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ARTICLE

Cyclotrimerization of phenylacetylene catalyzed by a cobalt half-sandwich complex embedded in an engineered variant of transmembrane protein FhuA

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An (η^5-cyclopentadienyl)cobalt(I) complex was covalently incorporated in an engineered variant of the transmembrane protein *Ferric hydroxamate uptake protein component: A,* FhuA ΔCVF^{tev}, using the thiol-ene reaction. CD spectrum shows the structural integrity of the biohybrid catalyst. MALDI-TOF of the segment containing the anchoring site for the cobalt complex Cys545 confirmed sucessful conjugation. This biohybrid catalyst catalyzed the cyclotrimerization of phenylacetylene to give a mixture of regiosomeric 1,2,4- and 1,3,5-triphenylbenzene in aqueous medium.

Introduction

Artificial metalloproteins consisting of a synthetic metal catalyst embedded in a protein scaffold expand the reaction scope of natural metalloenzymes.[1-6] Whitesides *et al.*[7, 8] introduced an achiral biotin-modified rhodium bis(phosphine) complex in the protein avidin and performed asymmetric hydrogenation of α-acetamidoacrylic acid to obtain the product with 44% *ee*. Later, Ward *et al.* improved the selectivity by protein engineering[2] and used this biotin-(strept)avidin method to generate a variety of artificial metalloproteins.[3, 8-12]
Watanabe *et al.* incorporated a rhodium complex in the spherical ferritin cage and utilized its discrete reaction space to polymerize phenylacetylene with narrow molecular weight distributions.[13] Hayashi *et al.* prepared an artificial metallo-protein consisting of a variant of the β-barrel protein nitrobindin (10 *β*-strands) and a rhodium half-sandwich complex **1** (Figure 1) that is covalently anchored *via* the maleimide function at the cyclopentadienyl (Cp) ligand. Exchanging amino acids in this metalloprotein by site-directed mutagenesis the stereoselectivity during phenylacetlyene polymerization was shifted from 93% *cis* to 82% *trans* content.[8, 9] By duplicating two *β*-strands the cavity size in nitrobindin was engineered to generate sufficient cavity space for the incorporation of bulky metal catalysts.[14] A cell-surface display whole-cell biohybrid system based on nitrobindin polymerized phenylacetylene with up to 80% *trans* content.[15]

The metal-catalyzed cyclotrimerization of acetylene derivatives produces benzene derivatives as two regioisomers. No trimerization products were observed using the metalloproteins mentioned above. This might be due to high temperatures required for rhodium catalysts to catalyze the cyclotrimerization. Catalysts based on cobalt promise to perform this reaction under milder conditions.[16, 17] Low-valent (η^5-cyclopentadienyl)cobalt(I) catalysts for the cyclotrimerization of terminal alkynes in aqueous media include dicarbonyl(η^5-cyclopentadienyl)-cobalt(I) in supercritical water[18], in ethanol/water mixture (3:2),[19] and in methanol/water mixture (1:4) at room temperature.[17]
Here we report on the covalent anchoring of a modified (η^5-cyclopentadienyl)cobalt(I) complex bearing a maleimide

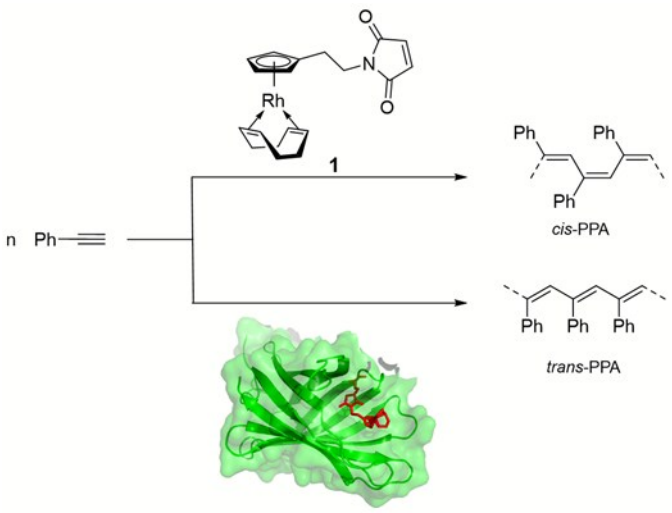

Figure 1: Polymerization of phenylacetylene using catalyst **1** to give *cis*-PPA and using nitrobindin-supported catalyst to give *trans*-PPA (PDB:3wjc).[9]

