to the same labeling reaction (−).

The presence of protein-bound porphyrin was confirmed by the Soret band, which shifted to 412 nm with **3** (compared to 416 nm for the native heme-containing protein). This wavelength is consistent with Fe^{II} oxygen-bound *Tt* H-NOX. The O_2 ligand was then removed by reduction with sodium dithionite under nitrogen atmosphere, which led to a Soret maximum at 423 nm (native *Tt* H-NOX 431 nm). Upon the introduction of CO into the headspace of the cuvette, the Soret band shifted to 419 nm (native *Tt* H-NOX 424 nm). Taken together, the spectra indicate that the silylalkyne attachment to the heme causes a hypsochromic shift of the Soret maxima in all *Tt* H-NOX ligation states. In addition, the gas-ligand binding capability confirms the functional integrity and proper incorporation of the heme derivative into the protein.

To determine whether a hemoprotein with an alkynylated heme cofactor can be further derivatized, the *Tt* H-NOX was subjected to copper(I)-catalyzed labeling with a fluorophore (Figure 3a). Compound **4** was saponified and the structurally compatible alkynes in this mixture were incorporated into the *Tt* H-NOX protein by using the same expression-based procedure. The resulting hemoprotein was successfully labeled through CuAAC with the azide-linked fluorophore 5-carboxamido-(6-azidohexanyl) tetramethylrhodamine (TAMRA azide) and the water soluble ligand tris(3-hydroxy-propyltriazolylmethyl)amine (THPTA).[16,21] Verification of the reaction was performed by SDS-PAGE with fluorescence scanning to detect the labeled heme and Coomassie staining for protein detection (Figure 3b). The Cu^{I} binding sites of the protein (the His_6 tag) were saturated by supplementing the reaction mixture with $ZnSO_4$.[21] Notably, the denaturing conditions during electrophoresis did not compromise the binding of the heme–fluorophore conjugate to the protein scaffold. Nonspecific binding of the fluorophore to the protein was excluded by performing the labeling reaction with *Tt* H-NOX protein without a terminal alkyne (Figure 3b, negative control). Overall the hemoprotein was site-specifically labeled by the CuAAC reaction.

In conclusion, gold-catalyzed C–H activation proved effective for the *meso*-alkynylation of a biologically relevant metalloporphyrin in a single synthesis step. Furthermore, the bioorthogonal product is amenable to incorporation into a gas-sensing heme protein. Since the mixture of alkyne regioisomers meets basic heme topology requirements for protein incorporation, we anticipate applicability to other heme proteins as well. Apart from the general usefulness of alkynylated porphyrins,[10] there are several potential applications for hemoproteins with engineered cofactors. First, the alkyne provides a handle for modulating porphyrin electron density, which affects cofactor redox potential and ligand affinity. This will be helpful for investigating the chemistry of natural heme proteins and for designing artificial variants with altered properties and reactivities.[2,22] Second, the selective incorporation of alkynes into biomolecules has become a prerequisite for state-of-the-art labeling and imaging technologies in biological settings.[5,6,23] We provided a representative example by engineering a functional gas-sensor hemoprotein that can be labeled with a fluorophore. Labeling of the protein cofactor instead of the protein scaffold adds another level of flexibility in chemical biology. Third, the alkyne tag facilitates the introduction of new spectroscopic signals and has recently been used as a Raman probe to visualize small molecules in cellular imaging.[24] We are currently pursuing the implementation of our semi-synthetic protein in the aforementioned applications.

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