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An Efficient, Step-Economical Strategy for the Design of Functional Metalloproteins

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Abstract

The bottom-up design and construction of functional metalloproteins remains a formidable task in biomolecular design. While numerous strategies have been used to create new metalloproteins, preexisting knowledge of the tertiary and quaternary protein structure is often required to generate suitable platforms for robust metal coordination and activity. Here we report an alternative and easily implemented approach (Metal Active Sites by Covalent Tethering or MASCoT) whereby folded protein building blocks are linked by a single disulfide bond to create diverse metal coordination environments within evolutionarily naïve protein-protein interfaces. Metalloproteins generated with this strategy uniformly bind a wide array of first-row transition metal ions (Mn^{II}, Fe^{II}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II} and vanadyl) with physiologically relevant thermodynamic affinities (dissociation constants ranging from 700 nM for Mn^{II} to 50 fM for Cu^{II}). MASCoT readily affords coordinatively unsaturated metal centers, including a five-His coordinated non-heme Fe site, and well-defined binding pockets that can accommodate modifications and enable coordination of exogenous ligands like nitric oxide to the interfacial metal center.

Introduction

Metalloproteins are amongst the most critical components of a living organism. All natural metalloproteins derive their particular function or competitive advantage via robust coordination of a particular metal ion or metallocofactor. Proteins that feature transition metal ions (e.g., Mn^{II}, Fe^{II} or Cu^{II}) often constitute a significant fraction (up to ~60%) of an organism's proteome.[1] Metalloproteins are key pharmaceutical targets[2] and progenitors of oncogenesis,[3] involved in immune response,[4,5] and play central roles in bioenergetics and metabolism.[6,7] The proteins involved in these processes have independently evolved unique metal coordination motifs that feature combinations of a small number of weak-field, amino-acid derived ligands, such as imidazole or carboxylates. How metalloproteins coordinate their respective metal ions with high affinities/selectivities and perform remarkably diverse functions despite a limited ligand repertoire remains a topic of great interest for biochemistry, inorganic chemistry and protein design.[8-10]

Efforts to emulate these features with designed metalloproteins provide a potential avenue towards understanding critical relationships between protein structure and metal coordination. The design of artificial metalloproteins is not unlike the development of human-made catalysts, pharmaceuticals or materials in that they involve the exploration of disparate synthetic approaches.[11-13] Some prominent strategies (Fig. 1a-d) include the incorporation of unnatural cofactors or

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51ligands onto preexisting host scaffolds,[14-21] redesign of existing scaffolds to enable 52metal coordination,[22-23] and *de novo* design using consensus sequence/structure 53motifs and/or computation.[24-29] In addition to these design efforts, directed evolution 54of natural metalloenzymes has also figured prominently in obtaining novel or 55improved reactivities.[30-31] In general, a requirement in most of these approaches is 56that the generation of a functional metal center relies on a preexisting site *in the* 57*interior* of a tertiary or quaternary structure donated by the host scaffold or derived 58from sequence/structural similarity to natural scaffolds. The significance of this 59constraint is nontrivial not only in terms of protein design but also in the context of 60natural evolution: how did the first functional metalloproteins come into existence 61without access to the large library of contemporary protein folds?[32] This 62evolutionary question is directly related to a synthetic one: can we construct and 63diversify functional metalloproteins without the internal constraints of a 64tertiary/quaternary structure (corresponding to evolutionary innovation) and with 65minimal engineering steps or mutations (corresponding to evolutionary efficiency)? 66Along these lines, we previously developed an approach, termed Metal Templated 67Interface Redesign (MeTIR) (Fig. 1e), in which a small protein building block (cyt 68cb_{562}) could be engineered on its surface to self-assemble via metal coordination 69into discrete oligomeric architectures.[33] The resultant interfaces in these assemblies 70could subsequently be tailored with computationally prescribed non-covalent 71interactions and reinforced with disulfide linkages to generate stable protein 72complexes that displayed functional properties such as allostery and *in vivo* 73catalysis.[34-37] While MeTIR represents a streamlined protein design process, it has 74still involved the incorporation of 10 to 15 surface mutations onto the target protein 75building block, thereby incurring a non-negligible design/genetic burden.