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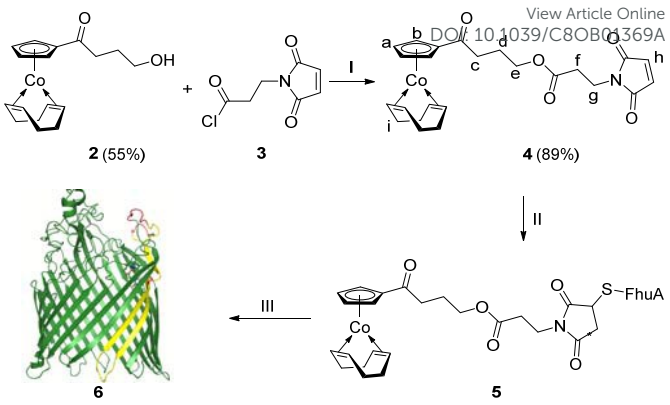
moiety in a variant of the membrane protein "*Ferric hydroxamate uptake protein component: A*" (FhuA) and its catalysis of the cyclotrimerization of phenylacetylene.

Results and discussion

The water-stable (η^5-cyclopentadienyl)cobalt(I) complex **2** was reported by Eaton *et al. to* catalyze the cyclotrimerization of terminal alkynes in aqueous media.[19] The maleimide moiety was attached *via* esterification of the alcohol residue in **2** and the acetyl chloride group of **3** in the presence of triethylamine (Scheme 1). Complex **4** was characterized by ^{1}H NMR, ^{13}C NMR and IR spectroscopy as well as elemental analysis (ESI, Figure S1 and S2). Comparing the NMR spectra of complex **2** and **4** revealed a downfield shift of the protons H_d and H_e and additional signals for the proton H_f, H_g and H_h (Scheme 1, ESI, Figure S1). IR spectroscopy showed additional CO vibrational bands at ν_{CO} = 1707 cm^{-1} (ESI, Figure S2).

Covalent conjugation of the maleimide group with the thiol group of cysteine 545 within FhuA proceeds by Michael-type addition reaction. Selective reaction of complex **4** under basic conditions was shown using protected *L*-cysteine as a model for the protein. The reaction was monitored by ^{1}H NMR spectroscopy following the olefinic signal of the maleimide group at δ = 6.79 ppm (Scheme 1; H_h).[20]

The *ferric hydroxamate uptake protein component: A* (FhuA), originally located in the outer membrane of *Escherichia coli* (*E. coli)*[21, 22] was engineered by removing the cork domain (amino acids Δ1-159).[23] A cysteine was introduced in position 545 (K545C) as single accessible cysteine for catalyst conjugation within the barrel structure. The surrounding of C545 was engineered to improve accessibility to the thiol and to avoid catalyst binding to coordinating residues (mutations N548V and E501F).[24] This FhuA variant has a molecular weight of ca. 63 kDa. To facilitate the characterization with mass spectrometry, two specific Tobacco Etch Virus (TEV)-protease cleavage sites were introduced within the loops 7 and 8 of FhuA. The digested protein fragment containing the metal catalyst has a molecular weight of approximately 6-7 kDa. The engineered variant of



Scheme 1: Synthesis of **4** and **6**. i) NEt_3, THF, 23 °C, 14 h; ii) SDS 1 (w/w)%, H_2O, 20 (v/v)% THF, 0.1 equiv. FhuA ΔCVF^{TEV}, 23 °C, 24 h; iii) NaP_i buffer (100 mM, pH = 8), PE-PEG (0.125 mM), EDTA (1 mM), H_2O, 23 °C, 3 d.

FhuA is named FhuA_Δ1-159_C545V548_F501_tev or in short FhuA ΔCVF^{tev}.

Conjugation of **4** to cysteine residue 545 (FhuA ΔCVF^{tev}) proceeded in an anaerobic environment by addition of **4** (10 equiv.) in degassed THF to FhuA ΔCVF^{tev} dissolved in degassed H_2O (containing 1(w/w)% sodium dodecyl sulfate, SDS). Refolding of the β-barrel structure was achieved by dialysis against a sodium phosphate buffer solution (NaP_i, 100 mM, pH = 8) containing either 2-methyl-2,4-pentanediol (MPD, 50 mM) or polyethylene-block-poly(ethylene glycol) (PE-PEG, 0.125 mM) as detergent (ESI, Scheme S2).

