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Short Self-assembling Peptides are Able to Bind to Copper and Activate Oxygen

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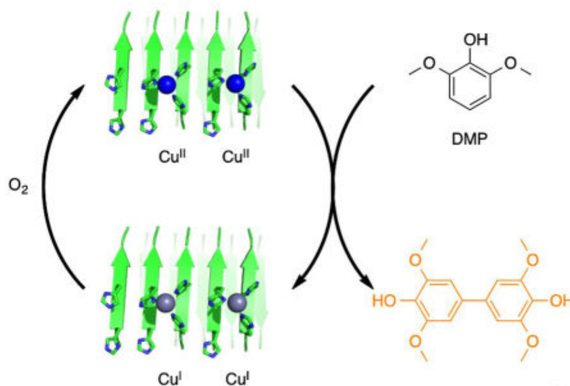
Abstract

We have shown that *de novo* designed peptides self-assemble in the presence of copper to create supramolecular assemblies capable of oxidation of dimethoxyphenol in the presence of dioxygen. Formation of the supramolecular assembly, akin to a protein fold, is critical for productive catalysis as the peptides possessing the same functional groups, but lacking the ability to self-assemble do not catalyze substrate oxidation. The ease with which we have discovered robust and productive oxygen activation catalysts suggests that these prion-like assemblies might have served as intermediates in evolution of enzymatic function and opens the path for development of new catalyst nanomaterials.

Graphical Abstract



De novo designed peptides self-assemble in the presence of copper to create supramolecular assemblies that catalyze oxidation of dimethoxyphenol by dioxygen.

Keywords

peptides; *de novo* design; catalysis; self-assembly; supramolecular chemistry

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Proteins extensively rely on metal cofactors to achieve structural stability and function. Indeed, it is estimated that up to a third of all proteins contain various metal ions.[1] Metalloenzymes are capable of efficiently tuning the properties of a metal ion to catalyze very difficult chemical transformations such as conversion of methane to methanol or oxidative depolymerization of lignin using simple oxidants such as dioxygen and hydrogen peroxide.[2] Recently we reported that short peptides designed from the first principles self-assemble in the presence of zinc to form amyloid-like fibrils, which efficiently catalyze hydrolysis of *p*-nitrophenyl esters.[3] The most catalytically active fibrils showed activity that rivals that of the natural enzymes by weight. The ease with which we were able to discover such activity – the original library included only 10 peptides – suggests that self-assembly of short peptides could have been a major pathway in enzyme evolution and opens the door to utilizing this approach to a large array of chemical reactions. In this paper we ask whether self-assembly of peptides can be used to discover supramolecular catalysts for oxygen activation.

Designing efficient catalysts for oxygen activation presents several challenges: 1. The ligand in the primary coordination sphere needs to properly tune the metal ion's redox potential; 2. The ligand has to be able to accommodate different oxidation states of the metal ion; 3. Catalysis should not involve formation of reactive oxygen species that can promote ligand decomposition. Thus it is hardly surprising that only a handful of successful protein/peptide designs capable of promoting redox reactions have been reported so far.[4]

The very simple coordination sphere produced by self-assembly of peptides previously reported by us[3] could potentially accommodate metal ions other than zinc. We set out to develop a copper based self-assembling system as copper ions are commonly employed in redox active enzymes, are hydrolytically stable at near neutral pH, and provide convenient spectroscopic handles.

As a model reaction in our studies, we have chosen oxidation of 2,6-dimethoxyphenol (DMP, Scheme 1). Oxidative dimerization of DMP produces colored product that can be easily assayed in a high throughput fashion. This reaction proceeds via Cu^{II}-mediated oxidation of the substrate; it is well benchmarked and extensively mechanistically characterized.[5] Additionally, oxidation of substituted phenols is commonly used in the polymer industry.[6]