76   Cysteine-derived disulfide bonds have been widely exploited to stabilize pre-77existing protein architectures (as in the case of MeTIR).[34-39] Dutton and colleagues 78pioneered the use of disulfide bonds for linking together *de novo* designed coiled-79coil peptides to form four-helix bundle "maquettes" that selectively and stably 80bound various macrocyclic metallocofactors.[26,40-42] We wondered whether such 81covalent linkages could also prove useful in the construction of metal active sites 82between two arbitrary proteins in the absence of additional stabilizing interactions. 83We surmised that the formation of a single disulfide linkage between two well-84folded proteins would give rise to a malleable protein-protein interface that can be 85conveniently engineered for the construction of metal coordination sites, with the 86advantages that a) the protein building blocks are stable and therefore amenable to 87extensive modifications (in contrast to peptidic building blocks), and b) their well-88defined surface features in combination with the restrictions imposed by the 89disulfide bond would yield robust active site environments (Fig. 1f). We term this 90strategy MASCoT (Metal Active Sites through Covalent Tethering). As we describe 91below, this strategy has allowed the simultaneous attainment of several functional 92features while requiring minimal design and engineering steps: 1) a singular metal 93coordination motif that can accommodate the entire mid-to-late first-row transition 94metal ion series with high affinities as well as a metal ion (Fe) in different oxidation 95states, 2) facile access to coordinative unsaturation and tolerance to large changes 96in both the primary and secondary coordination spheres, 3) a naturally rare penta-97histidine Fe coordination center that can reversibly bind small gaseous molecules, 98and 4) homo-oligomeric protein assemblies that display both local and global 99asymmetry.
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Results and Discussion

Implementation of MASCoT to construct the dimeric metal-binding complex CH₃

As a model building block for MASCoT, we employed cytochrome cb_{562}, a four-helix bundle heme protein, whose high stability, solubility and uniform α-helical composition proved instrumental in our earlier design efforts. To remove any 'structural memory' imposed during these efforts, our work herein has focused on the engineering of the original, unadultered cb_{562} scaffold.[43] With the idea that the largest interface between two copies of cyt cb_{562} would be obtained if the disulfide tether were located centrally on the protein surface, we opted for Cys substitution at position 96 which lies in the middle of Helix 4, the longest of the four α-helices. In order to obtain a stable metal binding site with a high coordination number, we placed a set of three His residues at positions 67 and 71 on Helix 3 and position 97 on Helix 4 that flank position 96. We predicted by inspection of the cyt cb_{562} crystal structure that this particular placement of three His residues would afford a stable coordination motif on the surface of each protein monomer, and that their combination via a Cys96-Cys96 disulfide tether would generate a clamshell-like metal-chelating motif in the nascent protein interface. Furthermore, we envisioned that the placement of the 3His motif immediately next to the disulfide bond would increase their net local concentration for efficient interfacial metal chelation and could enable the formation of strained coordination geometries. In essence, this strategy is quite analogous to the synthesis of a multi-dentate ligand scaffold, wherein the specific covalent connectivity of the donor atoms determines the flexibility and bite angle of the chelate, ultimately dictating the nuclearity and coordination geometry of the metal center.[44]

We first investigated the ability of the 3His motif to act as a standalone surface coordination site. Towards this end, we generated the [H67/H71/H97]cyt cb_{562} variant (denoted H_3), overexpressed it in *E. coli* and purified it as a soluble, monomeric protein. H_3 coordinates Co^{II}, Ni^{II}, and Zn^{II} ions with μM dissociation constants (K_d) (Table 1) in a 1:1 stoichiometry, as determined by analytical ultracentrifugation and metal-binding competition experiments (Supplementary Figs. 1 and 2). We obtained single crystals of Co^{II}-bound H_3 (Co-H_3) and determined its structure at 2.0-Å resolution (Fig. 2a). As planned, the engineered His residues ligate an octahedral Co^{II} center via their N_ϵ atoms in a facial arrangement reminiscent of the non-heme iron enzyme, EgtB, and other analogs from the DinB superfamily;[45] three aquo ligands are also well resolved. The backbone alignment of Co-H_3 with the parent cyt cb_{562} shows that the two proteins are essentially identical (RMSD = 0.34 Å), indicating that the core four-helix bundle structure remains unperturbed by surface mutations and metal coordination.

