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Substrate Selectivity Analyses of Factor Inhibiting Hypoxia-Inducible Factor**

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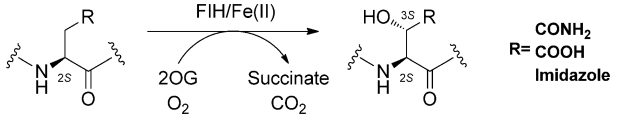
In prokaryotes and eukaryotes, 2-oxoglutarate (2OG) dependent oxygenases catalyze a wide range of oxidation reactions.[1] With oligomeric substrates, that is, proteins and nucleic acids, their identified reactions are limited to hydroxylations on carbon and *N*/*O*-methyl group demethylations through hydroxylation.[2] The factor inhibiting hypoxia-inducible factor (FIH) is a 2OG oxygenase contributing to the regulation of the hypoxic response in animals through β-hydroxylation of an Asn residue within the C-terminal transcriptional activation domain of hypoxia-inducible factor 1α (HIF-1α).[3] FIH also catalyzes the hydroxylation of conserved Asn residues in ankyrin repeat domain (ARD) proteins (Scheme 1).[4] ARDs comprise about 33 residues and



Scheme 1. FIH is a 2OG oxygenase catalyzing the post-translational hydroxylation of proteins including the hypoxia-inducible factor (HIF; R = $CONH_2$) and ankyrin repeat domain proteins (R = $CONH_2$, CO_2H, and imidazole).

hydroxylation can increase their conformational stability and may modulate protein–protein interactions.[5] Proteomic and bioinformatic analyses reveal that FIH catalyzed Asn hydroxylation is widespread and that FIH accepts a range of substrate sequences.[4a,b,5c,6]

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Recent work has revealed that FIH hydroxylates side-chains of residues other than Asn; FIH catalyzes the hydroxylation of Asp residues in the human cytoskeletal ankyrin family and His residues in the human tankyrase-2 ARD.[5c,7] We were interested in testing if FIH can catalyze the hydroxylation of other residues and therefore placed all 20 typical amino acids at the "hydroxylation position" in 20-mer peptides, based on a consensus ankyrin (CA) substrate (HLEVVKLLLEHGADV**N**AQDK)[5b] in which the residue at the potential hydroxylation site (given in bold font) was substituted. The peptides were incubated with FIH under standard conditions and matrix-assisted laser desorption ionization (MALDI) MS was used to identify modifications. In addition to the anticipated hydroxylation of the Asn (>95%) and Asp (85%) containing peptides, modifications were observed for the peptides containing His, Ser, Trp, Leu, and Ile residues (Figure 1, Figure S1, and Table ST1 in the Supporting Information). Shifts of +16 Da were observed for the Leu (CA_L, about 65% conversion) and Ile (CA_I, about 25% conversion) peptides. In contrast, the Ser peptide (CA_S) underwent mass shifts of −2 Da (about 20%) and −20 Da (about 30%). Interestingly for the His containing consensus AR peptide (CA_H), we observed not only a +16 Da shift, corresponding to hydroxylation,[7] but also lower extents of +14 Da and −2 Da shifts. We observed, to a small extent, a −2 Da mass shift for the Trp peptide. To investigate the relative efficiencies of the oxidation, the CA peptides were assayed simultaneously and then analyzed by LC-MS, for a +16 Da peak. The following order of efficiency was observed; CA_N ⩾ CA_H ≈ CA_S ≈ CA_D ⩾ CA_I ≈ CA_L (Figure S2).

