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Copper is a Cofactor of the Formylglycine-Generating Enzyme

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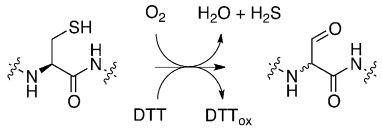
Formylglycine-generating enzyme (FGE) is an O_2-utilizing oxidase that converts specific cysteine residues of client proteins to formylglycine. We show that Cu^I is an integral cofactor of this enzyme and binds with high affinity (K_D = of 10^{-17} M) to a pair of active-site cysteines. These findings establish FGE as a novel type of copper enzyme.

Formylglycine-generating enzymes (FGEs) catalyze the O_2-dependent conversion of specific cysteine residues on client proteins to formylglycine (fGly; Scheme 1). This post-translational



Scheme 1. FGE catalyzes O_2-dependent conversion of cysteine residues to formylglycine (fGly), H_2S, and water. The enzyme requires an auxiliary reducing agent such as DTT to complete the four-electron reduction of O_2

modification is essential for the catalytic activity of phosphatases and sulfatases.[1] Reduced FGE activity in human cells leads to sulfatase deficiency.[2] In addition, FGE has emerged as a versatile tool for protein engineering, because it can introduce unique aldehyde functions into recombinant proteins.[1a,3] Initial biochemical and structural characterization of this enzyme raised an interesting mechanistic question: how does this enzyme activate O_2? None of the published crystal structures of this catalyst revealed any known redox cofactor.[4] The only redox-active features in the active site are two conserved cysteine residues, which by themselves can hardly activate O_2.[5,6] One-electron transfers between thiols and O_2 are prohibited by mismatched redox potentials, and ionic mechanisms are spin forbidden.

Recently we and others found that copper salts increase the in vitro activity of FGE up to 20-fold.[7] Although copper is a plausible agent in O_2 activation,[8] the interaction between FGE and transition metals remained mysterious. The general absence of copper in published crystal structures suggests that a potential FGE:copper complex would not be very stable.[4] On the other hand, Cu^I-dependent FGE activity is not affected by millimolar concentrations of EDTA,[7b] which is a strong ligand for Cu^{II} (pK_D = 18.8) or DTT (a strong ligand for Cu^I, pK_D = 15.3).[9] Hence, either FGE is an even stronger copper ligand, or it does not require direct metallation. In the later scenario copper might serve as an artificial source of electrons or activated oxygen species in the in vitro assay.[10]

Here we address this puzzle. We show that FGE from *Thermomonospora curvata* ($FGE_{curvata}$) binds Cu^I with an affinity similar to that of known high-affinity copper proteins.[9] We found that Cu^I binds to both active-site cysteines and remains bound throughout multiple catalytic cycles. We have previously shown that the active-site cysteines of $FGE_{curvata}$ readily form a disulfide bond under aerobic conditions.[7b] For unambiguous determination of the redox state of these two residues, we constructed an FGE variant in which all cysteines outside the active site were mutated to either serine or alanine (FGE_{4C}).[7b] This variant proved sevenfold more active than wild-type,[7b] and therefore was used in most of our subsequent experiments. FGE_{4C} also contained a disulfide bond after purification in the absence of reducing agents.[7b]

We used a published titration assay to estimate the Cu^I affinities of $FGE_{curvata}$ and FGE_{4C}.[9] A solution containing the 1:2 complex between Cu^I and bathocuproine disulfonate ($(Cu^I:(BCS)_2)$) was titrated with FGE_{4C} (Figure S1 in the Supporting Information). The transfer of Cu^I from the complex to FGE was monitored by measuring the decreasing absorption of $Cu^I:(BCS)_2$ at 483 nm. This qualitative experiment revealed that oxidized FGE_{4C} has no greater affinity for Cu^I than the control protein (BSA). In contrast, FGE_{4C} purified in reduced form showed significant Cu^I affinity (Figure S1). Because the oxidized and reduced forms of FGE_{4C} differ only by the redox state of the two active-site cysteines, we concluded that these thiols must be essential for Cu^I binding.