In order to test whether self-assembling peptides can indeed support copper-mediated substrate oxidation, we used a small focused library of peptides previously shown to self-assemble in the presence of zinc and catalyze ester hydrolysis. The results of the initial screening are presented in Figure 1. The most active peptide (**11**, Ac-IHIHIQI-$CONH_2$) showed activity that was more than an order of magnitude higher than baseline activity. The levels of activity are highly sequence dependent: the non-fibril forming control peptide (**14,** NH_2-IHIHIQI-COOH) that has the same primary sequence but lacks the caps on the termini shows activity that is lower than that of free copper ions in buffer. Similar to our previous work, peptides containing residues that promote β-strand secondary structure (Ile and Val, **7**, **8, 11** and **12**) showed high activity compared to peptides with residues of lower β-sheet forming propensity (Leu and Ala, **1**–**5**). CD spectra of **7** and **11** showed a clear β-sheet

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character in the presence of copper (Figure S1, Supporting Information). Replacing either His2 or His4 with alanine significantly reduces activity (Figure 1, peptides **9**, **10**). While both **9** and **10** form large aggregates with β-sheet character and bind copper, albeit with different stoichiometry (Figures S2, S3), they demonstrate lower activity under all conditions. This suggests that the His-X-His motif provides the most optimal functional group arrangement among the peptides studied in this work. The nature of the residue in position 6 is also important for catalytic activity, with peptides containing Gln and Tyr in this position producing the most active catalysts. Under the same conditions amyloid beta peptide (Aβ 1–40), which is known to self-assemble and bind copper,[7] does not catalyze DMP oxidation. Thus self-assembly of histidine-containing peptides alone is not sufficient for catalysis.

We chose **11**, the most active peptide, for in depth characterization. Based on the Job's plot of activity (Figure S3) and copper titrations (Figure S4), we determined the ratio of Cu^{II} to **11** complex to be approximately 1:2. The rate enhancement of DMP oxidation relative to Cu^{II} in buffer is pH-dependent and reaches more than 65-fold at pH 6 (Figure S5). The catalyst undergoes multiple turnovers resulting in a complete oxidation of the substrate in less than 24 hours (Figure 2). The histidine complex of copper is completely inactive; imidazole and N-acetyl-histidine-amide show low activity, on par with that of non-self-assembling peptides **4** and **5** under the same conditions (Figure 1, Figure S6). A non-self-assembling hexahistidine peptide **16** (Figure S2), is also completely inactive, suggesting that multivalent coordination of histidines is not sufficient for activity. Thus **11** provides an appropriate coordination sphere for copper to engage in productive catalysis. Next, we performed ultracentrifugation experiment to determine the nature of the active species. Centrifugation for 1 hour at 100,000 *g* completely removes peptide **11** species from the solution as concluded from the absorbance of the supernatant (Figure S7). The supernatant shows no activity in DMP oxidation suggesting that large peptide aggregates are responsible for catalysis. Similarly, filtration of the copper-peptide complex through a 0.22 μm membrane (Figure S8) produces filtrate that has no activity. On the other hand, dialysis of the peptide solution fully preserves its oxygen activating activity (Figure S9). The inactive peptide **14** bound to copper, as shown by EPR data discussed below, did not show any secondary structure (Figures S1, S6) and did not precipitate in ultracentrifugation experiment under the same conditions (Figure S10).

Ultracentrifugation has also provided us with an opportunity to quantitatively characterize binding of copper to the fibrils. Measurement of the equilibrium concentrations of copper in the supernatant after the centrifugation shows that under the conditions used, ~90% of the copper is bound by the peptide (Figure S10).

