

Design, Optimization and Characterization of a *de novo* Gold Hydroaminase

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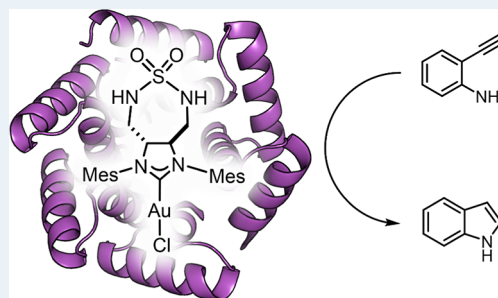
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Supporting Information

ABSTRACT: Artificial metalloenzymes (ArMs) offer a versatile and evolvable platform to extend the biocatalytic repertoire. Here we report the assembly of an ArM resulting from supramolecular anchoring an *N*-heterocyclic carbene Au(I) complex into a *de novo* designed tandem repeat protein (TRP). We identified a variant that, compared to the free cofactor, led to a higher catalytic activity for the Au-catalyzed hydroamination of 2-ethynylaniline. Structure-guided mutagenesis of this variant improved the activity, resulting in a double mutant displaying up to 4-fold higher catalytic rates than the original TRP. Biophysical and crystallographic analysis revealed distinct cofactor binding poses, with single mutations reshaping the active site and correlating with improved catalytic performance. Importantly, the TRP scaffold imparted robustness, preserving catalytic activity under acidic conditions, in the presence of organic cosolvent, and at elevated temperatures, where the free cofactor was deactivated. This work highlights the potential of *de novo* designed proteins to harbor non-natural metal cofactors and points to design principles for stabilizing sensitive catalysts under chaotropic conditions.

KEYWORDS: artificial metalloenzymes, gold catalysis, supramolecular anchoring, computational design, cofactor binding site design



INTRODUCTION

Metal cofactors expand the chemical space available to natural enzymes, catalyzing diverse and challenging reactions, from photosynthesis to oxygen transfer.¹ Most metalloenzymes rely on first-row transition metals such as iron or copper to catalyze their chemistry. Synthetic chemists have access to a much larger repertoire of chemical elements, and often rely on precious metals bound to synthetic ligands to catalyze synthetically relevant transformations. Such organometallic catalysts are not found in living systems, and thus the unique chemistry they catalyze is mostly absent from Nature.

The field of artificial metalloenzymes (ArMs) has significantly advanced in recent years, enabling the development of ArMs that catalyze a wide-range of abiotic transformations through incorporation of synthetic metal catalysts.^{2–6} However, much of the existing literature relies on a small group of natural *apo* proteins which display a high affinity for inhibitors, cofactors or metals as a basis for tethering such catalysts. Particularly successful examples include the biotin–streptavidin technology, serum albumins, multidrug resistance regulator LmrR, hemoprotein reconstitution and the use of human carbonic anhydrase inhibitors.^{7–15} Owing to significant advances in *de novo* protein design,^{16–22} there has recently been much interest in the use of computational tools to design new protein scaffolds for biocatalytic purposes.^{22–28} Beyond representing a significant achievement, these *de novo* designed proteins embody many features of biotechnological and

industrial relevance. For instance, their increased thermal stability, pH tolerance, and solvent compatibility make them promising candidates for applications in biocatalysis.

Capitalizing on our extensive experience with supramolecular anchoring using the biotin–streptavidin technology, we set out to design an ArM based on noncovalent interactions to localize an abiotic organometallic cofactor within a *de novo* designed protein. With the aim of exploring novel chemical space, we selected an *N*-heterocyclic carbene gold(I) complex and set out to anchor it within a *de novo* designed host protein.

Accordingly, we selected a computationally designed tandem repeat protein (TRP), tailored to bind to a sulfamide-containing NHC ligand (sulfaNHC). This ligand was originally developed for the assembly of a Ru-based metathesis catalyst,²⁹ and we surmised that the cavity designed for ring-closing metathesis may be large enough to accommodate the Au(I)-catalyzed hydroamination of 2-ethynylaniline.

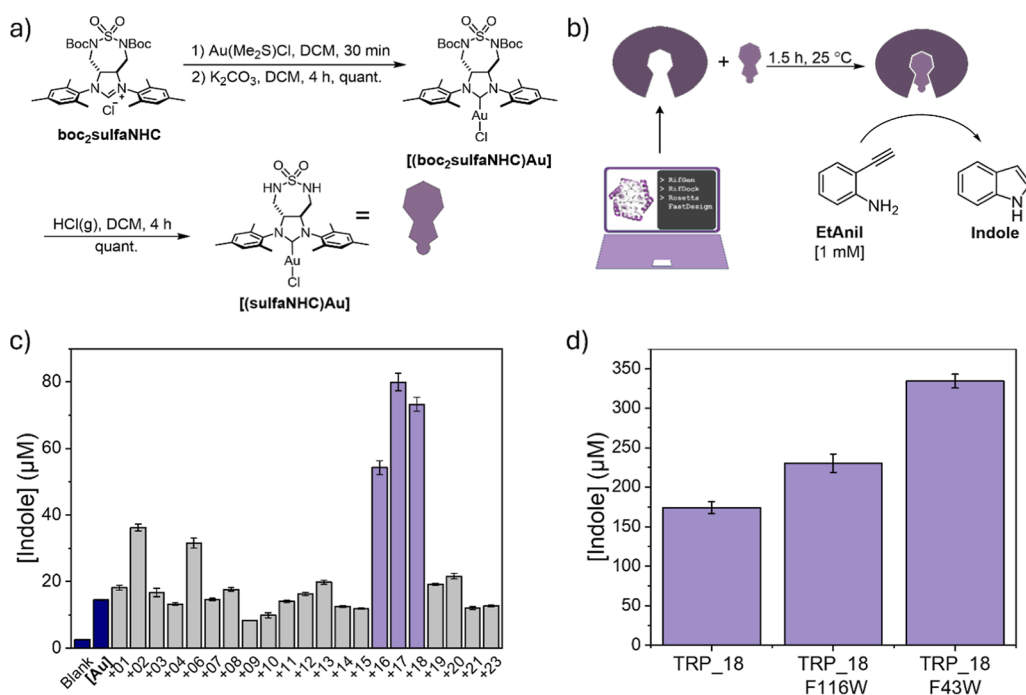
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Scheme 1. Artificial Metalloenzyme Assembly and Initial Screening^a