Next, we incorporated the T96C mutation into H_3 to generate the clamshell CH_3 variant. As shown by SDS PAGE analysis, purification of CH_3 under oxidizing conditions exclusively afforded a dimeric species (Supplementary Fig. 3), which was subsequently crystallized both in the absence and presence of various divalent metal ions (Fe^{II}, Co^{II}, Cu^{II}). The resulting crystal structures, with resolutions ranging from 2.75 Å to 1.33 Å (Supplementary Table 3), reveal marked topological differences between apo-CH_3 and the metal-bound forms (M-CH_3) (Fig. 2b and c) arising from the flexibility of the disulfide linkage. Apo-CH_3 displays a roughly perpendicular arrangement of two protein monomers with an interprotein dihedral angle (θ_{ip}) of 112°. There is minimal interfacial contact between the two monomers (buried surface area = 174 Å²),[46] suggesting that the apo-CH_3 likely has a fluxional

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151structure in solution and that the observed conformation is stabilized by crystal
152packing interactions. In contrast, all three M-**CH₃** complexes possess a singular,
153compact conformation (RMSD = 0.261 - 0.399 Å) with an antiparallel arrangement
154of the protein monomers (θ_{ip} = 163°) and a single, mononuclear metal center
155adjacent to the disulfide linkage. The dimer topology deviates considerably from C_2
156symmetry as highlighted by the structure of the Co-**CH₃** complex (Fig. 2c), which
157features close protein-protein contacts across only one half of the interface (Fig.
1582e).
159   The topological asymmetry is also projected onto the metal center that
160possesses an unusual penta-His coordination sphere completed by five of the six
161designed His residues, whereby a single H97 side chain remains unbound (Fig. 2d).
162In Fe- and Co-**CH₃** complexes, the metal coordination is completed by a single aquo
163ligand, whereas in Cu-**CH₃**, a sixth ligand is not observed as expected from the d^9
164electronic configuration of the Cu^{II} center (Fig. 3a). In each of the three complexes,
165we observe an ideal square pyramidal arrangement (angular structural parameter
166τ_5 = 0.03 - 0.08)[47] of the His ligands with nearly identical M^{II}-N coordination
167distances (2.0-2.2 Å), including the axial His-Cu bond in Cu-**CH₃** (2.2 Å). We also
168prepared a vanadyl adduct of **CH₃** by reacting the protein with a stoichiometric
169amount of $[V^{IV}{\equiv}O][SO_4]$ and characterized it by electron paramagnetic resonance
170(EPR) spectroscopy in solution (*vide infra*). The ^{51}V hyperfine parameters of frozen
171solutions of $(V^{IV}{\equiv}O)$-**CH₃** are consistent with a monomeric vanadyl species bound to
172five His ligands.[48] The square-pyramidal, 5-His coordination motif is exceedingly rare
173in natural proteins, with only a handful of representatives in the Protein Data Bank,
174including the urease maturation protein, UreE, and integral membrane
175hydroxylases.[49, 50] In all M-**CH₃** crystal structures, the primary and secondary
176coordination spheres display local B-factors that are 131 - 154% smaller than the
177mean protein B-factors, consistent with a high degree of structural rigidity found
178within the protein-protein interface (Supplementary Fig. 4). From a design
179perspective, our observations indicate that a properly placed disulfide linkage in a
180sterically encumbered protein-protein interface can impose a strong structural
181restraint, engendering a robust, coordinatively unsaturated metal coordination
182geometry that is normally achieved only within the well-defined interior of a folded
183protein. Importantly, they show that it is possible with MASCoT to create substantial
184local and global structural asymmetry in a homo-oligomeric system.[8,51]
185   We next sought to gauge the affinity of the penta-His motif for divalent
186transition metal ions. Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)
187analyses of M-**CH₃** samples that were extensively buffer exchanged confirmed a 1:1
188M^{2+}:**CH₃** stoichiometry (Supplementary Fig. 5). Metal competition titrations of **CH₃**
189using the fluorescent metal chelator, Fura-2,[52] revealed K_d's ranging from low μM for
190Mn and low-to-sub nM for Co, Ni, Zn to low fM for Cu, in accord with the expected
191trend of the Irving-Williams series (Table 1) (for discussion on Fe, see below).
192Affinities determined for Co-**H₃** and Co-**CH₃** differ by 3 orders of magnitude
193(corresponding to ca. 4 kcal/mol), providing a quantitative measure on the
194enhancement of metal binding thermodynamics due to increased denticity/chelate
195effect afforded by disulfide crosslinking. The values found for **CH₃** represent some of
196the highest metal affinities reported for His-rich designed metalloproteins,[53-55]
197consistent with the high denticity of the **CH₃** scaffold.
198
199**Modulation of the primary and secondary coordination spheres in CH₃**