To probe Cu^{II} coordination environment in different peptides we used low-temperature EPR spectroscopy. The g_{II} and A_{II} values for Cu^{II} are commonly used to determine the composition of the copper coordination sphere. EPR spectra of Cu^{II} bound to the most active peptides (Ac-IHIHIQI-$CONH_2$ and Ac-VHVHVYV-$CONH_2$) are of the classic type 2 with $g_{II} = 2.27$ and $A_{II} = 167$ G, values that are consistent with either 3N1O or 2N2O coordination environment in the equatorial plane based on the Blumberg-Peisach plots (Figures 3, S11).[8] Peptide **14** (NH_2-IHIHIQI-COOH) that does not aggregate, has no β-

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sheet structure at pH 8 as shown by CD and ultracentrifugation (Figures S6 and S10) and is inactive in DMP oxidation assay, has a distinctly different EPR spectrum (Figure S11). These results suggest a major change in the coordination environment of copper despite essentially identical primary peptide sequence. EPR spectra also shed light on the possible reaction mechanism. Addition of DMP to Cu^{II} – **7** results in gradual diminishing of the EPR signal (Figure 3) consistent with the reduction of Cu^{II} to Cu^{I} in line with the previously proposed mechanism.[5c, 5d]

The contribution of self-assembly to the overall activity can be examined by comparing the properties of peptides **4**, **5** and **11**. The identities of metal-binding residues are the same in all cases but the residues in the "hydrophobic" positions are varied. Peptides **4** and **5** that have lower β-sheet forming propensity bind copper but show no aggregation nor β-sheet character in the presence of the metal ion (Figures S6, S7, S10). The copper coordination environments in **5** and **11** are different as judged by the EPR data (Figures S11, S12). The observed differences in activity can be explained by the high stability of the β-sheet assemblies that lock the histidines into a conformation that is more suitable for the appropriate copper coordination akin to how a protein fold modifies metal ions properties. Comparison of peptides **4** and **11** is particularly instructive. Isomerization of the side chain of amino acid residues not involved in the primary coordination sphere of the copper ion (leucine vs. isoleucine) results in self-assembly and a *ca.* 5-fold increase in the initial rate of phenol oxidation.

We established that oxidation of DMP promoted by Cu^{II}-peptide is oxygen-dependent, as removal of oxygen prevented formation of the product, but introduction of O_2 into the reaction mixture immediately resulted in DMP oxidation (Figure S13). Oxygen, superoxide and H_2O_2 do not oxidize DMP on the timescale of several hours, however formation of the product occurs immediately upon mixing Cu^{II}-peptide and DMP, emphasizing the crucial role of Cu^{II} in this reaction. Addition of catalase (Figure S14) or superoxide dismutase (Figure S15) does not substantially diminish the reaction rate mechanistically supporting the Cu^{II}-promoted oxidation and effectively excluding the possibility of radical oxidation of the substrate. Among the different metal ions tested (Cu^{II}, Fe^{III}, Fe^{II}, Mn^{II}, Ni^{II}, Co^{II}) only Cu^{II} catalyzes DMP oxidation to any appreciable extent, suggesting that redox potential of Cu^{II} and coordination environment supported by fibril core are optimal for catalysis (Figure S16).

Supramolecular approaches to designing efficient catalysts for chemical reactions have been extremely productive.[9] Self-assembling properties of peptides have been previously used to create multidentate ligands for transition metal catalysts,[10] hydrogels with esterase activities,[11] and light-capturing materials.[12] The ability to genetically encode large peptide libraries opens the path for discovery and optimization of peptide catalysts using high-throughput techniques.[13] In this paper we show that self-assembly of short *de novo* designed peptides results in formation of efficient supramolecular catalysts capable of oxygen activation. Moreover, we show that supramolecular assemblies are capable of supporting oxygenation catalysis that does not rely on radicals. This finding underscores the fact that amyloid-like assemblies formed by even very short peptides can facilitate a number of various chemical transformations in a highly sequence-specific manner. Considering recent findings that amyloid-supported metal sequestration and catalysis is more likely to be