^a(a) $[(\text{sulfaNHC})\text{Au}]$ cofactor assembly from the ligand precursor $(\text{boc})_2\text{sulfaNHC}$; see Supporting Information Section 2, Schemes S1 and S2 for synthetic details; (b) Incubation of designed TRP Variants and the gold cofactor yields an active hydroaminase; see Table S1 (Supporting Information Section 1) and Figure S2 (Supporting Information Section 4), for information regarding protein purification; (c) Screening of the 23 TRP designs for indole production from EtAnil by means of UPLC; the controls “Blank” (no cofactor, no protein) and $[\text{Au}]$ ($[(\text{sulfaNHC})\text{Au}]$ cofactor only) are displayed in navy; indole production for the top three mutants is indicated in purple; error bars represent standard deviation between duplicate measurements; see Supporting Information Section 6.1.1 for further reaction details; (d) Hydrominase activity was compared for two tryptophan-containing mutants TRP_18 F116W and TRP_18 F43W. TRP_18 F43W displayed a 1.9-fold improvement and was selected as a parent for further mutagenesis; error bars represent standard deviation between triplicate measurements; see Supporting Information Section 6.1.2 for further reaction details; for all the above data, see Figures S8–11 for analytical methods and calibration curves (Supporting Information Section 6.2), and Figures S20–S24 for data from the library screening (Supporting Information Section 7).

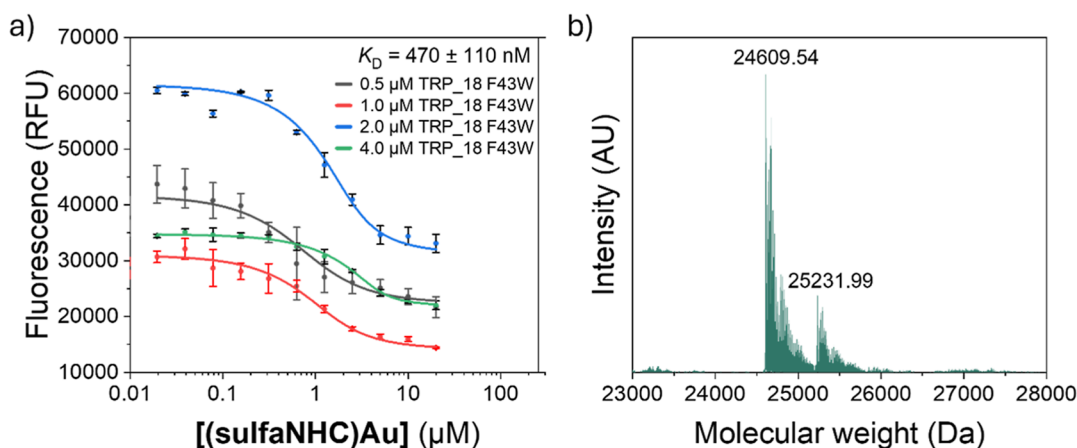


Figure 1. Structural characterization of $[(\text{sulfaNHC})\text{Au}] \cdot \text{TRP_18 F43W}$ (a) analysis of tryptophan quenching data for TRP_18 F43W upon addition of the $[(\text{sulfaNHC})\text{Au}]$ cofactor, providing a $K_D = 470 \pm 110$ nM. See Supporting Information Section 8.2 for assay details and Figure S25 for controls. (b) Deconvoluted, smoothed mass spectrum of $[(\text{sulfaNHC})\text{Au}] \cdot \text{TRP_18 F43W}$. Both the *apo* TRP_18 F43W (calc. 24610.08 Da, detected: 24609.54 Da) and *holo* $[(\text{sulfaNHC})\text{Au}] \cdot \text{TRP_18 F43W}$ (calc. 25233.26 Da, detected: 25231.99 Da), are detected. This leads to a mass shift which is consistent with the molecular weight of the cofactor without its chloride ligand (calc. 623.18 Da, detected: 622.45 Da). See Supporting Information Section 8.1 for details.