For a more quantitative estimation of the copper affinity of FGE (K_D) we recorded the absorption at 483 nm as a function of FGE_{4C} or $FGE_{curvata}$ concentration and fitted the resulting curves to an equation describing the equilibrium between the $Cu^I:(BCS)_2$ and FGE:Cu^I complexes (Supporting Information).[9] All titration buffers contained 2 mM cysteamine to keep the enzymes in reduced form. Because cysteamine is a comparably

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Table 1. Kinetic parameters and Cu^{I} affinities of FGE variants and auxiliary thiols.[a]

	k_{cat} [min^{-1}]	K_M [μM]	k_{cat}/K_M [min^{-1} M^{-1}]	pK_{D,Cu^I}
$FGE_{curvata}$	1.6 ± 0.1	580 ± 40	2900 ± 50	17.1[b]
FGE_{4C}	4.2 ± 0.5	230 ± 40	20000 ± 4000	17.1[b]
FGE_{S266A}	0.006	520 ± 240	49 ± 8	17.7[b]
FGE_{S290K}	n.d.	n.d.	6.6 ± 0.2	16.7[b]
FGE_{C269S}	n.d.	n.d.	≤1	n.d.
FGE_{C274S}	n.d.	n.d.	≤1	n.d.
cysteamine				14.1[c]
DTT				15.1,[c] 15.6[b]
DTBA				15.8[c]

[a] Michaelis–Menten parameters for FGE-catalyzed oxidation of a Cys-containing peptide to the fGly-containing product (Supporting Information). Apparent dissociation constants (K_D) of Cu^{I} complexes with FGE variants or low-molecular-weight thiols were determined by using a published titration assay.[9] [b] Values determined in the presence of 2 mM cysteamine. [c] Values determined in the absence of additional thiols. n.d.: low specific activities prevented accurate determination of these parameters.

weak Cu^{I} binder ($K_D = 10^{-14.1}$ M; Table 1), its presence should not affect the apparent Cu^{I} affinity of FGE dramatically. As a test of this assumption we determined the apparent Cu^{I} affinity of DTT in the absence ($K_D = 10^{-15.1}$ M) and in the presence ($K_D = 10^{-15.6}$ M) of cysteamine. Both values are in fair agreement with a published value ($K_D = 10^{-15.3}$ M).[9] With this assay we determined an apparent dissociation constant (K_D, Table 1) of the Cu^{I} complexes with FGE_{4C} or $FGE_{curvata}$. As both proteins bind Cu^{I} with similar strengths we concluded that none of the Cys residues outside the active site contributes to copper binding. Similar complex stabilities have been reported for copper chaperones from human (Atox1, $K_D = 10^{-17}$ M),[9] *Saccharomyces cerevisiae* (Atx1, $K_D = 10^{-17}$ M)[9] and *Bacillus subtilis* (CopZ, $K_D = 10^{-17}$ M),[11] thus suggesting that FGE should be well equipped to procure copper in a cellular context.[12]

Despite the remarkable copper affinity of FGE, it remains puzzling that this enzyme is fully active in a millimolar DTT solution. The apparent Cu^{I} affinity of DTT is 100 times lower than that of FGE. A 1000-fold excess of DTT should therefore destabilize the FGE:Cu^{I} complex. This is not what we observed. Reactions containing 2 μM FGE, 2 μM Cu^{I}, and 2 mM of either cysteamine ($K_D = 10^{-14.0}$ M), DTT ($K_D = 10^{-15.1}$ M), or dithiobutylamine (DTBA, $K_D = 10^{-15.8}$ M)[13] displayed approximately the same rate of product formation (Figure S2), thus showing that the Cu^{I} affinity of the redox buffer does not influence catalytic activity.

One possible explanation for this behavior could be that the Cu^{I} affinity of FGE in the presence of substrate is at least two orders of magnitude higher than for the resting enzyme. We could not directly measure the Cu^{I} affinity of the enzyme:substrate complex because the substrate peptide (Abz-SAL-**C**SPTRA-NH_2) is a proficient Cu^{I} binder in its own right, thus saturating concentrations of this peptide are incompatible with the titration assay. A substrate analogue containing Ser in place of Cys (Abz-SAL**S**SPTRA-NH_2) did not interfere with this assay, but also proved a poor FGE ligand (Figure S3). Consequently, the presence of this peptide did not change the Cu^{I} affinity of either FGE_{4C} or FGE_{S266A}.

As an alternative strategy to gauge the influence of the substrate on Cu^{I} binding by FGE, we analyzed the ability of FGE_{4C} and inactive FGE variants to exchange Cu^{I} during catalysis. For this, we designed four variants of FGE_{4C} by using a structural model based on the crystal structure of human FGE (Figure 1).