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200   Given the robustness of the metal coordination environment in **CH₃** and the
201 fact it is built in a minimally engineered interface formed by the outer surfaces of
202 protein building blocks, we reasoned that it should readily accommodate changes in
203 its primary and secondary coordination spheres. We first targeted positions 70
204 (natively Gly residues) which lie just above the equatorial M(His)$_4$ plane (Fig. 2d)
205 and could, upon substitution with an appropriate residue, enable the formation of an
206 active-site pocket above the metal center. We opted for replacement of G70 with a
207 Tyr residue (to create variant **CH₃Y**) because of its large but polar side chain. The
208 metal-binding capacity and thermodynamics of **CH₃Y** closely match those of **CH₃**
209 (Table 1, Supplementary Fig. 5), suggesting that the G70Y mutation must not
210 significantly perturb the quaternary protein structure or the coordination
211 environment. Furthermore, EPR spectra collected on frozen solutions of (V^{IV}≡O)- or
212 Cu^{2+}-bound **CH₃** and **CH₃Y** variants and pertinent Hamiltonian parameters are
213 nearly identical for each metal ion (Fig. 3, Supplementary Tables 5-6). These
214 findings are corroborated by the crystal structures of several M-**CH₃Y** complexes (M
215 = Fe, Cu, V^{IV}≡O) (resolutions of 1.1 to 1.8 Å) which are isomorphous with their G70
216 counterparts (RMSD$_{C\alpha}$ = 0.418 Å) (Fig. 3). The exceptions are the well-resolved Y70
217 side chains that span the dimeric interface above the metal ion and displace
218 numerous ordered water molecules that are observed in M-**CH₃** (Fig. 3a), effectively
219 creating a small hydrophobic pocket in the interfacial crevice.
220   We subsequently targeted the primary coordination sphere with the
221 particular aim of creating a stable Mn binding site. Mn lies at the bottom of the
222 Irving-Williams series due to its relatively low Lewis acidity and lack of crystal-field
223 stabilization energy, rendering the engineering of high-affinity Mn coordination sites
224 inherently challenging.[56] In light of the predominance of carboxylate-rich
225 coordination motif in natural Mn-proteins,[6-7,56-58] we hypothesized that the
226 replacement of one or more of the designed His residues in **CH₃** and **CH₃Y** with Glu
227 would increase the Mn affinity of these constructs. Two variants, **CH₂E** and **CH₂EY**,
228 were thus created through the H97E mutation. **CH₂E** and **CH₂EY** were found to
229 retain significant fractions of Mn (0.56 and 0.72 equiv per protein dimer,
230 respectively) following incubation with 1 equiv of Mn and subsequent buffer
231 exchange (20 mM MOPS, pH 6.5, 150 mM Tris-HCl), a trait not observed for the **CH₃**
232 and **CH₃Y** congeners (<0.03 equiv per protein dimer) (Supplementary Fig. 5).
233 Accordingly, these variants were able to compete with the chelating indicator Mag-
234 Fura-2[57] for Mn binding, allowing us to determine K_d's of 16 μM (pH 7) or 5 μM (pH
235 8.5) for Mn-**CH₂E** and 5 μM (pH 7) or 700 nM (pH 8.5) for Mn-**CH₂EY** (Fig. 4a) (Table
236 1). These values approximate those of natural Mn transcription factors and
237 metalloenzymes.[57-58]
238   Both the structurally unique coordination spheres and secondary-sphere H-
239 bonding networks help rationalize the high-affinity Mn coordination observed for
240 **CH₂E** and **CH₂EY**. The overall topologies of Mn-**CH₂E** and Mn-**CH₂EY** (Fig. 4b)
241 diverge markedly from those of metal-bound **CH₃** or **CH₃Y** structures (RMSD = 3.7 -
242 3.9 Å), with the major structural differences arising from a near-perfect antiparallel
243 arrangement of the individual protein monomers (θ_{ip} = 176° and 174° for Mn-**CH₂E**
244 and Mn-**CH₂EY**, respectively). The Mn coordination sphere in Mn-**CH₂E** (Fig. 4c)
245 includes three meridional His side chains, and a single κ^1-bound Glu that completes
246 a square planar ligand arrangement around a *trans*-(OH₂)₂Mn^{II} unit. These aquo
247 ligands are in turn engaged in strong H-bonding interactions with the two other
248 engineered His and Glu side chains. In contrast, the Mn^{II} coordination sphere
249 determined for Mn-**CH₂EY** (Fig. 4d) includes all four designed histidine residues and