Coupling efficiency of up to 91% was determined with the fluorescence titration method of the cysteine using ThioGlo®.[20, 24-27] The structural integrity of the β-barrel protein host was confirmed by circular dichroism (CD) spectroscopy. CD spectra showed absorptions characteristic for β-barrel structure with a minimum at λ_{min} = 215 nm and a maximum at λ_{max} = 192 nm (Figure 2). Refolded metal-free protein and metalloprotein were compared displaying similar characteristics (ESI, Figure S3). Similar spectra were observed for different metalloproteins based on FhuA ΔCVF^{tev}.[20, 24-27]

Digestion of FhuA ΔCVF^{tev} and biohybrid catalyst **6** was performed with TEV-protease resulting in three fragments with

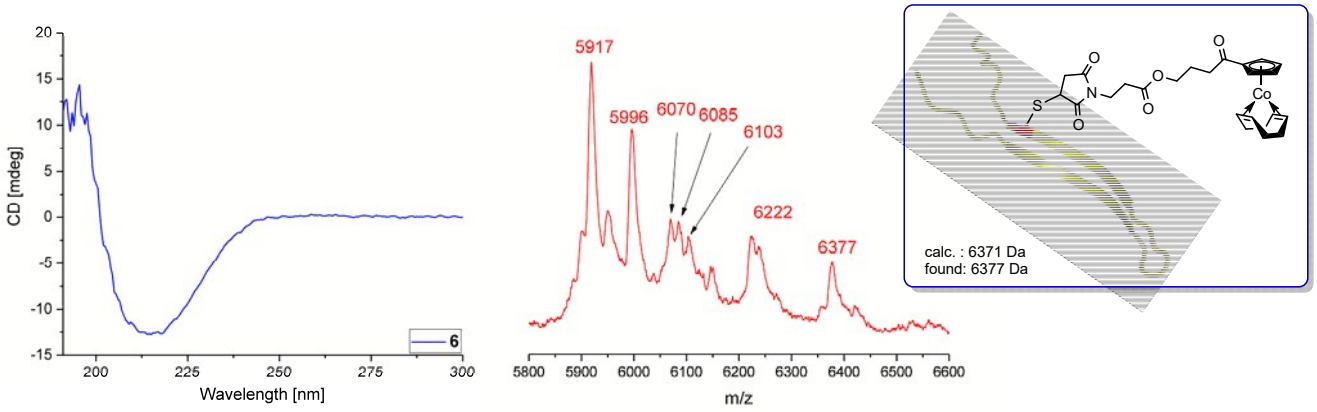


Figure 2: CD spectrum of metalloprotein **6** with MPD (left), MALDI-TOF mass spectrum of smallest fragment after digestion with TEV protease (right)).

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5.9, 17.3 and 40.3 kDa. The 5.9 kDa fragment contains the cysteine C545 and the metal catalyst was analyzed by Matrix-Assisted-Laser-Desorption/Ionisation Time-of-Flight mass spectroscopy (MALDI-TOF MS). The mass spectrum obtained shows the metal-free protein at m/z = 5917 Da (calc. 5902 Da) (ESI, Figure S4). Metalloprotein **6** was identified at m/z = 6377 Da (calc. 6371 Da) along with fragmentation of the metal catalyst (Figure 2; ESI Figure S5).

Catalytic activity of **4** in the cyclotrimerization was demonstrated with phenylacetylene. Due to insolubility of **4** in water, an organic co-solvents was necessary to achieve catalytic activity (Tabel 1, Entry 1). THF, ethanol, isopropanol and *tert*-butanol were probed (ESI, Table S1). Complex **4** turned out to be insoluble in *tert*-butanol and only moderately soluble in ethanol and isopropanol (ESI, Table S1). THF showed good solubility and thus the highest conversion (39%).

Increasing catalyst loadings (1.0, 2.5, 5.0, 7.5 mol%) resulted in increase in phenylacetylene conversion to give the triphenylbenzene regioisomers (ESI, Table S2). The variation of pH from 6 to 8) did not influence selectivity or activity (Table 1, Entry 9 and 11). Temperature, solvents, pH-value and additives were varied without complex **4** present. In these cases, no conversion of phenylacetylene was observed (Table 1, Entry 8, 10, 12-14). These results indicate that **4** is necessary.

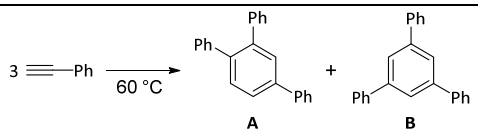
The cyclotrimerization of phenylacetylene in an aqueous solution (H_2O/THF, 9:1) catalyzed by **4** resulted in 18% conv. (24 h), 39% conv. (48 h), 51% conv. (72 h), 82% conv. (168 h) and >99% conv. (504 h) (Table 1, Entry 2-6). The regioselectivity was between 63% and 70% favoring the isomer 1,2,4-triphenylbenzene (Table 1, Entry 2-6). When sodium phosphate buffer (100 mM, pH = 8) was added to the reaction mixture, neither the conversion nor the regioselectivity was affected (Table 1, Entry 9 and 11).