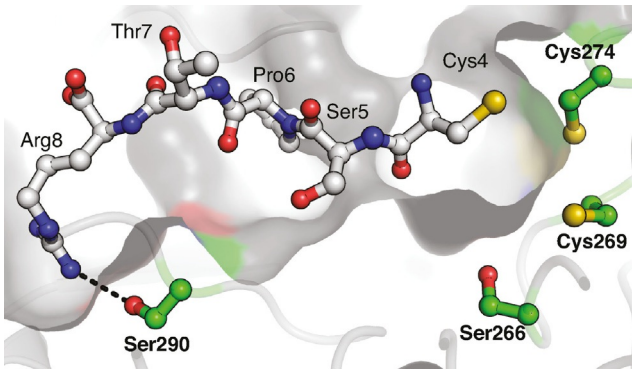


Figure 1. Structural model of $FGE_{curvata}$ based on the structure of human FGE (PDB code: 2AIJ).[4c,14] Residues 4–8 of the substrate were modeled according to a similar substrate bound to the human enzyme. This model suggests that Ser290 makes a 3.0 Å hydrogen bond to Arg8 on the substrate (dashed line).

We produced two variants, each of which had one of the active site cysteines mutated to serine (FGE_{C269S} and FGE_{C274S}). Both were inactive and unable to bind Cu^{I} (Table 1); this suggested that both thiols are important for Cu^{I} binding and for catalysis. The third variant had a conserved active-site serine at position 266 substituted to alanine (FGE_{S266A}). This reduced k_{cat} 270-fold, but did not affect K_M, and did not interfere with copper binding ($K_D = 10^{-17.6}$ M). The mutation in the fourth variant (FGE_{S290K}) was designed to impair substrate binding. Ser290 is at the bottom of the substrate binding groove, more than 15 Å from the catalytic site (Figure 1).[4c] We mutated this residue to lysine in order to block substrate binding through steric and coulombic repulsion. As expected, the corresponding protein could not be saturated with substrate, and the catalytic efficiency (k_{cat}/K_M) was reduced 440-fold. Cu^{I} affinity reduced only 2.5-fold ($K_D = 10^{-16.7}$ M; Table 1).

We tested the ability of these variants to compete with FGE_{4C} for Cu^{I} during catalysis in reactions containing 0.5 μM FGE_{4C}, 0.5 μM $CuSO_4$, 2 mM DTT, 50 mM EDTA, and 200 μM substrate (Figure 2). The reactions were started by addition of FGE_{4C}. After one minute the reaction mixtures were supplemented with a ninefold excess of FGE_{C269S}, FGE_{C274S}, FGE_{S266A}, FGE_{S290K}, or BSA (Figure 2). FGE_{S266A} reduced FGE_{4C} activity approximately 15-fold (m_2/m_1, Figure 2A), consistent with redistribution of limiting Cu^{I} among 0.5 μM FGE_{4C} and 4.5 μM FGE_{S266A}. Addition of more Cu^{I} immediately restored full FGE_{4C} activity (Figure S4), thus confirming Cu^{I} as the limiting factor.

The rate at which Cu^{I} redistributed between FGE_{4C} and FGE_{S266A} (0.07 ± 0.02 min^{-1}, Figure 2) provides an estimation of how fast the FGE_{4C}:Cu^{I} complex decays (k_{off,Cu^I}; Figure 2). This