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250a κ^2-bound glutamate, collectively reminiscent of the non-heme Fe site found in the
251photosynthetic reaction center of *R. sphaeroides*.[59] The remaining E97 residue is H-
252bonded to the non-coordinating N_δ of H67$_A$. Apparently, the steric pressure exerted
253by the adjacent Y70 residues effectively prevents alternative rotameric
254configurations of H67$_B$, guiding its coordination to the Mn ion in Mn-**CH₂EY**.
255   Unlike the other divalent metal ions examined in this work, solution studies
256on Fe^{II} have been complicated by adventitious redox reactions involving the ferric
257heme cofactor that, for example, have prevented the quantitative determination of
258the K_d's for the Fe complexes of all variants discussed thus far. Hence, we
259engineered heme-free variants of **CH₃Y** and **CH₂EY**, denoted **CH₃Y*** and **CH₂EY***,
260respectively, in which the heme binding pocket has been engineered with several
261hydrophobic and largely bulky residues (M7W, C98R, C101A, H102I, R106L)[60] that
262occlude the cofactor. Crystal structures of Fe- and (V^{IV}≡O)-**CH₃Y*** complexes reveal
263the expected penta-His primary coordination environment (Fig. 5, Supplementary
264Fig. 7) and affords Co^{II}, Cu^{II} and Zn^{II} affinities that are comparable to those for **CH₃Y**
265(Table 1). Most notably, the K_d for the Fe-**CH₃Y*** complex is 37(3) nM, which
266compares well with natural and designed non-heme Fe^{II} metalloproteins, such as α-
267ketoglutarate dioxygenase enzymes (K_d = 7.5 µM), cytoplasmic Fe^{II}-sensors (K_d =
2681.2 µM), and the *de novo* designed protein DF2 (K_d = 17.8 µM).[58,61] The removal of
269the redox-active heme group also enables unobstructed analyses of the redox
270properties of the bound non-heme Fe center. In this regard, preliminary
271electrochemical measurements of the Fe-**CH₂EY*** system reveal a quasireversible
272Fe$^{III/II}$ redox couple centered at 0.49 V (vs NHE) at pH 6.0 (Supplementary Figure 8),
273indicating that the dimeric protein scaffold can accommodate multiple oxidation
274states of a metal center.
275

276**Nitric oxide-binding properties of the Fe-CH₃Y* complex**
277   One of the unique functions of metalloproteins is the binding of small
278diatomic molecules such as O$_2$, CO and NO, which is essential for their storage,
279transport and chemical activation in living systems. Despite their prevalence in
280biology, there are few *de novo* designed metalloproteins with a demonstrated ability
281to bind small gaseous molecules and such systems rely almost exclusively on the
282use of privileged cofactors like the heme group.[8,26,62-64] There is only one previous
283report demonstrating NO coordination to an engineered non-heme iron protein.[64]
284Having established the formation of a robust Fe^{II} coordination site and a nascent
285binding pocket in Fe-**CH₃Y***, we explored its NO binding properties through a
286battery of spectroscopic and structural investigations. Addition of a suitable NO
287donor (diethylammonium NONOate) to anaerobic solutions of Fe-**CH₃Y*** resulted in
288the development of an intense amber hue and visible charge transfer bands
289characteristic of a mononuclear, intermediate-spin {FeNO}^{7} unit (Fig. 5c).[65] In
290addition, sharp features emerged at $g \sim 4.0$ in the X-band EPR spectra of similarly
291prepared solutions that confirmed the presence of an Fe center with a S_{tot} = 3/2
292ground state (Fig. 5c-inset).[66-67] Mössbauer spectra collected on frozen solutions of
293^{57}Fe-enriched Fe-**CH₃Y*** revealed a single quadrupole doublet whose parameters
294diverge from those of ^{57}FeSO$_4$ collected in an identical buffer system
295(Supplementary Fig. 9). Addition of NONOate to these solutions gave rise to
296magnetic field-dependent multiline absorption features arising from unquenched
297magnetic interactions with the electronic spin manifold (Fig. 5d).[67-71] These features
298could be well simulated with a S_{tot} = 3/2 spin-Hamiltonian, a positive zero field
299splitting (>10 cm^{-1}) and low rhombicity (E/D ~ 0), which are similar to those found in

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300 synthetic non-heme {FeNO}[7] complexes.[67,69-71] In contrast, addition of NONOate to
301 solutions of [57]FeSO$_4$ in the absence of **CH$_3$Y*** gives rise to complex spectra
302 suggestive of multiple Fe-containing species (Supplementary Fig. 10).