RESULTS AND DISCUSSION

The assembly of the cofactor was achieved by metalation and deprotection of the previously reported $\text{boc}_2\text{sulfaNHC}$ ligand from our group.²⁹ This ligand had been designed with a hydrogen bonding sulfamide moiety, enabling it to be

anchored non-covalently to proteins via specifically designed hydrogen bond interactions, Scheme 1a.²⁹ A library of TRPs was designed using the RifGen/RifDock suite of programs³⁰ to dock the related ruthenium cofactor and its hydrogen bonding residues inside a protein cavity, followed by Rosetta FastDe-

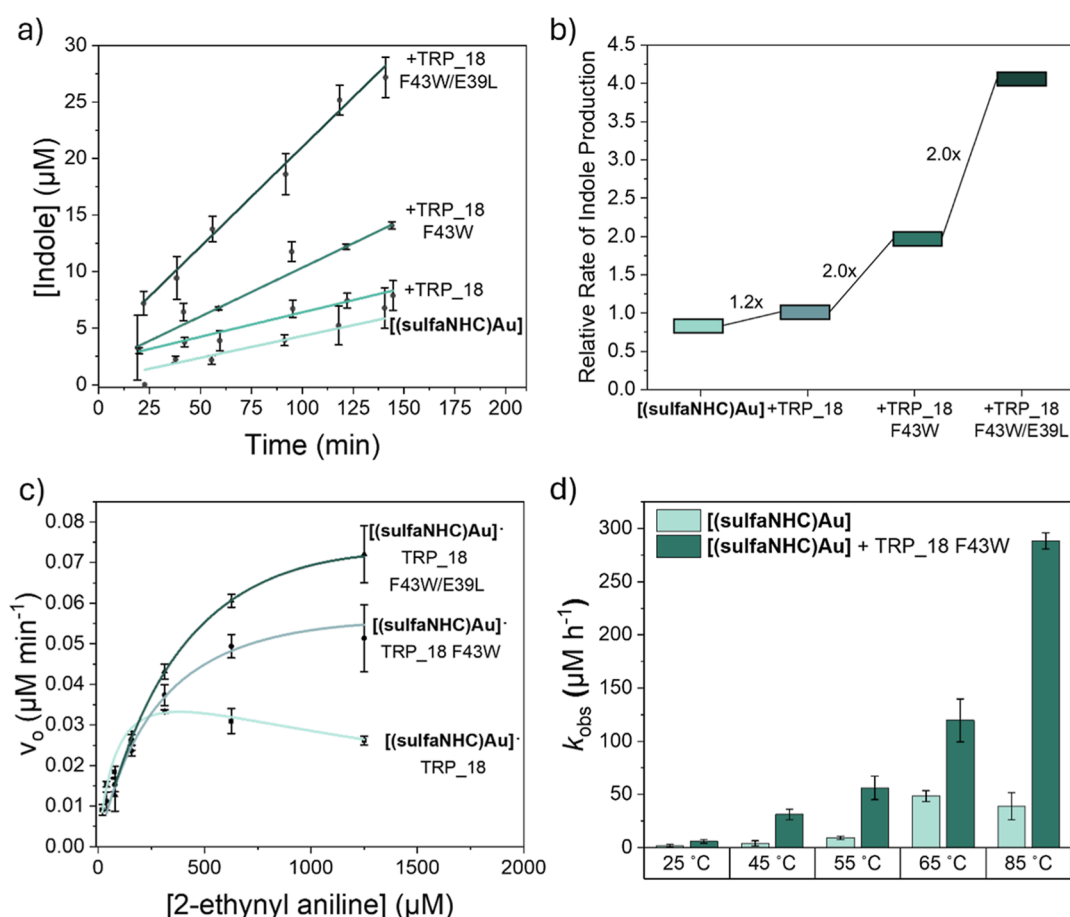


Figure 2. Catalytic performance of the ArMs. (a) Initial rates resulting from mutagenesis of the TRP_18 scaffold. See [Supporting Information Section 6.1.3](#) for reaction details. (b) Relative initial rate improvements, revealing a 4-fold increase in activity for the evolved double mutant [(sulfaNHC)Au]·TRP_18 F43W/E39L compared to [(sulfaNHC)Au]·TRP_18. (c) Michaelis–Menten saturation kinetic profile of [(sulfaNHC)Au]·TRP_18 and its mutants. See [Supporting Information Section 6.7](#) and [Figure S16](#) details. (d) Rate enhancement through heating the artificial hydroaminase. See [Supporting Information Section 6.1.4](#) and [Figure S5](#) for details. Prior to kinetic data collection, quenching studies ensured the viability of the method, [Supporting Information Section 6.3](#) and [Figure S12](#). For all data, error bars represent standard deviation between triplicate measurements.

sign³¹ to optimize the amino acid sequence and the backbone around the docked cofactor. With the cofactor [(sulfaNHC)-Au] in hand, we expressed and purified by affinity chromatography 21 of the 23 TRP constructs, as two of these could not be expressed in high enough yield, [Supporting Information Section 4.3](#) and [Figure S1](#). Following incubation with [(sulfaNHC)Au], we screened these ArMs, [(sulfaNHC)Au]·TRP_1–23, for the hydroamination of 2-ethynylaniline (EtAnil) to indole, [Scheme 1b,c](#).