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a rule rather than an exception,[3, 14] we expect *de novo* designed self-assembled catalytic peptides to combine the power of highly controlled metal coordination sphere common in homogeneous catalysis with practical advantages of heterogeneous catalysts. Moreover, synergistic interactions observed in these systems provide additional opportunities for high throughput screening for catalytic function. Finally, the diversity of reactions catalysed by simple peptide assemblies lends further support to the amyloid-first hypothesis of emergence of enzymatic function.[15]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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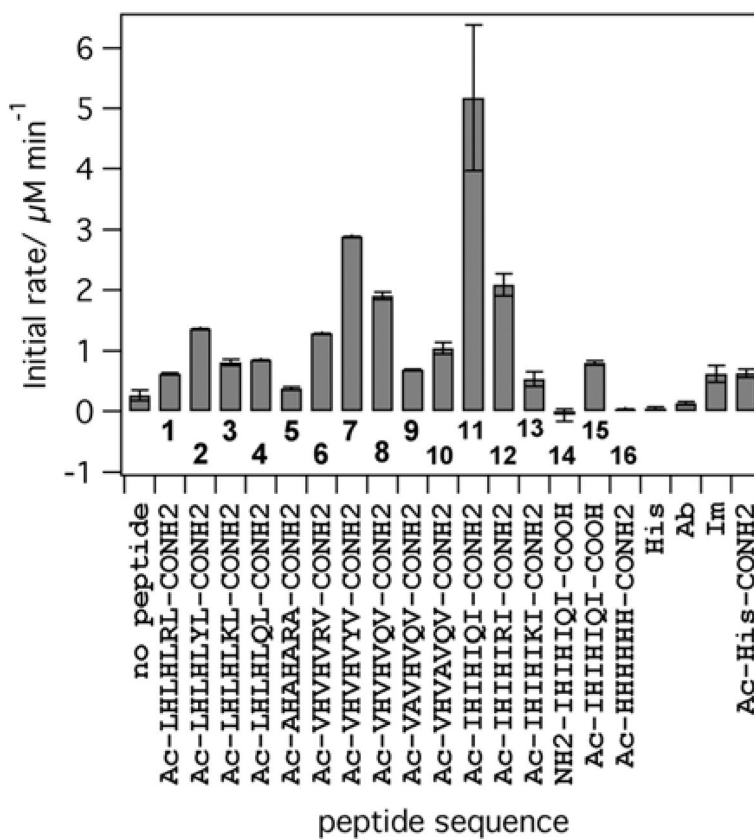

Figure 1.

Initial rates of product formation catalyzed by Cu^{II} (10 μM) in the absence/presence of various peptides (20 μM). All reactions are in 25 mM Hepes, pH 7.9 at room temperature, [DMP] = 500 μM. His = histidine (40 μM), Im = imidazole (40 μM), Ac-His-CONH2 = N-acetyl-histidine-amide (40 μM), Ab = Amyloid beta (10 μM).

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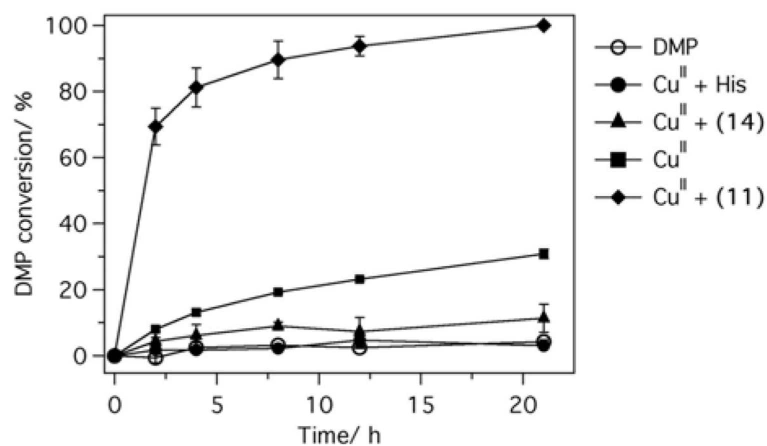

Figure 2.

Conversion of dimethoxyphenol catalyzed by Cu^{II} in buffer, Cu^{II} in the presence of histidine (His), peptides **11** and **14**. Reaction was initiated by adding DMP (200 μM). Aliquots were taken at various time points and immediately analysed by HPLC. Buffer: 25 mM Hepes, pH 8, [Cu^{II}] = 40 μM, [peptide] = 80 μM, [His] = 160 μM.