303   To gain complementary structural insight into this protein-bound Fe-nitrosyl
304 complex, we soaked pre-formed crystals of Fe-**CH$_3$Y*** with diethylammonium
305 NONOate and observed the development of an amber hue within each individual
306 crystal (Supplementary Fig. 11). The 2.0-Å resolution X-ray diffraction data obtained
307 from these crystals show that (FeNO)-**CH$_3$Y*** is isostructural with Fe-**CH$_3$Y*** (RMSD =
308 0.11 Å) and contains a distinct electron density above the Fe center which is
309 consistent with a bound NO ligand (Fig. 5a and b). Structural modeling and
310 refinement of the Fe-NO moiety revealed a Fe-N distance of 1.8 Å and a rather
311 acute Fe-N-O angle of 135°, which is unusual among synthetic non-heme {FeNO}[7]
312 complexes that have been crystallographically characterized (typical angles range
313 from 147 to 179°).[67, 69-71] We cannot rule out the possibility of X-ray induced
314 reduction of the Fe center to generate an {FeNO}[8] species that would be
315 anticipated to display such an acute Fe-N-O angle.[70,71] Alternatively, it is noteworthy
316 that the NO ligand occupies a rather confined pocket defined by the Tyr70 side
317 chains which may impose steric constraints on the ligand geometry.

318   Although NO readily coordinates the ferrous center of Fe-**CH$_3$Y***, extended
319 exposure of this complex to a O$_2$-rich atmosphere does not induce similar
320 coordination of O$_2$. Monitoring these oxygenation reactions with [57]Fe Mossbauer and
321 EPR methods reveals that only 18% of the Fe content in Fe-**CH$_3$Y*** is slowly oxidized
322 over 24 hours to a single high-spin Fe^{III} species with spectroscopic parameters
323 inconsistent with a ferric superoxide (Supplementary Fig. 12).[72] We surmise that the
324 neutral-, nitrogen-rich coordination environment provided by **CH$_3$Y*** serves to
325 elevate the Fe$^{III/II}$ reduction potential into a regime that is incompatible with effective
326 metal-to-O$_2$ charge transfer that is thought to be essential for O$_2$ coordination in Fe
327 metalloenzymes.[1, 72] The high reduction potential measured for the related Fe-
328 **CH$_2$EY*** species (0.49 V) is suggestive of a thermodynamically unfavorable Fe-O$_2$
329 interaction. The hexa-histidine site of calprotectin similarly affords a coordination
330 environment that selectively stabilizes the ferrous redox state.[73] Given our ability to
331 introduce H-bonding interactions in related constructs (Fig. 4), ongoing efforts are
332 directed at increasing the donor strength of the ligating His residues via secondary
333 sphere tuning to facilitate robust O$_2$ coordination and subsequent activation.
334 Nonetheless, the demonstrated competence of Fe-**CH$_3$Y*** to coordinate a diatomic
335 ligand (NO) in a structurally well-defined manner is a testament to the functional
336 promise of this scaffold.
337

338 Conclusions

339   The construction of functional proteins in the laboratory is a multi-step
340 process much like the synthesis of a complex natural product from simpler
341 molecules. Both processes involve a careful choice of the building blocks and of the
342 design/synthetic strategy to assemble them, as well as a considerable amount of
343 optimization of the design/synthetic steps to maximize the yield and functional
344 properties of the target structure. While the elegance and heroism of >30-step
345 syntheses are undisputed in organic chemistry, it has been also recognized that a
346 large number of steps is detrimental to the feasibility and practicality of a synthetic
347 route. Thus, under the principle of "step economy", the focus has considerably
348 shifted to the invention of new types of reactions which minimize the number of
349 steps to reach the desired target.[13] It is also preferable if new synthetic strategies

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350also allow for increased chemical diversification at each step, which translates into 351a wider array of functional molecules that can be obtained with minimal additional 352effort. This synthetic principle has obvious parallels to the natural evolution of 353proteins as well: if an evolutionary route involves fewer genetic perturbations to 354produce a protein with diversifiable functions, it will likely be more efficient than 355one that requires more perturbations to obtain a protein with a non-diversifiable 356function.