The initial screening revealed that [(sulfaNHC)Au]·TRP_16, 17 and 18 afforded the highest amount of indole after 23 h. However, further characterization revealed that, unlike [(sulfaNHC)Au]·TRP_18, [(sulfaNHC)Au]·TRP_17 did not exhibit saturation behavior and instead afforded reduced activity with excess protein, [Supporting Information Section 6.4](#) and [Figure S13](#). Therefore, TRP_18 was selected for further studies. Testing reactivity with and without the *N*-terminal His₆-purification tag (His-tag) revealed little activity difference, so the His-tag was retained for the majority of catalytic experiments, [Supporting Information Section 6.5](#) and [Figure S14](#).

After confirming the catalytic activity of [(sulfaNHC)Au]·TRP_18, we set out to (i) quantify its dissociation constant for cofactor binding, and (ii) improve its hydroaminase activity by

mutagenesis and screening. To monitor binding, we relied on tryptophan fluorescence quenching.^{32,33} Since the original TRP_18 scaffold contained no tryptophan in its binding site, we tested two mutants, TRP_18 F116W and TRP_18 F43W, both of which contain an active site tryptophan residue, and were previously shown to be viable variants for [(sulfaNHC)-Ru] binding.²⁹ Both [(sulfaNHC)Au]·TRP_18 F116W and [(sulfaNHC)Au]·TRP_18 F43W revealed improved activity compared to [(sulfaNHC)Au]·TRP_18 after 23 h, with [(sulfaNHC)Au]·TRP_18 F43W being the most active. Accordingly, TRP_18 F43W was selected for further studies, [Scheme 1d](#).

Affinity measurements by tryptophan fluorescence quenching of TRP_18 F43W revealed strong binding of [(sulfaNHC)Au] with a $K_D = 470 \pm 110$ nM, [Figure 1a](#). Moreover, native mass spectrometry further confirmed cofactor binding with a mass shift of +622.45 Da, corresponding to the molecular weight of [(sulfaNHC)Au], [Figure 1b](#).

In order to further increase the hydroaminase activity we performed two rounds of site-saturation mutagenesis, using the ruthenium-bound X-ray crystal structure as a guide (PDB: 8S6P)²⁹ for selecting 12 positions for mutagenesis (E4, E39, Y50, E74, L78, F81, E109, E144, A148, E179, L183, V187).