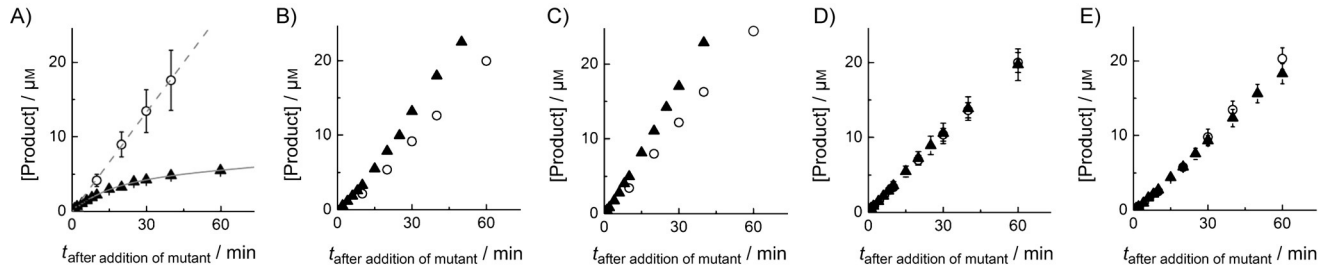


Figure 2. FGE_{4C}-catalyzed product formation as a function of time (○) in reactions containing 0.5 μM FGE_{4C}, 200 μM substrate, 0.5 μM $CuSO_4$, 2 mM DTT, 50 mM EDTA, 50 mM NaCl and 50 mM Tris (pH 8). The reaction mixtures were supplemented with 5 μM A) FGE_{S266A}, B) FGE_{C274S}, C) FGE_{C269S}, D) BSA, or E) FGE_{S290K}, one minute after initiation with FGE_{4C} (▲). In A), ▲ data were fitted to the function $[P] = A(1-\exp(-k_{off,Cu^I}t)) + m_1 t$ (——), and the ○ data were fitted to $[P] = m_2 t$ (-----), where A is the concentration of product formed until Cu^I redistribution between FGE_{4C} and $FGES_{266A}$ is complete (4.0 μM), k_{off,Cu^I} is the rate at which the FGE_{4C}:Cu^I complex decays (0.07 min^{-1}), m_1 is the residual activity after Cu^I redistribution ($m_1/[FGE_{4C}] = 0.05$ min^{-1}), and m_2 is the activity of Cu^I-complemented FGE_{4C} ($m_2/[FGE_{4C}] = 0.8$ min^{-1}). The data are averaged values from two or more independent experiments.

rate is eleven times slower than the catalytic turnover ($m_2/[FGE_{4C}] = 0.8$ min^{-1}), thus suggesting that metal binding and unbinding cannot be part of the catalytic cycle. The same competition experiment showed that neither BSA, FGE_{C269S}, FGE_{C274S}, nor FGE_{S290K} can extract Cu^I from FGE_{4C}. For BSA and the two cysteine variants this result is consistent with their complete lack of Cu^I affinity (Table 1). FGE_{S290K}, however, is a strong Cu^I binder (Table 1), but its ternary complex with copper and substrate is weak. The observation that FGE_{S290K} cannot sequester Cu^I in the FGE_{4C}-catalyzed reaction is consistent with the idea that substrate-binding increases the apparent Cu^I affinity of FGE. This finding, in combination with the observation that the Ser-containing substrate analogue is a poor FGE ligand, indicates that the thiol function of the substrate might be the third copper ligand in the active site.

In a final experiment we used EPR spectroscopy to probe the redox state of copper bound to FGE_{4C}. Freeze-quenched reactions containing FGE_{4C}, Cu^I, EDTA, DTT, and substrate yielded a featureless EPR spectrum, not significantly different from that measured with a control sample (without added copper; Figure 3). Apparently, the accumulating copper species during catalysis is EPR silent. In contrast, a sample without DTT showed the clear EPR signal of Cu^{II}, whereas a control reaction

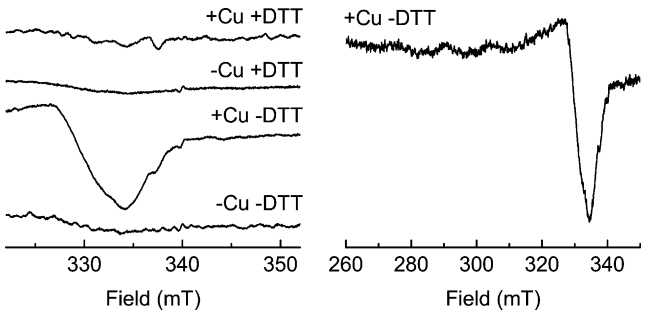
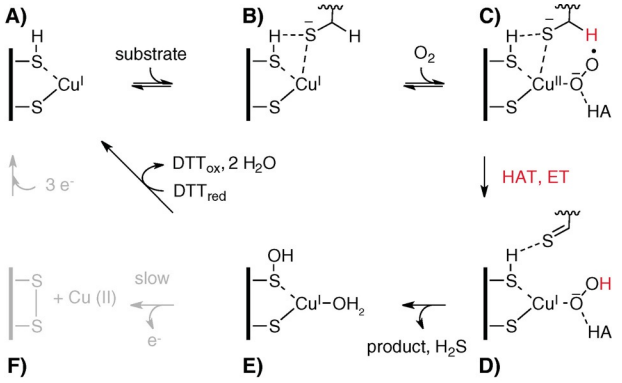