We investigated modification to the Leu containing CA_L peptide by MS and NMR spectroscopy. MS fragmentation analyses of the product assigned hydroxylation to the Leu (HLEVVKLLLEHGADV**L**AQDK, Figure S3). ^{1}H-^{13}C HSQC NMR analyses of a purified mixture of modified/unmodified peptides reveals hydroxylation at the β-carbon as evidenced by characteristic shifts (α-proton: δH 4.27, δC 59.7 ppm; β-proton: δH 3.81, δC 74.8 ppm; Figure S4). 2D ^{1}H–^{1}H COSY and TOCSY analyses reveal coupling of α- and β-hydrogen atoms (J = 6 Hz) with correlation to the γ-hydrogen atom (δH 1.87, δC 24.3 ppm) and two δ-methyl groups (δH 1.01, 0.95 ppm and δC 16.2, 17.0 ppm). We then investigated modification of the CA_S peptide (Figure S5). LC-MS analyses of the product show that species giving rise to +16, −2, and −20 Da (at low levels) mass shifts co-elute with the unmodified peptide (retention time 2.5 minutes); the major

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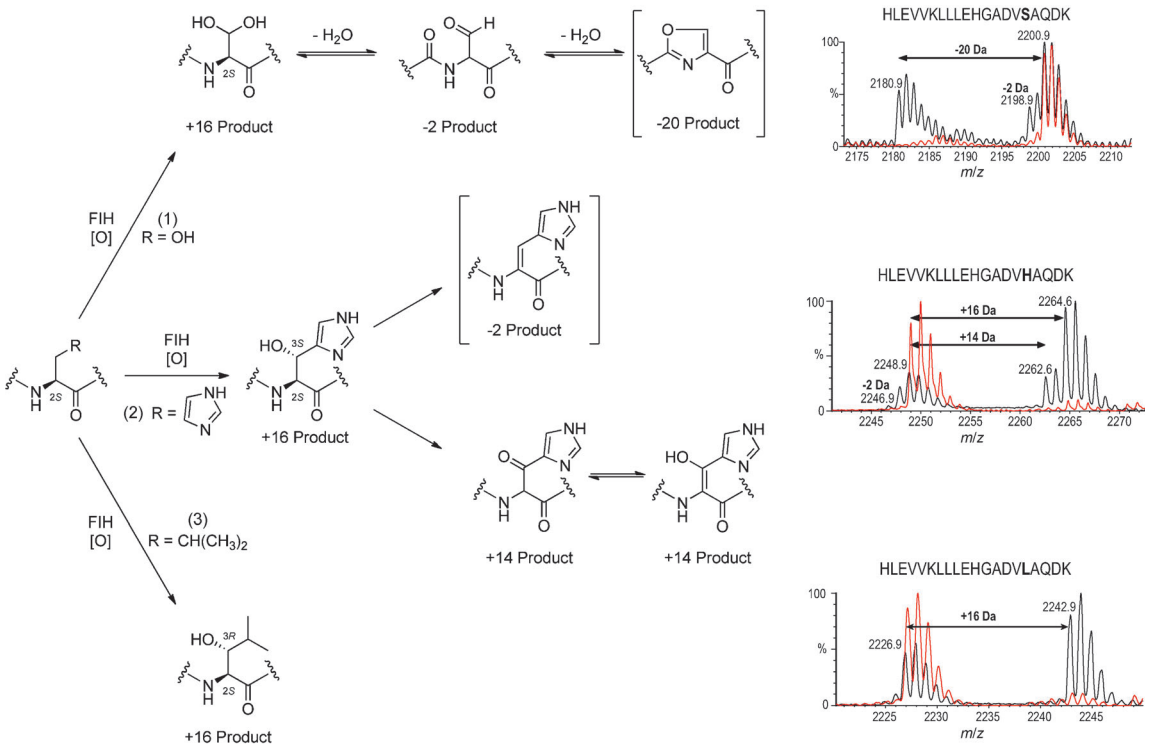

Figure 1. FIH is promiscuous with respect to the residues it can oxidize. FIH catalyzes hydroxylation of 1) Ser, 2) His, and 3) Leu residues in CA peptides; dehydration may occur during MS analysis. Peptides were analyzed by MALDI, with the red spectra corresponding to control incubations absent of FIH (but containing Fe^{II} and 2OG) with the black spectra corresponding to incubations containing FIH. Assignments were made by MS fragmentation and NMR analyses (see the Supporting Information).