357   In the spirit of step economy, we have introduced MASCoT as a readily 358accessible strategy for the design of oligomeric metalloproteins. A key rate-limiting 359step in the design of functional proteins is the generation of a stable, yet sufficiently 360malleable protein architecture through the implementation of numerous non-361covalent interactions. This step was streamlined in MASCoT through the tethering of 362two arbitrary but well-folded protein building blocks via a single disulfide bond, 363thereby creating a new structural context between two proteins through only one 364mutation. The elaboration of the resulting interface led to the generation of unusual 365tetra- and penta-dentate metal coordination motifs that uniformly accommodate a 366wide variety of coordinatively saturated or unsaturated metal ions, enable 367substantial alterations in their primary and secondary coordination spheres and 368bind small gaseous molecules. The fact that each of these functional features are 369difficult to design on their own but are simultaneously achieved through MASCoT 370through minimal engineering attests to the expediency of the covalent tethering 371strategy to build new functional sites in protein interfaces. As this strategy is 372predicated upon the use of well-folded protein building blocks and natural amino 373acids, its application in the laboratory evolution of enzymatically-active 374metalloproteins operative in oxidative or hydrolytic processes can be readily 375envisioned.[35]

376

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602**Author contributions**
603J.R. co-conceived the project, designed and performed experiments, analyzed data 604and co-wrote the paper. M. F and M. T. G. performed EPR and Mössbauer 605experiments. F.A.T. conceived and directed the project and wrote the paper. All 606authors discussed the results and commented on the manuscript.
607

608**Competing Financial Interests**
609The authors declare no competing interests.
610

611**Methods and Data Availability**
612The principal data supporting the findings of this work are available within the 613figures and the Supplementary Information file. Coordinates and structure factor 614files for Co-**H₃** (6DYI), apo-**CH₃** (6DYB), Co-**CH₃** (6DYC), Fe-**CH₃** (6DYE), Cu-**CH₃** 615(6DYD), Fe-**CH₃Y** (6DYG), Cu-**CH₃Y** (6DYF), $V^{IV}O$-**CH₃Y** (6DYH), Mn-**CH₂E** (6DY6), Fe-616**CH₂E** (6DY4), Mn-**CH₂EY** (6DY8), Fe-**CH₃Y*** (6DYJ), FeNO-**CH₃Y*** (6DYK), and $V^{IV}O$-617**CH₃Y*** (6DYL) have been deposited to the Protein Data Bank with the corresponding 618PDB ID codes. Additional data that support the findings of this study are available 619from the corresponding author on request.
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625**Figure Captions**
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627**Fig. 1 | Design strategies for the construction of functional** 628**metalloproteins**. **a-e**, Schematic representations of previously reported 629metalloprotein design and engineering strategies. **f**, The MASCoT strategy described 630herein. MASCoT utilizes intermolecular disulfide linkages to create flexible protein-631protein interfaces that serve as an evolutionarily-naïve surface on which to forge 632diverse metal-binding sites. This strategy does not require unnatural amino acids or 633cofactors, benefits from an expedient workflow and is potentially generalizable to a 634wide range of protein monomers.

635**Fig. 2 | Implementation of MASCoT. a**, Cartoon representation of M-**H₃** and 636(bottom) closeup of the coordination environment of Co-**H₃**. **b**, Cartoon 637representation of apo-**CH₃**. **c**, Cartoon and surface representations of M-**CH₃**. **d**, 638Closeup of the coordination environment of Co-**CH₃**. **e**, Salient interfacial 639interactions that characterize the asymmetric quaternary structure. Water ligands 640are shown as red spheres. In **a** and **e**, the $2F_o$-F_c electron density maps are shown in 641gray and contoured at 2.0 σ and the anomalous difference maps are shown in 642magenta and contoured at 8.0 σ. In **a-c**, heme cofactors are not shown for clarity.

643**Fig. 3 | Structural, spectroscopic and analytical data on Cu-, Fe-, and $V^{IV}O$** 644**bound metalloproteins**. Closeup views of Cu binding sites in **a**, Cu-**CH₃** and **b**, Cu-

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645**CH₃Y**. The $2F_o$-F_c maps are shown in gray and contoured at 2.0 σ. **c**, EPR spectra of
646Cu-bound metalloproteins and simulations (gray-dotted lines). Conditions: 400 µM
647Cu, pH 6.5 MOPS, 150 mM NaCl, X-Band, 40 K, 20 mW. **d**, Cu-binding isotherm for
648Fura-2-**CH₃** competition experiments. Protein samples (20 µM/monomer) were
649combined with 10 µM Fura-2 (pH 6.5, 20 mM MOPS, 150 mM NaCl) and Cu was
650added sequentially. The gray line represents a simulated isotherm in which **CH₃**
651does not compete with Fura-2 for Cu. Closeup views of metal binding sites **e**, in Fe-
652**CH₃Y** and **f**, in V^{IV}O-**CH₃Y**. The $2F_o$-F_c maps are shown in gray and contoured at 1.4
653σ. **g**, EPR spectra of V^{IV}O-bound metalloproteins and simulations (gray-dotted lines).
654Conditions: 400 µM V^{IV}O, pH 6.5 MOPS, 150 mM NaCl, X-Band, 10K, 100 µW. **h**, Fe-
655binding isotherm for Fura-2-**CH₃Y*** competition experiments. Protein samples (20
656µM/monomer) were combined with 10 uM Fura-2 (pH 6.5, 20 mM MOPS, 150 mM
657NaCl) and Fe was added sequentially under anaerobic conditions. The gray line
658represents a simulated isotherm in which **CH₃Y*** does not compete with Fura-2 for
659Fe.