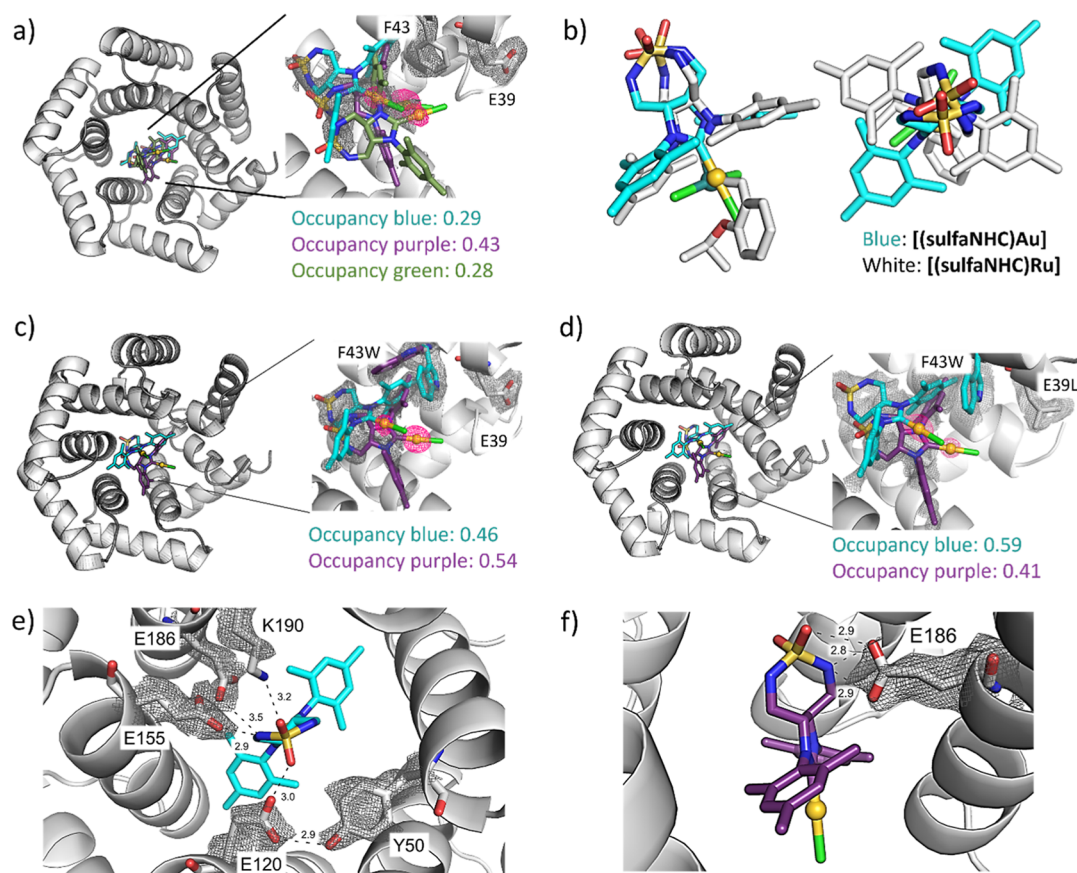


Figure 3. TRP_18 mutants cocrystallized with [(sulfaNHC)Au]. Electron densities are displayed as a gray mesh ($\sigma = 1$) with anomalous density in magenta ($\sigma = 2$). (a) Overview of [(sulfaNHC)Au]·TRP_18 (PDB 9H10, 2.05 Å resolution). (b) Superposition of [(sulfaNHC)Au] (PDB 9RBE) and [(sulfaNHC)Ru] (PDB 8S6P). (c) Overview of [(sulfaNHC)Au]·TRP_18 F43W (PDB 9RBE, 2.1 Å resolution). (d) Overview of [(sulfaNHC)Au]·TRP_18 F43W/E39L (PDB 9RB0, 2.2 Å resolution). (e) Overview of the blue binding mode in [(sulfaNHC)Au]·TRP_18 F43W/E39L, with electron density displayed for key binding residues. (f) Overview of purple binding mode in [(sulfaNHC)Au]·TRP_18 F43W/E39L, with electron density displayed for E186. See Supporting Information Section 5.3 and Figure S4 for more binding site details. All crystal production, data collection and processing details can be found in Supporting Information Section 5, Figure S3 and Tables S2–S4.

The first round led to the identification of the mutant TRP_18 F43W/E39L which improved indole production by 1.6-fold over TRP_18 F43W after 23 h. Unfortunately, the second round with TRP_18 F43W/E39L as parent led to no further improvement, Supporting Information Section 7.2. We therefore set out to analyze the origin of activity enhancement. Importantly, we could exclude tighter binding as the cause of activity improvement as TRP_18 F43W/E39L bound the [(sulfaNHC)Au]-cofactor less tightly than TRP_18 F43W ($K_D = 860 \pm 91$ nM, Supporting Information Section 8.2.2 and Figure S26).

One limitation of gold(I) NHC systems is the tendency of these catalysts to form dimers at high concentrations which can impede catalysis.^{34,35} We noticed that the relative improvement in activity of the ArM [(sulfaNHC)Au]·TRP_18 over the free cofactor [(sulfaNHC)Au] depended on cofactor concentration, with higher cofactor concentrations leading to greatly enhanced improvements in comparison to lower concentrations. For example, 16 μ M cofactor resulted in a 15.9-fold improvement in activity upon incorporation inside the 20 μ M protein, while 1 μ M only produced a 3.5-fold improvement after 17 h, Supporting Information Section 6.6 and Figure S15. This suggests that one key feature for the improvement in activity of the ArM may be due to the protein minimizing the cofactor dimerization.

In summary, the three mutants identified by iterative saturation mutagenesis, i.e. [(sulfaNHC)Au]·TRP_18, [(sulfaNHC)Au]·TRP_18 F43W and [(sulfaNHC)Au]·TRP_18 F43W/E39L, afforded turnover numbers (TONs) of 13, 25 and 38, respectively after 24 h, Figure S21. To complement these assays, we investigated whether a rate enhancement could be observed in our system. In order to test this, we determined initial rates over the first 80 min of the reaction. We carried this out at low cofactor: protein ratios (2 μ M [(sulfaNHC)Au]: 20 μ M TRP), ensuring a binding efficiency of cofactor to protein of >95% and thus minimizing the effects of binding affinity on ArM activity. The three hydroaminase mutants displayed varying initial rates (k_{init}), Figure 2a,b, with each mutant performing 2-fold better than the previous. We thus conclude that the origin of improvement after 23 h is at least in part kinetic; the second coordination sphere environment affects the rate of the hydroamination to various degrees for each mutant along the evolutionary trajectory.

To further scrutinize the cause of this rate enhancement, we performed Michaelis–Menten kinetics, Figure 2c. The results indicate that the origin of rate enhancement can be traced back to an enhancement in k_{cat} by 2.56-fold, and the minimization of substrate inhibition, which severely impacts the activity of the original variant [(sulfaNHC)Au]·TRP_18 under the screening

conditions (1 mM substrate), [Supporting Information](#) Section 6.7 and [Figure S16](#).

One attractive feature of *de novo* designed proteins is their resilience toward chaotropic agents including temperature, pH and cosolvent. The reactions were typically carried out room temperature, acidic pH and moderate MeCN concentration (pH 5.0, 12.5% (v/v) MeCN). We also tested the system at higher MeCN concentrations: Beyond 30% MeCN, only baseline activity was observed, [Supporting Information](#) Section 6.9 and [Figure S19](#). We were also curious to test the effect of temperature on the catalytic performance. For most systems, the reaction rate increases by 2-fold every 10 °C, but this law breaks down at high temperatures due to enzyme denaturation.^{36,37} In contrast to the free cofactor [(sulfaNHC)Au], whose activity eroded above 65 °C, the TRP_18 F43W ArM maintained a temperature-dependent increase in k_{obs} , [Figure 2d](#). This effect was also observed for the evolved variant [(sulfaNHC)Au]·TRP_18 F43W/E39L, suggesting that the thermostable TRP scaffold shields the cofactor from heat-induced deactivation, [Figures S6 and S7](#). This highlights the potential for thermostable *de novo* scaffolds to protect fragile catalysts from temperature-induced deactivation.