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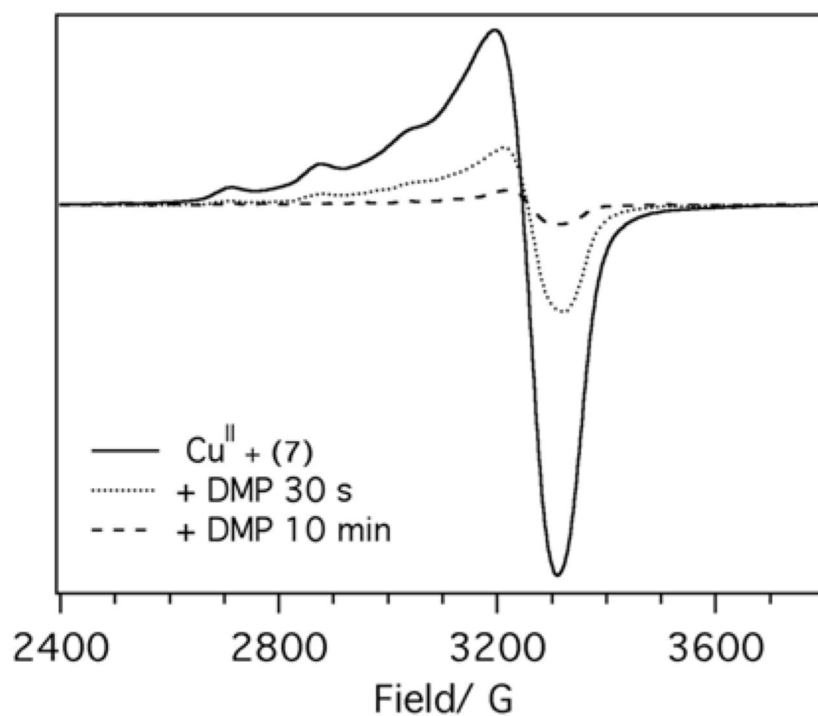

Figure 3.

X-band EPR spectra of Cu^{II} (150 μM) in the presence of peptide **7** (300 μM) acquired in pH 7.6 buffer (25 mM Hepes, 10 % glycerol) at 10 K. Frequency 9.39 GHz, power 5.024 mW, modulation frequency 100 kHz, modulation amplitude 8 G, conversion time 40 ms, time constant 163.8 ms. After acquiring the spectrum (solid line), DMP was added to the sample to a final concentration of 1 mM (5 μL of 70 mM solution), the sample was mixed and frozen (+DMP, 30 s, dotted line). After recording another EPR spectrum, the sample was thawed and incubated at room temperature for 10 min (+DMP, 10 min, dashed line).

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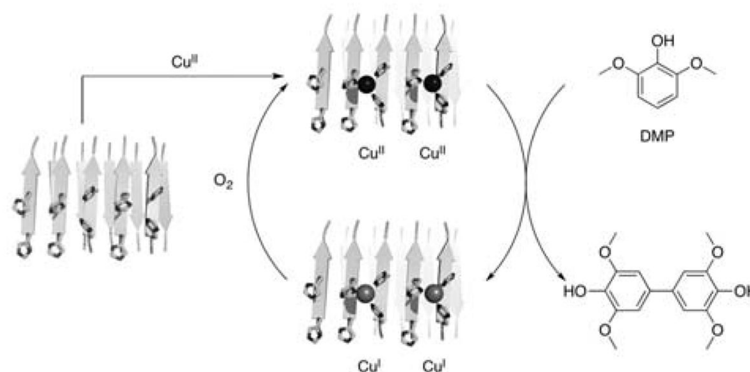

Scheme 1.

Copper-mediated oxidation of dimethoxyphenol (DMP) by dioxygen. Fibril structures shown in this scheme are not derived from experimental data; they are based on a previously reported computational model for zinc containing peptide assemblies.[3]