FhuA ΔCVF^{tev} limits the reaction conditions such as temperature (up to 64 °C)[27] and the choice and amount of organic co-solvent.[27] 60 °C were selected as reaction temperature together with 10 (v/v)% THF as co-solvent. FhuA ΔCVF^{tev} tolerates up to 40 (v/v)% THF without denaturation.[27]

Generally utilizing the biohybrid conjugate **6**, no additional THF is necessary for the catalysis because the biohybrid catalyst is perfectly soluble in water (Table 1, Entry 16). Due to low water solubility of the substrate, the reaction is performed in an emulsion-like fashion as for the polymerization of phenylacetylene.[9] Addition of 10 (v/v)% co-solvent increased the solubility of phenylacetylene in the aqueous phase and the activity (Table 1, Entry 17). Using PE-PEG as detergent, the metalloprotein precipitated and only low conversions were observed (Table 1, Entry 18 and 19). In contrast, MPD as the detergent did not lead to precipitation of the metalloprotein during catalysis and **6** showed increased activity (TON = 130).

In all catalytic runs with **4** and **6** neither dimerization nor polymerization products of phenylacetylene were observed by ^{1}H NMR spectroscopy and GC MS. Using catalyst **1**, only formation of poly(phenylacetylene) was observed without any

Table 1: Cyclotrimerization reaction of phenylacetylene



A **B**

Entry[a]	Catalyst (mol%)	Solvent	pH	Buffer, Detergent	Time [h]	A/B[b]	Conv.[b] [%]	TON
1[c,e]	**4** (5)	H_2O	-	-	72	-	-	-
2[c]	**4** (5)	H_2O/THF	-	-	24	70/30	18	4
3[c]	**4** (5)	H_2O/THF	-	-	48	67/33	39	8
4[c]	**4** (5)	H_2O/THF	-	-	72	64/36	51	10
5[c]	**4** (5)	H_2O/THF	-	-	168	67/33	82	16
6[c]	**4** (5)	H_2O/THF	-	-	504	63/37	>99	20
7[f]	**4**	-	-	-	72	69/31	-	-
8[c]	-	H_2O/THF	6	NaP_i	72	-	-	-
9[c]	**4** (5)	H_2O/THF	6	NaP_i	72	63/37	45	9
10[c]	-	H_2O/THF	8	NaP_i	72	-	-	-
11[c]	**4** (5)	H_2O/THF	8	NaP_i	72	62/38	48	10
12[c]	-	H_2O	8	NaP_i, SDS	72	-	-	-
13[c]	-	H_2O	8	NaP_i, MPD	72	-	-	-
14[c]	-	H_2O	8	NaP_i, PE-PEG	72	-	-	-
15[d]	**6** (0.1)	H_2O	8	NaP_i, SDS	24	69/31	5	50
16[d]	**6** (0.1)	H_2O	8	NaP_i, MPD	24	70/30	5	52
17[d]	**6** (0.1)	H_2O/THF	8	NaP_i, MPD	24	69/31	13	130
18[d]	**6** (0.1)	H_2O	8	NaP_i, PE-PEG	24	-	<1	-
19[d]	**6** (0.1)	H_2O	8	NaP_i, PE-PEG	48	-	<1	-

a) V = 0,5 mL, 60 °C; b) determined by GC MS with a minimum of three runs, internal standard: mesitylene (1.0 mL, 15 mM in THF) selectivity = ±4%, conversion = ±4%; c) 31.5 mM phenylacetylene; d) 63 mM phenylacetylene; e) THF was removed before H_2O and phenylacetylene were added; f) neat conditions: THF was removed in vacuum before addition of phenylacetylene (70 µL), no conversion determined.