Next, we turned to X-ray crystallography to investigate cofactor positioning and its possible effect on rate enhancement. Cococrystallization relying on sitting-drop vapor-diffusion yielded crystals of [(sulfaNHC)Au]·TRP_18 diffracting to 2.05 Å (PDB 9H10). The gold cofactor occupies a similar metal position as was recently reported for the related [(sulfaNHC)Ru]·TRP structure (PDB: 8S6P), but rotated by ~57°, [Figure 3b](#).³⁹ While the ruthenium structure indicated possible hydrogen bonding contacts with K190 and Y50, the positioning of the [(sulfaNHC)Au] cofactor significantly affects the potential supramolecular interactions that hold the cofactor in place. Indeed, the anomalous density maps indicate three distinct binding poses of the cofactor—displayed in blue, purple, and green in [Figure 3a](#). The blue pose is closest in orientation to the previously reported ruthenium structures, and appears to be stabilized by hydrogen bonds to E155, K190 and E120, with additional hydrophobic contacts to V151 and F81 π - π interactions which held the ruthenium cofactor in place are not observed in the gold structure, as they are hampered by the [(sulfaNHC)Au] cofactor's position. The purple pose engages E186 and packs against A148 and L8, and the green pose contacts T117. Residue Y50 adopts two conformations in this structure, one of which clashes with the blue pose, [Figure S4](#) and [Supporting Information](#) Section 5.3. Refined occupancies are 0.29 (blue), 0.43 (purple) and 0.28 (green). Comparison with the *apo* TRP_18 structure (PDB 9RAA) reveals only minimal structural changes upon cofactor binding ($C\alpha$ RMSD 0.187 Å).

Next, we determined the X-ray crystal structure of [(sulfaNHC)Au]·TRP_18 F43W to 2.1 Å resolution. With just one mutation, the third (green) cofactor pose disappeared, and the occupancies of both others increased, with the blue occupancy increasing the most (46:54, blue: purple). The overall $C\alpha$ RMSD between *apo* (PDB 9RAR) and *holo* (PDB 9RBE) structures was 0.35 Å. The main cofactor binding residues remain consistent with the previous structure, although Y50 now adopts a major conformation; forming H-bonding interactions with E120, rather than clashing with the blue cofactor pose, [Figure S4](#) and [Supporting Information](#) Section 5.3. Interestingly, the F43W mutation exhibits poor electron density which overlaps with the cofactor. Con-

sequently, two rotameric states were modeled for this residue, one of which clashes with the blue cofactor. The two rotamers are color-coded to correspond with the different cofactor poses, inset [Figure 3c](#).

Finally, we solved the X-ray structure of [(sulfaNHC)Au]·TRP_18 F43W/E39L to 2.2 Å resolution, [Figure 3d](#). The same two binding poses persist, and the blue pose is further enriched (blue/purple, 0.59:0.41). In this variant, F43W is well ordered and adopts the nonclashing rotamer. This suggests that the mutation E39L, which is adjacent to F43W on the helix, stabilizes this rotamer position. Since the two residues appear to be too far to interact directly (e.g., via C-H... π interactions), we hypothesize that E39L indirectly stabilizes F43W by rigidifying the local backbone and optimizing rotamer placement. The overall $C\alpha$ RMSD between *apo* (PDB 9RAQ) and *holo* (PDB 9RB0) structures of TRP_18 F43W/E39L was found to be 0.355 Å. The binding sites associated with the blue and purple poses in TRP_18 F43W/E39L are depicted in [Figure 3e,f](#).

The progressive enrichment of the blue pose, [Figure 3](#), correlates with increased hydroaminase activity, supporting the hypothesis that evolution of the binding pocket favors a specific, catalytically active pose. In light of the results from the Michaelis–Menten analysis, this conformation likely enhances activity by reducing susceptibility to substrate inhibition. It is also possible that these mutations contribute directly to rate enhancement by another means. For example, the F43W residue, stabilized by E39L, is positioned adjacent to the gold center and may regulate substrate access via π - π interactions with the benzene ring of 2-ethynylaniline.

To further probe the effect of this “blue” binding pose on rate, we mutated the residues thought to be involved in its stabilization (E155A and K190A). As expected, the E155A mutation was found to reduce the activity of the enzyme for both mutants –39% for TRP_18 F43W and 69% for TRP_18 F43W/E39L, perhaps reflecting the increased occupancy of the blue binding mode in the F43W/E39L mutant. However, the K190A mutation had minimal effect on the hydroaminase activity for [(sulfaNHC)Au]·TRP_18 F43W/K190A, suggesting that hydrogen bonding for K190A may have little impact on activity and thus presumably binding, [Supporting Information](#) Section 6.8 and [Figures S17 and 18](#).

To assess whether the observed cofactor binding poses could have been anticipated to enhance activity, a DFT-optimized transition state for indole ring formation was aligned with the crystal structures. Both the “blue” and “purple” cofactor states accommodate the transition state with minimal steric clash across all variants, whereas the “green” state exhibits minor clashes, which may explain its absence in the higher-activity variants, [Figure S27](#). Notably, the “blue” state uniquely supports two plausible substrate orientations. Since no direct interactions between the transition state and either the protein or the Au center were observed, differences in activity between cofactor binding poses are likely driven by subtle effects, such as hydrophobic interactions or substrate recruitment.