portion of the −20 Da species, however, elutes at a longer time (2.9 minutes). LC-MS analyses of the purified −20 Da species collected at 2.9 minutes again showed the presence of all +16, −2, and −20 Da species, suggesting that the three products equilibrate under LC-MS conditions (under MALDI conditions, the +16 Da species was not observed). MS/MS analyses assigned the modifications as arising from Ser. These observations led to the proposal that FIH catalyzes Ser hydroxylation to give a β-geminal diol product (+16 Da) which is in equilibrium with a formylglycine/enol mixture (−2 Da). The product can condense, at least under the LC-MS conditions, with a neighboring amide to form a cyclic product resulting in a −20 Da shift (Figure 1). After methoxyamine treatment, the +16, −2, and −20 Da peaks were replaced by a single species with a +29 Da shift relative to the formylglycine product, consistent with oxime formation.[8] Treatment of the product with biotin hydrazide,[9] gave a species with a +244 Da mass shift, consistent with hydrazone formation (Figure S5). We then analyzed the purified CA_S product (approximately a 2.3:1 mixture of modified/unmodified peptides) by NMR spectroscopy (Figure S6). In D_2O solution (pD 5) resonances at δH 5.22 ppm (a singlet) and at δC 88.5 ppm were observed in the ^1H–^{13}C HSQC spectroscopy; these correlate with the proton and carbon shift of a hydrated aldehyde. The lack of proton–proton coupling is likely due to deuteration at C-α through enolization. In 95% H_2O (5% D_2O), the proton spectrum exhibits overlapping doublets at 5.22 and 5.23 ppm (each $J = 5$ Hz), both with carbon correlations in the HSQC spectrum at 88.8 ppm. The proton doublets correlated in the 2D TOCSY spectrum to resonances at 4.29 and 4.27 ppm, which were assigned as the α-protons in both 2*R* and 2*S* forms of the hydrated aldehyde.[10] These protons correlate to δC 58.9 and 58.1 ppm, respectively, in the HSQC spectrum recorded in 95% H_2O; neither of these cross-peaks are apparent in D_2O, consistent with these carbon atoms being deuterated in excess D_2O (Figure S6). The presence of both D- and L-formylglycine was also evidenced by the observation that in 95% H_2O, TOCSY correlations to amide NH proton shifts (8.28 and 8.20 ppm) were observed from the β-proton doublets, and that two Val α-proton HSQC correlations (δH/δC 4.13/59.3 ppm and 4.15/59.3 ppm) were observed in both H_2O and D_2O for the single valine (Val 15) at the −1 position relative to the hydroxyserine residue.

We have previously observed FIH-catalyzed His-hydroxylation of tankyrase-2 ARD.[7] Here, we observed that the consensus AR peptide containing a His residue at the target hydroxylation position (CA_H) not only undergoes hydroxylation (+16 Da), but also modifications manifesting +14 and −2 Da shifts (Figure 1). The presence of these shifts was also demonstrated by LC-MS (Figure S7 i). We propose that the β-hydroxylated CA_H product is further oxidized to give a β-oxo-His-residue, likely existing as a mixture of keto and enol forms, giving rise to the observed +14 Da increment. Alternatively, the β-hydroxy-His-residue may dehydrate to form α,β-dehydrohistidine to give the −2 Da mass shift, or desaturation may occur directly as known for other 2OG oxygenases.[11] To test the FIH dependency of formation of the