660**Fig. 4 | Mn binding by CH₂E and CH₂EY. a**, Mn-binding isotherm for Mag-Fura-2-
661**CH₂EY** competition experiments. Protein samples (20 µM/monomer) were combined
662with 10 µM Mag-Fura-2 (pH 8.5, 20 mM Tris, 150 mM NaCl) and Mn^{2+} was added
663sequentially. The gray line represents a simulated isotherm in which **CH₂EY** does
664not compete with Mag-Fura-2 for Mn^{2+}. **b**, Comparison of the quaternary structures
665of Mn-**CH₂E** and Co-**CH₃** highlighting the difference in inter-protein dihedral angles
666and emphasizing the tolerance of the MASCoT approach to gross structural
667perturbations. **c**, Closeup view of the Mn coordination sphere present in Mn-**CH₂E**. **d**,
668Closeup view of the Mn coordination sphere present in Mn-**CH₂EY**. $2F_o$-F_c maps are
669shown in gray and contoured at 2.0 σ. Panels **c** and **d** illustrate that secondary
670sphere point mutations can diversify metal-binding coordination spheres.

671**Fig. 5 | Nitric oxide binding properties of Fe-CH₃Y*. a**, Closeup view of FeNO-
672**CH₃Y*** with superimposed $2F_o$-F_c (gray, 1.6 σ) and NO F_o-F_c omit maps (magenta,
67310.0 σ) that illustrate well-ordered electron density arising from an Fe-bound NO
674ligand. **b**, FeNO-**CH₃Y*** coordination environment with salient metrical parameters
675of a protein-bound {FeNO}[7]. **c**, UV-visible spectra of Fe-**CH₃Y*** (black) and FeNO-
676**CH₃Y*** (orange). (inset) X-band EPR spectra of FeNO-**CH₃Y***. **d**, [57]Fe Mössbauer
677spectra of FeNO-**CH₃Y*** at 4.2 K in the presence of 50-mT magnetic field. The
678absence of [57]Fe-containing contaminants indicates that NO addition to Fe-**CH₃Y*** in
679solution yields a single, homogenous {FeNO}[7] subunit.

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	H$_3$	CH$_3$	CH$_3$Y	CH$_2$E	CH$_2$EY	CH$_3$Y*[d]
Mn^{II}	n.d.[b]	> 2 x 10^{-5}	n.d.[b]	1.6(4) x 10^{-5}	4.7(8) x 10^{-6}	n.d.[b]
[a]Mn^{II}$_{pH\ 8.5}$	n.d.[b]	n.d.[b]	n.d.[b]	4.8(5) x 10^{-6}	7(3) x 10^{-7}	n.d.[b]
Fe^{IIc}	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	3.7(3) x 10^{-8}
Co^{II}	7(2) x 10^{-6}	7(3) x 10^{-9}	8(1) x 10^{-9}	3.4(4) x 10^{-8}	n.d.[b]	1.9(3) x 10^{-9}
Ni^{II}	6(4) x 10^{-7}	9(2) x 10^{-10}	1.5(5) x 10^{-9}	7(1) x 10^{-9}	n.d.[b]	n.d.[b]
Cu^{II}	n.d.[a]	4(1) x 10^{-14}	2.1(8) x 10^{-13}	1.4(2) x 10^{-13}	n.d.[b]	5(2) x 10^{-14}

Table 1 | Dissociation constants (K_d) (in M) determined for the metal complexes of various protein constructs. All titrations were performed in a metal-free buffer solution (15 mM MOPS) at pH 6.5 with 150 mM NaCl unless stated otherwise. [a]Titration was performed in a metal-free buffer solution (15 mM Tris-HCl) at pH 8.5 with 150 mM NaCl. [b]Not determined. [c]Titration was performed under anaerobic conditions. [d]**CH$_3$Y*** refers to a variant of **CH$_3$Y** wherein the heme cofactor has been removed via steric occlusion.