To further evaluate substrate recruitment, the substrate was docked into the *holo* crystal structures using PLACER, while keeping the cofactor approximately fixed.³⁸ In these calculations, the cofactor remained well localized only in the “blue” state, whereas the “purple” and “green” states showed increased positional uncertainty. In all states and variants, the substrate was placed near the Au atom with moderate confidence, but no clear interactions were identified that

would enforce a specific substrate orientation or positioning within the pocket. Given the relatively small size of the EtAnil substrate, this is not unexpected.

Attempts to model substrate binding using structure prediction tools such as AlphaFold3, RoseTTAFold 3, and Chai-1 were unsuccessful, as these methods failed to recapitulate the experimentally observed cofactor binding states.^{39–41} This same problem had previously been observed with the ruthenium cofactor.²⁹

MATERIALS AND METHODS

Chemicals and Substrates

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Water was purified by a Milli-Q Advantage system. DNA sequences encoding the designed proteins were purchased from IDT as subcloned genes in pET29b vector. Oligonucleotides for cloning were obtained from Microsynth AG. Enzymes and chemicals for molecular biology (Q5 High-Fidelity DNA Polymerase, High GC Enhancer, T4 DNA ligase, DpnI, Bsa-HF-v2) as well as competent cells (TOP10, DH5 α , LEMO21) were obtained from New England Biolabs (NEB).

Cofactor Synthesis

The precursor **boc₂sulfaNHC** was synthesized following previously reported procedures,²⁹ and used to produce the [(sulfaNHC)Au] cofactor in two steps. First, **boc₂-sulfaNHC** was dissolved in CH₂Cl₂ and reacted with chloro(dimethylsulfide)gold(I) and K₂CO₃ at room temperature overnight to afford the protected complex [(**boc₂sulfaNHC**)Au], which was filtered. Boc-deprotection was then achieved by purging HCl gas through a solution of the protected complex in CH₂Cl₂ at room temperature, yielding the active [(sulfaNHC)Au] cofactor as a white solid. Full experimental details are provided in the Supporting Information (Section 2).

Protein Expression and Purification

Tandem repeat proteins (TRPs) were expressed in *E. coli* LEMO21 (DE3) using autoinduction media (ZYM-5052 or ZYP-5052) at 16–20 °C for 48–72 h. Cells were harvested by centrifugation and frozen at –20 °C prior to lysis. Pellets were resuspended in lysis buffer, incubated at 37 °C, sonicated, and clarified by centrifugation. TRP variants were purified by Ni-NTA affinity chromatography, washed extensively, and eluted with imidazole-containing buffer. For screening applications, freezing and thawing was used to lyse cells instead of sonication, and eluates were used directly. For large-scale preparations, proteins were dialyzed into storage buffer, concentrated, aliquoted, and flash-frozen. When required, His-tags were removed by TEV protease digestion followed by a second Ni-NTA step. Final polishing was performed by size-exclusion chromatography on a Superdex 16/200 75 pg column, yielding monomeric TRP proteins of the expected molecular weight ($\approx$ 25 kDa). Full protocols are provided in the Supporting Information (Section 4).

Site-Saturation Mutagenesis

Mutant libraries were generated by touchdown PCR using NNK (round 1) and NDT/VHG/TTG (round 2) degenerate codons. Products were analyzed by agarose gel electrophoresis, pooled, digested with DpnI, heat inactivated and purified using a commercial PCR cleanup kit. Libraries were assembled by Golden Gate cloning and transformed into *E. coli* TOP10 or DH5 α . Colonies were pooled, and plasmid DNA was extracted using a commercial miniprep kit. The resulting plasmid libraries were then transformed into LEMO21 cells for protein expression. Plasmid transformations were performed using standard heat-shock procedures. Full experimental details are provided in the Supporting Information (Section 3).

Activity Measurements

A solution of [(sulfaNHC)Au] cofactor (2–10 μ M), MES buffer (40–100 mM, pH 5) and NaCl (150–300 mM) was combined with the TRP variants (10–20 μ M) and preincubated for 1.5 h at room

temperature. Reactions were initiated by addition of 2-ethynylaniline (1 mM, final MeCN 12.5% (v/v)) and carried out at the temperatures indicated, with aliquots taken at defined time points. Aliquots were quenched with MeOH containing an internal standard and analyzed by UPLC, and initial rates were determined from linear fits of product formation versus time. During library screening, indole formation was also monitored using a modified Ehrlich's test, which correlated well with UPLC quantification. Full experimental procedures, quenching validation, and analytical methods are provided in the Supporting Information (Section 6).

Michaelis–Menten Kinetics

Reactions were performed in MES buffer (100 mM, pH 5) containing NaCl (150 mM), TRP₁₈ variants (15 μ M), and [(sulfaNHC)Au] (2 μ M). Reactions were initiated by addition of 2-ethynylaniline at varying concentrations (final MeCN 12.5% (v/v)) and incubated at 25 °C. Aliquots were taken at defined time points, quenched, and analyzed by UPLC. Reactions were conducted in triplicate, and initial rates were extracted to generate Michaelis–Menten plots using a substrate-inhibition model. Full experimental details, data, and fitting procedures are provided in the Supporting Information (Section 6.7).