Figure 3. X-band (~9.52 GHz) continuous-wave EPR spectra of 50 μM FGE_{4C} and 0.5 mM substrate in the absence or presence of 50 μM $CuSO_4$ and/or 5 mM DTT. Temperature: 150 K; modulation amplitude: 1 mT; microwave attenuation: 15 dB (6.346 mW); 160 scans each. Spectra were acquired on a Bruker Elexsys 500 spectrometer equipped with a super-high Q resonator. Left: detail of the region of the strongest Cu^{II} signal component. Right: full spectrum of the Cu^{II} species in the absence of DTT.

containing no DTT and no added copper was again EPR silent (Figure 3). Accumulation of a Cu^{II} species is consistent with the previous observation that FGE_{4C} in absence of DTT oxidizes to the disulfide form, which does not bind copper.

The combination of catalytic and structural analyses of FGE described here and elsewhere[4a–c, 7, 15] strongly implicates FGE as a copper-metalloenzyme: the active form strongly binds one equivalent of Cu^I in the active site;[7b] the Cu^I:protein complex remains intact throughout the entire catalytic cycle (Figure 2); other transition metals cannot complement FGE,[7] thus suggesting that the cofactor engages in redox chemistry;[8] FGE reduces O_2 by using two electrons from the substrate and two electrons from an auxiliary reducing agent such as DTT;[4a, 7b] in the presence of DTT the rate-limiting step is hydrogen-atom abstraction from the substrate;[7b] the accumulating species during catalysis is an EPR-silent species (Figure 3); in the absence of an appropriate reducing agent turnover is much slower,[4a, b, 7] and a Cu^{II}-containing species accumulates (Figure 3); this oxidized species can slowly turn over by using the substrate thiol as an electron source;[7b] and finally, addition of a proper reducing agent to this slow reaction immediately reactivates the enzyme.[7b, 15]

In our view these observations are best explained the following mechanistic proposal (Scheme 2). FGE in the cuprous state (**A**) binds substrate (**B**) and O_2 to form a cupric superoxo



Scheme 2. Plausible catalytic mechanism of FGE-catalyzed formylglycine formation.

intermediate (**C**); hydrogen atom transfer (HAT) and electron transfer (ET) from the substrate reduce this intermediate to a Cu^{I} hydroperoxo complex (**D**); the resulting thioaldehyde hydrolyzes to form the fGly-containing product and hydrogen sulfide, and the hydroperoxo complex collapses into a stable but oxidized form of FGE (**E**). In the presence of DTT this species is quickly reduced to the active resting state (**A**). In absence of a proper reducing agent, species **e** decays to the disulfide form of FGE (**F**), which does not bind copper. Therefore Cu^{I} leaves the active site and oxidizes to Cu^{II}. A much slower three-electron process restores the reduced FGE:Cu^{I} complex (**A**).

The absence of any EPR signature during turnover may be explained by the following scenarios: 1) the cupric superoxo species (**C**) is not formed or does not accumulate to a significant extent, even though the following HAT is rate-limiting; 2) species **c** does accumulate but has a diamagnetic singlet ground-state because of a highly covalent interaction between Cu^{II} and superoxide;[16] or 3) the accumulating Cu^{II} species has a triplet ground-state that does not produce an EPR signature in the X-band spectrum.[17] The reactivity of previously characterized Cu^{II} superoxo complexes shows that diamagnetic species generally do not cleave C–H bonds,[16,18] whereas paramagnetic species do.[19] Based on this, we predict that FGE forms an EPR-silent paramagnetic Cu^{II} superoxo species that mediates homolytic C–H bond cleavage.

A similar sequence of events has been implicated in the catalytic mechanisms of the copper enzymes polysaccharide monooxygenase (PMO)[20] and peptidylglycine α-hydroxylating monooxygenase (PHM).[8a,21] In PHM a Cu^{II} superoxo species has been shown to cleave the Cα–H bond of a C-terminal glycine residue. Electron transfer from a neighboring Cu^{I} center forms the Cu^{I} hydroperoxo species, which immediately eliminates water to form Cu^{II}-oxyl, which in turn hydroxylates the substrate radical. In PMOs a Cu^{II} superoxo species has been proposed to extract a hydrogen atom from the anomeric carbon (C1) in polysaccharides. Electron transfer from an auxiliary reducing agent forms a Cu^{I} hydroperoxo complex, followed by Cu^{II}-oxyl formation, and by hydroxylation of the substrate radical. FGE, PMO, and PHM oxidize their substrates by two electrons, and therefore depend on a reducing agent to fully reduce oxygen. PHM activity depends on ascorbate,[8a] in vitro FGE activity depends on thiols, and PMOs seem to accept electron donors such as gallic acid or the reduced form of cellobiose dehydrogenase.[20a] Future investigations will investigate to what extent these three reactions follow analogous catalytic mechanisms.