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+14/−2 Da species, we re-incubated about 50% hydroxylated CA_H with Fe^{II} and 2OG. MALDI analyses show that with co-factors but without FIH, there is no increase in intensity for the two species after incubation, whereas with FIH, the intensities of the +14/−2 Da species increased (Figure S7ii), implying that formation of β-oxo-His and dehydrohistidine residues is FIH-dependent. Due to the low conversion efficiency for modification of Ile- and Trp-containing peptides, we were unable to isolate sufficient amounts of modified material for NMR or MS analyses. It is likely that the +16 Da modification of the Ile containing peptide CA_I represents hydroxylation as occurs for Leu oxidation. For CA_W, only a −2 Da (instead of +16 Da) shift was observed (about 23%) after incubation with FIH (Figure S8i). It is possible that the −2 Da modification results from FIH-catalyzed β-hydroxylation followed by dehydration, facilitated by a conjugated system (Figure S8ii). However, because there was no evidence for a +16 Da species, direct desaturation is also possible, as precedented for other 2OG oxygenases.[1]

The observation that FIH catalyzes Leu/Ile residue hydroxylation is notable because all other reported FIH substrates have polar side-chains that can bind in a pocket adjacent to the active site iron.[5c] In these structures, the substrate Asn amide side-chain is positioned to hydrogen bond with the FIH/Gln-239 amide side-chain. In the case of the ankyrin fragments containing appropriately positioned His and Asp residues, the hydroxylated residue is positioned similarly; though in the former case some movement of FIH Gln-239 was observed. To investigate the structural basis of the promiscuity of FIH, we determined structures of FIH·Zn^{II}·*N*-oxalylglycine (NOG) complexed with CA_L or CA_S (Figures 2 and 3). The structures reveal how FIH accommodates different residue types for hydroxylation at its active site. All the peptides appear to adopt similar main-chain conformations, where the hydroxylated residue is at the apex of a γ-turn. There are differences in the position of FIH-active site residues, notably with respect to Arg-238/Gln-239; the side chain of the latter is positioned to form two hydrogen bonds with Asn substrates (Figures S9 and S10).[2,4c] In the CA_L structure (Figure S9e), the Arg-238 side-chain is positioned further from the hydrophobic Leu side-chain. The Leu side-chain C_α-C_β-C_γ angle is different relative to the Asn substrate (Figure S9a), such that both C_β hydrogen atoms are reasonably close to the active site metal (Zn-pro-3*R* hydrogen 3.3 Å and Zn-pro-3*S* hydrogen 4.0 Å compared to 4.1 Å for the Asn pro-3*S* hydrogen-Fe in PDB: 1H2K), making prediction of which Leu hydrogen is hydroxylated, difficult (Figure 3).

Structural studies on the FIH-HIF peptide complex predict that the pro-3*S* hydrogen on the oxidized Asn-methylene projects towards the metal, consistent with the production of the 2*S*,3*S* hydroxylated product.[2] In the Ser substrate (CA_S) complex, the Ser side-chain was refined in equal occupancy conformations; in one the alcohol is positioned to coordinate the metal (2.9 Å) whilst the other side-chain conformation is similar to the direction of Asn/Asp/His residues at this position resulting in the pro-3*S* hydrogen pointing towards the metal (Figure 3b and Fig-

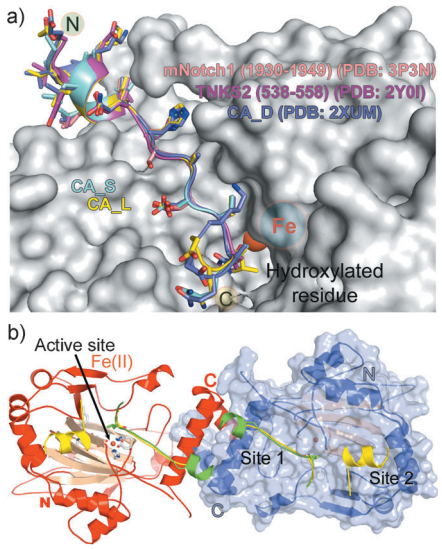

Figure 2. FIH binds peptides with different hydroxylated residues in a similar manner. a) Ribbon representation of superimposed peptide substrates when bound to FIH (gray); salmon, mouse Notch1 (mNotch1, 1930–1949, PDB: 3P3N); purple, Tankyrase-2 (TNKS2, 538–558, PDB: 2Y0I); blue, CA_D (PDB: 2XUM); yellow, CA_L; cyan, CA_S; and orange sphere, Fe. b) The FIH homodimer (molecule A in red and molecule B in blue) in a complex with HIF-1α CAD-(786–826) (yellow) and CA_L (green) with the double-stranded β-helix core (light salmon) and Fe (orange sphere) showing N- and C-terminal binding sites 1 and 2.[2]