Protein Crystallography

His-cleaved TRP variants (4–12 mg·mL^{–1}) were incubated overnight at 4 °C with a 2-fold molar excess of [(sulfaNHC)Au] and centrifuged. Crystallization drops were set up at 1:1 or 1:2 ratios of protein solution to mother liquor. Crystals typically formed within 1–2 days at 18 °C. Crystals were then cross-linked with glutaraldehyde and flash-frozen. *Apo* crystals did not require cross-linking. X-ray diffraction data were collected at the diamond light source (λ = 0.9763 Å) and processed with AIMLESS. Structures were solved by molecular replacement (PHASER, PDB 9GVF), refined iteratively in REFMAC and COOT, and cofactor occupancies validated with phenix.refine. Figures were prepared in PyMOL. Further crystallization conditions, as well as full refinement statistics are provided in the Supporting Information (Section 5).

Tryptophan Quenching

Tryptophan fluorescence quenching assays were performed in 96-well black plates by titrating [(sulfaNHC)Au] (dilution series in MeCN) into TRP variants (0.4–3.5 μ M) in MES buffer (100 mM, pH 5), 150 mM NaCl. Samples were equilibrated before measuring fluorescence (λ_{ex} = 290 nm, λ_{em} = 335 nm) on a Tecan Infinite M1000 Pro. Inner filter effect and cofactor solubility were verified by UV/vis ($\leq$ 0.1 AU at excitation/emission wavelengths). Binding curves were fitted globally using a single-site binding model to determine K_D values.

Native Mass Spectrometry

TRP₁₈ F43W (80 μ M) was incubated with [(sulfaNHC)Au] (40 μ M) in MES buffer (100 mM, pH 5), 150 mM NaCl at room temperature for 1 h. The assembled ArM was buffer-exchanged into 50 mM ammonium acetate (pH 6.8) using centrifugal filters and diluted $\sim$ 10⁵-fold prior to direct infusion into a Bruker maXis-II ESI-QTOF. Spectra were acquired in positive mode, processed with Compass Data Analysis, baseline-subtracted, smoothed, and deconvoluted using a maximum entropy algorithm. Internal calibration was performed using a low-concentration tuning mix.

CONCLUSION

In this study, we report the design and mutagenesis of an artificial gold hydroaminase, which relies on supramolecular interactions to anchor a [(sulfaNHC)Au] cofactor within the binding pocket of a *de novo* designed TRP. The artificial hydroaminase was optimized by mutagenesis for the production of indole from 2-ethynylaniline. The final double mutant exhibited a 4-fold higher rate than the original scaffold. Moreover, the remarkable stability of the scaffold allows the reaction to be carried out at low pH, with 12.5% (v/v) MeCN, and at elevated temperatures.

X-ray crystallography provides a structural basis for the observed rate enhancement resulting from mutagenesis. The original [(sulfaNHC)Au]·TRP_18 structure revealed a binding mode similar to that of the previously reported ruthenium–sulfamide artificial metathase, with the sulfamide and metal found in approximately the same position in both systems. However, the gold cofactor was rotated compared to the ruthenium analogue, and also displayed two additional cofactor orientations. The origin of these conformational differences between the gold and ruthenium cofactors remains unclear, given that the ligand is identical in both systems. These differences may arise from the F116W mutation introduced in the ruthenium metathase, or from the metal identity itself, which may influence the cofactor binding in unpredictable ways.

Mutation of two close-lying residues stabilized one cofactor pose over the two others observed in [(sulfaNHC)Au]·TRP_18. As the changes in occupancy correlated with enhanced activity, we hypothesize that the stabilized pose is more catalytically competent than the alternatives. Indeed, multiple cofactor poses inside an active site expose the catalytic center to different environments, which may explain a difference in activity between mutants. These insights offer clear avenues for activity improvements by further stabilizing the active orientation through mutagenesis.

Overall, this study represents one of the first attempts to anchor an organometallic cofactor within a *de novo* designed protein solely through supramolecular interactions. This strategy poses challenges, particularly in the absence of a covalent interaction to securely anchor the abiotic cofactor. Importantly, a single mutation can shift the cofactor's position within the active site, introducing an element of unpredictability, while also allowing for adaptability and potentially improved activity. We hope this work will inspire further development of computationally designed ArMs using non-covalent anchoring strategies. By expanding beyond natural protein hosts, this strategy could open new catalytic possibilities and lead to ArMs with enhanced activity for new-to-nature reactions.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscatal.5c08100>.

Contains synthetic and experimental procedures, Supporting Information figures, further crystallographic information, protein and DNA sequences. Supporting Information Crystal structures of TRP_18 (*apo* 9RAA, *holo* 9H10), TRP_18 F43W (*apo* 9RAR, *holo* 9RBE) and TRP_18 F43W/E39L (*apo* 9RAQ, *holo* 9RB0) are available on the PDB (PDF)

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Author Contributions

Majority of catalytic experiments, biophysical characterization and crystallography (E.M.), ligand and cofactor synthesis and characterization (B.L.), directed evolution (J.U. and E.M.), select catalytic experiments (J.U. and S.B.), crystallographic advice and discussions (R.P.J.), *de novo* protein design and computational calculations (I.K.), manuscript writing (E.M.), conceptualization and supervision (T.R.W. and D.B.).

Notes

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