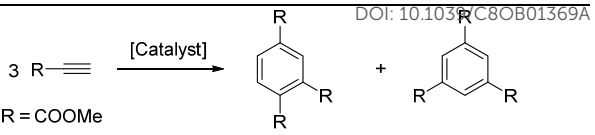
evidence for cyclotrimerization. Field *et al.* showed for a series of Co(I), Rh(I) and Ir(I) catalysts that phenylacetylene can be cyclotrimerized as well as dimerized.[28] Steric and electronic effects of the ligand system as well as the substrate influence the regioselectivity of the cyclotrimerization. If steric and electronic effects are minimized regioselectivity with the statistical ratio of approximately 3:1 favoring the 1,2,4-substituted benzene derivative will be observed. The cyclotrimerization of phenylacetylene showed similar regio-selectivities with approximately 2:1 favoring the 1,2,4-triphenylbenzene. Neither **4** nor **6** showed large effects on the regioselectivity. Catalyst **2** and the (η^5-cyclopentadienyl)-cobalt(I) catalyst used by Butenschön *et al.* in aqueous media show similar regioselectivities.[29] In contrast to catalyst **2** (ethanol/water mixture; 3:2)[19] and the (η^5-cyclopentadienyl) cobalt(I) catalyst (methanol/water mixture; 1:4) used by Butenschön *et al.*[29] we decreased the amount of organic co-solvent necessary by incorporating catalyst **4** in the protein scaffold to obtain water-soluble biohybrid catalyst **6**.

Several other terminal alkynes of varying water-solubility were tested in the cyclotrimerization under conditions shown in Table 1 (ESI, Table S4). Propargyl alcohol, *N,N*-dimethyl-2-propynylamine, *N,N,N*-trimethyl-2-propynyl ammonium iodide, propiolamide and 5-hexynenitile did not show any conversion with catalyst **4** or **6**.

Regarding the activity, catalyst **2** was not used for the cyclotrimerization of phenylacetylene.[19] With respect to phenylacetylene **2** and **4** did not show any difference in activity or selectivity. Butenschön *et al.* observed TON's up to 17 (5 mol%, 8 h, rt, TOF = 2.1 h^{-1}) for phenylacetylene.[17] Catalyst **4** (5 mol%, 24 h, 60 °C, TOF = 0.2 h^{-1}) showed low activity even at elevated temperatures. The metalloprotein **6** (0.1 mol%, 24 h, 60 °C, TOF = 5.4 h^{-1}) on the other hand were not active, but due to low catalyst concentrations activity increased in aqueous media with TON's up to 130.

Acetylenes with a carbonyl group in α-position to the C-C triple bond were converted with **4** as well. Methyl propiolate for example showed a regioselectivity of 68:32 favoring the 1,2,4-substituted benzene derivative (Table 2, Entry 1). Biohybrid catalyst **6** selectively converted methyl propiolate into the 1,3,5-substituted benzene derivative (>99%) (Table 2, Entry 3). Other acetylenes bearing a carbonyl group in the α-position such as ethyl propiolate or *tert*-butyl propiolate showed the same selectivity. Since FhuA without any metal co-factor showed similar activity and selectivity as did catalyst **6** (Table 2, Entry 2-3) it became apparent that the metal catalyst may not be involved in the cyclotrimerization of methyl propiolate. Previously, primary and secondary amines were reported to act as organo catalysts.[30-32] Acetylenes bearing a Michael-acceptor in α-position undergo triple Michael-type addition leading exclusively to the 1,3,5-regioisomer. The protein catalyzes these substrates and therefore the metal complex does not participate in the reaction. Experiments with *L*-lysine and *L*-histidine as c amino acids support this suggestion by converting methyl propiolate exclusively to the 1,3,5-substituted regioisomer (ESI, Table S3).

Table 2: Cyclotrimerization of methyl propiolate



Entry[a]	Catalyst	A/B[e]	Conv.[f] [%]
1[b]	**4** (1)	68/32	19
2[c]	FhuA (0.1)	1/99	40
3[d]	**6** (0.1)	1/99	39

a) V = 0.5 mL, 60 °C; b) a stock solution of **4** (0.1 M in THF); c) 22 mM methyl propiolate; d) 22 mM methyl propiolate; e) determined by GC MS with a minimum of three runs, int. std.: mesitylene (1.0 mL, 15 mM in THF) errors are standard deviation: selectivity = ±4%; f) determined by ^{1}H NMR; int. std. acetone (4 µL), error: conversion = ±5%;

Conclusion

A modified (η^5-cyclopentadienyl)cobalt(I) catalyst was anchored in the engineered transmembrane protein FhuA ΔCVF^{tev} with a cysteine anchoring site C545 and characterized by MALDI-TOF mass spectrometry and CD spectroscopy. The cyclotrimerization of phenylacetylene with the water-soluble biohybrid catalyst **6** gave a 2:1 mixture of 1,2,4- and 1,3,5-triphenylbenzenes. The regioselectivity appears to be not affected by the protein environment, as the formation of the intermediate cobaltacyclopentadiene ("cobaltole") seems to be not influenced by the bioconjugation.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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