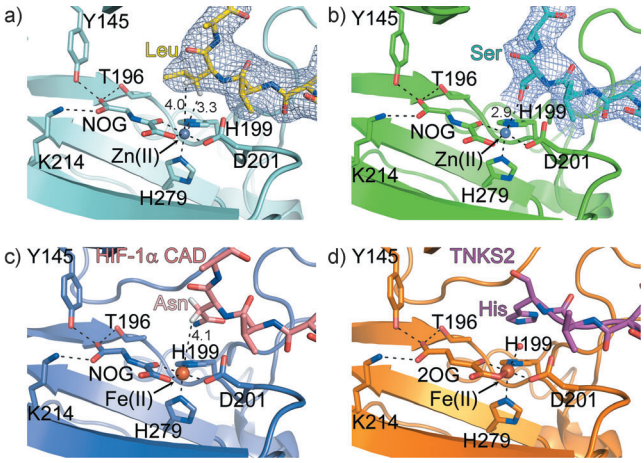

Figure 3. Flexibility in substrate binding at the FIH active site. Views from the active site when bound to substrates showing m*Fo*-D*Fc* difference maps (contoured to 3σ, light blue mesh) around the new substrates (CA_L and CA_S) residues. The difference electron density was calculated using phases derived from the model, with peptide residues omitted to remove model bias. The active site metals, Zn (substituting for Fe) or Fe are slate or orange spheres, respectively. a) The CA_L Leu-substrate (yellow), note both pro-3*R* and pro-3*S* hydrogen atoms (modelled) are close to the metal (3.3 and 4.0 Å, respectively); b) The CA_S (cyan), side chain has two conformations, one of which is positioned to coordinate the metal (Ser Oγ-Zn, 2.9 Å); c) HIF-1α CAD (salmon) with hydroxylated Asn-803 showing that the pro-3*S* hydrogen modeled at the C_β of Asn-803 is positioned for oxidation (pro-3*S* hydrogen-Fe, 4.1 Å); d) TNKS2 (purple) peptide containing His-553 at the hydroxylated position.

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ure S9b). We considered the possibility that this structure represents a geminal diol product; however this is unlikely because the structure was obtained under "non-reactive" conditions (Zn and NOG substituting for Fe and 2OG, respectively).

The differences in position of the substrate Leu residue side-chain compared with the side-chain of other polar substrate residues suggested that the stereoselectivity of C_β hydroxylation might be different. Because the NMR analyses could not determine the C_β stereoselectivity of the products, we carried out an amino acid analysis after hydrolysis of the FIH-catalyzed products. Reported results for the CA_N and CA_D peptides demonstrate the formation of the 2*S*,3*S* product[5c] in line with results for HIF-1α CAD Asn hydroxylation.[2] Similarly and consistent with crystallographic studies,[7] the amino acid analysis of the hydroxylated CA_H (Figure S11 i) also manifested formation of the 2*S*,3*S* product implying analogous modes of binding. Analysis of the hydroxylated CA_L product (Figure S11 ii) demonstrates formation of a 2*S*,3*R* product with the same relative stereochemistry as the previously assigned 2*S*,3*S* product (note the change in C_β assignment due to the Cahn—Ingold–Prelog rules).