In conclusion, the data show that FGE is a copper-dependent oxidase. Although the reduced enzyme:Cu^{I} complex is very stable, it is highly sensitive to oxidation. The apparent instability under aerobic conditions might explain the previous difficulties in observing Cu^{I}-containing FGE by crystallography. Our discovery raises novel questions about the in vivo copper delivery to FGE, and highlights a potential connection between oxidative stress, copper homeostasis, and sulfatase deficiency in humans.

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Keywords: copper · EPR · formylglycine · oxidases · oxygen activation · protein engineering

[1] a) M. J. Appel, C. R. Bertozzi, *ACS Chem. Biol.* **2015**, *10*, 72–84; b) B. Schmidt, T. Selmer, A. Ingendoh, K. von Figura, *Cell* **1995**, *82*, 271–278; c) S. Jonas, B. van Loo, M. Hyvönen, F. Hollfelder, *J. Mol. Biol.* **2008**, *384*, 120–136.

[2] a) M. P. Cosma, S. Pepe, I. Annunziata, R. F. Newbold, M. Grompe, G. Parenti, A. Ballabio, *Cell* **2003**, *113*, 445–456; b) T. Dierks, B. Schmidt, L. V. Borissenko, J. Peng, A. Preusser, M. Mariappan, K. von Figura, *Cell* **2003**, *113*, 435–444.

[3] I. S. Carrico, B. L. Carlson, C. R. Bertozzi, *Nat. Chem. Biol.* **2007**, *3*, 321–322.

[4] a) T. Dierks, A. Dickmanns, A. Preusser-Kunze, B. Schmidt, M. Mariappan, K. von Figura, R. Ficner, M. G. Rudolph, *Cell* **2005**, *121*, 541–552; b) B. L. Carlson, E. R. Ballister, E. Skordalakes, D. S. King, M. A. Breidenbach, S. A. Gilmore, J. M. Berger, C. R. Bertozzi, *J. Biol. Chem.* **2008**, *283*, 20117–20125; c) D. Roeser, A. Preusser-Kunze, B. Schmidt, K. Gasow, J. G. Wittmann, T. Dierks, K. von Figura, M. G. Rudolph, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 81–86; d) D. Roeser, B. Schmidt, A. Preusser-Kunze, M. G. Rudolph, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2007**, *63*, 621–627.

[5] a) G. W. Luther III, A. J. Findlay, D. J. MacDonald, S. M. Owings, T. E. Hanson, R. A. Beinart, P. R. Girguis, *Front. Microbiol.* **2011**, *2*, 1–9; b) R. J. Huxtable, *Biochemistry of the Elements, Vol. 6: Biochemistry of Sulfur,* Plenum, New York, **1986**, pp. 199–268.

[6] M. D. Toscano, K. J. Woycechowsky, D. Hilvert, *Angew. Chem. Int. Ed.* **2007**, *46*, 3212–3236; *Angew. Chem.* **2007**, *119*, 3274–3300.

[7] a) P. G. Holder, L. C. Jones, P. M. Drake, R. M. Barfield, S. Bañas, G. W. de Hart, J. Baker, D. Rabuka, *J. Biol. Chem.* **2015**, *290*, 15730–15745; b) M. Knop, P. Engi, R. Lemnaru, F. P. Seebeck, *ChemBioChem* **2015**, *16*, 2147–2150.

[8] a) J. P. Klinman, *J. Biol. Chem.* **2006**, *281*, 3013–3016; b) S. T. Prigge, B. A. Eipper, R. E. Mains, L. M. Amzel, *Science* **2004**, *304*, 864–867.

[9] Z. Xiao, J. Brose, S. Schimo, S. M. Ackland, S. La Fontaine, A. G. Wedd, *J. Biol. Chem.* **2011**, *286*, 11047–11055.