Overall, the results reveal the potential for a high degree of promiscuity for FIH catalysis, although the efficiency of hydroxylation varies. FIH is able to catalyze the oxidation of residues with polar, neutral, acidic, basic, hydrophobic, and hydrophilic side-chains, all of which can be accommodated within its active site, albeit with some conformational changes in binding that occur for the hydrophobic substrates. Other studies reveal factors other than residue identity and local sequence context influence whether a particular AR residue is hydroxylated by FIH in cells;[5a,c] thus the results suggest that the range of post-translational modifications catalyzed by 2OG oxygenases from animals, specifically FIH and related JmjC enzymes, may be considerably wider than that perceived to date,[12] although it is unlikely to encompass the total range of reaction types catalyzed by those observed for plant and microbiological 2OG oxygenases. From a biocatalytic perspective, the results suggest that FIH engineering to optimize specific types of residue oxidation or induce new activities may be possible. The observation that FIH catalyzes oxidation at a serine to a formylglycine residue is notable because it opens up new possibilities for post-translational modification through reaction of the formyl glycine residue with appropriate nucleophiles.[13] It also raises the possibility that 2OG oxygenases may directly enable functional modifications (e.g. protein cross-linking) through reaction of nucleophiles with the introduced carbonyl group. It is also notable that the formylglycine product of serine oxidation can undergo epimerization, suggesting that oxygenases could catalyze protein side-chain epimerization. Precedent for this comes from epimerization and rearrangement/reaction of small molecule substrates catalyzed by bacterial 2OG oxygenases.[1a,14]

Experimental Section

Peptide synthesis: Peptide synthesis used a CSBio synthesizer, aminomethylstyrene resin (0.05 mol, Polymer Laboratories) and rink amide linkers. Peptides were cleaved from the resin using CF_3COOH and purified by HPLC (Vydac 218TP C_{18} column, 250 × 22 mm, particle size 10–15 μm, Grace, UK), eluting with a gradient of MeCN in 0.1 % aq. CF_3COOH, then lyophilized.

FIH incubation assay: Small scale assays used 100 μM FIH, 100 μM Fe^{II}, 100 μM peptide, 2 mM ascorbate, and 2 mM 2OG (37 °C for 2 h or 22 °C for 16 h). Incubations were desalted using a C_{18} spin column (Pierce) prior to MALDI-TOF MS/LC-MS analyses. Assays to determine the order of hydroxylation efficiency used 125 μM FIH, 100 μM peptide, 500 μM Fe^{II}, 2 mM ascorbate, and 2 mM 2OG (37 °C for 2.5 h). The peptides were analyzed by LC-MS and the ratio (% hydroxylation) of non-hydroxylated to hydroxylated (+16 Da) peptides was determined.

Mass spectrometric evidence for formylglycine (FGly) formation: For confirmation of FGly formation, the desalted reaction product was reacted with an equal volume of methoxyamine (10 mM) or biotin hydrazide (5 mM; at room temperature and for 30 minutes) followed by MS.

See the Supporting Information for other methods.

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[1] a) I. J. Clifton, M. A. McDonough, D. Ehrismann, N. J. Kershaw, N. Granatino, C. J. Schofield, *J. Inorg. Biochem.* **2006**, *100*, 644–669; b) R. P. Hausinger, *Crit. Rev. Biochem. Mol.* **2004**, *39*, 21–68.

[2] J. M. Elkins, K. S. Hewitson, L. A. McNeill, J. F. Seibel, I. Schlemminger, C. W. Pugh, P. J. Ratcliffe, C. J. Schofield, *J. Biol. Chem.* **2003**, *278*, 1802–1806.

[3] a) K. S. Hewitson, L. A. McNeill, M. V. Riordan, Y. M. Tian, A. N. Bullock, R. W. Welford, J. M. Elkins, N. J. Oldham, S. Bhattacharya, J. M. Gleadle, P. J. Ratcliffe, C. W. Pugh, C. J. Schofield, *J. Biol. Chem.* **2002**, *277*, 26351–26355; b) D. Lando, D. J. Peet, J. J. Gorman, D. A. Whelan, M. L. Whitelaw, R. K. Bruick, *Genes Dev.* **2002**, *16*, 1466–1471; c) D. Lando, D. J. Peet, D. A. Whelan, J. J. Gorman, M. L. Whitelaw, *Science* **2002**, *295*, 858–861.

[4] a) M. E. Cockman, D. E. Lancaster, I. P. Stolze, K. S. Hewitson, M. A. McDonough, M. L. Coleman, C. H. Coles, X. Yu, R. T. Hay, S. C. Ley, C. W. Pugh, N. J. Oldham, N. Masson, C. J. Schofield, P. J. Ratcliffe, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 14767–14772; b) M. E. Cockman, J. D. Webb, H. B. Kramer, B. M. Kessler, P. J. Ratcliffe, *Mol. Cell. Proteomics* **2009**, *8*, 535–546; c) M. L. Coleman, M. A. McDonough, K. S. Hewitson, C. Coles, J. Mecinovic, M. Edelmann, K. M. Cook, M. E. Cockman, D. E. Lancaster, B. M. Kessler, N. J. Oldham, P. J. Ratcliffe, C. J. Schofield, *J. Biol. Chem.* **2007**, *282*, 24027–24038; d) J. E. Ferguson III, Y. Wu, K. Smith, P. Charles, K. Powers, H. Wang, C. Patterson, *Mol. Cell. Biol.* **2007**, *27*, 6407–6419; e) S. E. Wilkins, S. Karttunen, R. J. Hampton-Smith, I. Murchland, A. Chapman-Smith, D. J. Peet, *J. Biol. Chem.* **2012**, *287*, 8769–8781.

[5] a) A. P. Hardy, I. Prokes, L. Kelly, I. D. Campbell, C. J. Schofield, *J. Mol. Biol.* **2009**, *392*, 994–1006; b) L. Kelly, M. A. McDonough, M. L. Coleman, P. J. Ratcliffe, C. J. Schofield, *Mol. Biosyst.* **2009**, *5*, 52–58; c) M. Yang, W. Ge, R. Chowdhury, T. D. W. Claridge, H. B. Kramer, B. Schmierer, M. A. McDonough, L. Gong, B. M. Kessler, P. J. Ratcliffe, M. L. Coleman, C. J. Schofield, *J. Biol. Chem.* **2011**, *286*, 7648–7660.

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[6] S. Linke, C. Stojkoski, R. J. Kewley, G. W. Booker, M. L. Whitelaw, D. J. Peet, *J. Biol. Chem.* **2004**, *279*, 14391 – 14397.
[7] M. Yang, R. Chowdhury, W. Ge, R. B. Hamed, M. A. McDonough, T. D. W. Claridge, B. M. Kessler, M. E. Cockman, P. J. Ratcliffe, C. J. Schofield, *FEBS J.* **2011**, *278*, 1086 – 1097.
[8] I. S. Carrico, B. L. Carlson, C. R. Bertozzi, *Nat. Chem. Biol.* **2007**, *3*, 321 – 322.
[9] 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.
[10] J. E. Baldwin, M. Bradley, R. M. Adlington, W. J. Norris, N. J. Turner, *Tetrahedron* **1991**, *47*, 457 – 480.
[11] R. C. Wilmouth, J. J. Turnbull, R. W. Welford, I. J. Clifton, A. G. Prescott, C. J. Schofield, *Structure* **2002**, *10*, 93 – 103.
[12] P. A. Del Rizzo, S. Krishnan, R. C. Trievel, *Mol. Cell. Biol.* **2012**, *32*, 4044 – 4052.
[13] D. Rabuka, J. S. Rush, G. W. deHart, P. Wu, C. R. Bertozzi, *Nat. Protoc.* **2012**, *7*, 1052 – 1067.
[14] I. J. Clifton, L. C. Hsueh, J. E. Baldwin, K. Harlos, C. J. Schofield, *Eur. J. Biochem.* **2001**, *268*, 6625 – 6636.

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