[10] H. Speisky, M. Gómez, C. Carrasco-Pozo, E. Pastene, C. Lopez-Alarcon, C. Olea-Azar, *Bioorg. Med. Chem.* **2008**, *16*, 6568–6574.

[11] A. Badarau, C. Dennison, *J. Am. Chem. Soc.* **2011**, *133*, 2983–2988.

[12] T. D. Rae, P. J. Schmidt, R. A. Pufahl, V. C. Culotta, T. V. O'Halloran, *Science* **1999**, *284*, 805–808.

[13] J. C. Lukesh, M. J. Palte, R. T. Raines, *J. Am. Chem. Soc.* **2012**, *134*, 4057–4059.

[14] K. Arnold, L. Bordoli, J. Kopp, T. Schwede, *Bioinformatics* **2006**, *22*, 195–201.

[15] J. H. Peng, S. Alam, K. Radhakrishnan, M. Mariappan, M. G. Rudolph, C. May, T. Dierks, K. von Figura, B. Schmidt, *FEBS J.* **2015**, *282*, 3262–3274.

[16] P. Chen, D. E. Root, C. Campochiaro, K. Fujisawa, E. I. Solomon, *J. Am. Chem. Soc.* **2003**, *125*, 466–474.

[17] a) M. P. Lanci, V. V. Smirnov, C. J. Cramer, E. V. Gauchenova, J. Sundermeyer, J. P. Roth, *J. Am. Chem. Soc.* **2007**, *129*, 14697–14709; b) P. J. Donoghue, A. K. Gupta, D. W. Boyce, C. J. Cramer, W. B. Tolman, *J. Am. Chem. Soc.* **2010**, *132*, 15869–15871.

[18] a) D. J. E. Spencer, N. W. Aboelella, A. M. Reynolds, P. L. Holland, W. B. Tolman, *J. Am. Chem. Soc.* **2002**, *124*, 2108–2109; b) N. W. Aboelella, E. A. Lewis, A. M. Reynolds, W. W. Brennessel, C. J. Cramer, W. B. Tolman, *J. Am. Chem. Soc.* **2002**, *124*, 10660–10661; c) N. W. Aboelella, S. V. Kryatov, B. F. Gherman, W. W. Brennessel, V. G. Young, Jr., R. Sarangi, E. V. Rybak-Akimova, K. O. Hodgson, B. Hedman, E. I. Solomon, C. J. Cramer, W. B. Tolman, *J. Am. Chem. Soc.* **2004**, *126*, 16896–16911; d) N. W. Aboelella, B. F. Gherman, L. M. R. Hill, J. T. York, N. Holm, V. G. Young, Jr., C. J. Cramer, W. B. Tolman, *J. Am. Chem. Soc.* **2006**, *128*, 3445–3458; e) K. Fu-

jisawa, M. Tanaka, Y. Moro-oka, N. Kitajima, *J. Am. Chem. Soc.* **1994**, *116*, 12079 – 12080.

[19] a) R. L. Peterson, R. A. Himes, H. Kotani, T. Suenobu, L. Tian, M. A. Siegler, E. I. Solomon, S. Fukuzumi, K. D. Karlin, *J. Am. Chem. Soc.* **2011**, *133*, 1702 – 1705; b) A. Kunishita, M. Kubo, H. Sugimoto, T. Ogura, K. Sato, T. Takui, S. Itoh, *J. Am. Chem. Soc.* **2009**, *131*, 2788 – 2789; c) S. Kim, J. Y. lee, R. E. Cowley, J. W. Ginsbach, M. A. Siegler, E. I. Solomon, K. D. Karlin, *J. Am. Chem. Soc.* **2015**, *137*, 2796 – 2799; d) B. N. Sánchez-Eguía, M. Flores-Alamo, O. Orio, I. Castillo, *Chem.Commun.* **2015**, *51*, 11134 – 11137.

[20] a) W. T. Beeson, V. V. Vu, E. A. Span, C. M. Phillips, M. A. Marletta, *Annu. Rev. Biochem.* **2015**, *84*, 923 – 946; b) C. M. Phillips, W. T. Beeson, J. H. Cate, M. A. Marletta, *ACS Chem. Biol.* **2011**, *6*, 1399 – 1406.

[21] J. P. Evans, N. J. Blackburn, J. P. Klinman, *Biochemistry* **2006**, *45*, 15419 – 15429.

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