DOI: 10.1002/cbic.201000641

The 2-Oxoglutarate-Dependent Oxygenase JMJD6 Catalyses Oxidation of Lysine Residues to give 5*S*-Hydroxylysine Residues

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This paper is dedicated to Anthony (Tony) C. Willis on the occasion of his retirement in gratitude for many years of assistance with amino acid analysis.

Collagen prolyl- and lysylhydroxylases were the first enzymes catalysing post-translational hydroxylations on the carbons of proteins to be identified. Both collagen prolyl- and lysylhydroxylases are Fe^{II}- and 2-oxoglutarate (2OG)-dependent oxygenases. Subsequently, 2OG oxygenases have been shown to catalyse other protein hydroxylation reactions.[1,2] The aspartyl and asparaginyl residues of proteins containing epidermal growth-factor-type domains undergo carbon hydroxylation.[1] Intracellularly localised proteins also undergo hydroxylation on carbon. Following the identification of the hypoxia-inducible-factor (HIF) prolyl and asparaginyl hydroxylases,[3] the asparaginyl hydroxylase, factor inhibiting HIF (FIH), has been shown to catalyse multiple hydroxylations in ankyrin repeat domains.[3] There is also evidence for alternative substrates for the HIF prolylhydroxylases.[4,5] Further N^{ε}-methyl lysine demethylation is catalysed by a widespread family of histone demethylases[6] (JmjC subfamily) in a mechanism that probably involves initial hydroxylation of the N^{ε}-methyl group.[1,7,9]

There are variations in the regio- and stereochemical specificity of the studied 2OG-dependent hydroxylases that catalyse post-translational modifications. In the case of collagen prolylhydroxylation, different modifications to the same type of residue are associated with different functions (Scheme 1). For example, *trans*-4-prolyl hydroxylation stabilises the collagen triple helix,[10] whereas *trans*-3-prolyl hydroxylation is proposed to destabilise collagen to enable further modifications. In the case of the epidermal growth-factor-type domain protein, hydroxylation of aspartyl and asparaginyl residues occurs to give the 3*R* products,[11] whereas in HIF and ankyrin repeat domains, hydroxylation of asparginyl residues occurs to give the 3*S* products.[5,12] Thus, in addition to identifying the residue that is oxidised, it is important to define the regio- and stereochemical outcome of hydroxylation reactions. JMJD6 has been recently reported to catalyse hydroxylation at C-5 of lysyl residues in splicing regulatory proteins.[13] Here we report that JMJD6-catalysed C-5 lysyl hydroxylation occurs to give the hydroxylysine product with 5*S* stereochemistry. This result contrasts with the collagen lysyl hydroxylases that give products with the 5*R* stereochemistry.[14]

Webby et al. have reported that the splicing regulatory protein LUC7-like2 (LUC7L2) undergoes JMJD6-catalysed C-5 hydroxylation at Lys 269 and that a peptide fragment of $LUC7L2_{267–278}$ is a JMJD6 substrate.[13] To define the stereochemistry of the JMJD6-catalysed product, we began by synthesising a $LUC7L2_{267–278}$ peptide with a 2*S*,5*R*-hydroxylysine at residue 269, that is, with the 5*R* stereochemistry as observed in collagen lysyl hydroxylase catalysis,[14] and comparing it by means of NMR and MS analyses with a hydroxylated $LUC7L2_{267–278}$ peptide produced by JMJD6 catalysis. To prepare the synthetic standard, suitably protected 2*S*,5*R*-hydroxylysine was synthesised as previously reported[15] and incorporated by solid-phase synthesis to give the peptide NP**K(OH)**RSRSREHRR (**K(OH)** = 5*R*-hydroxylysine). For comparison, $LUC7L2_{267–278}$ was synthesised and incubated with recombinant human JMJD6 produced in *Escherichia coli*. The synthetic and enzymatically produced peptides were then compared by 1D TOCSY (Figure 1). These analyses, confirmed the regiochemistry of JMJD6-catalysed hydroxylation as occurring at C-5. However, at least with the small amount of enzymatically produced material available, the NMR analyses were unable to unambiguously distinguish between 5*R* and 5*S* products because the differences in the coupling constants for the relevant positions are small; for 2*S*,5*R*-hydroxylysine, the *J* values for H-6 and H-6′ are 13.3, 3.1 Hz (H-6, 2.97 ppm) and 13.3, 9.6 Hz (H-6′, 2.73 ppm), respectively;[16] for 2*S*,5*S*-hydroxylysine, the values are 13.1, 1.7 Hz (H6, 2.96 ppm) and 13.1, 10.0 Hz (H-6′, 2.73 ppm).[16] We therefore carried out amino acid analyses after hydrolysis of the hydroxylated peptide to investigate the stereochemistry of JMJD6-catalysed hydroxylation.

Initially, we optimised a method[17] for amino acid analysis that employs derivatisation of a mixture of all four stereoisomers of racemic C-5 hydroxylysine with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), followed by positive ion electrospray ionisation (ESI) MS analysis. Separation of the two sets of diastereomers (2*S*,5*S*/2*R*,5*R* and 2*S*,5*R*/2*R*,5*S*) was achieved by optimisation of HPLC conditions. The extracted ion chromatogram corresponded to the mass of hydroxylysine derivatised on both its amino groups ($[M+H]^+$ 503.1 Da).

We then carried out acid hydrolysis of the $LUC7L2_{267–278}$, product of JMJD6 catalysis for amino acid analysis. For compar-

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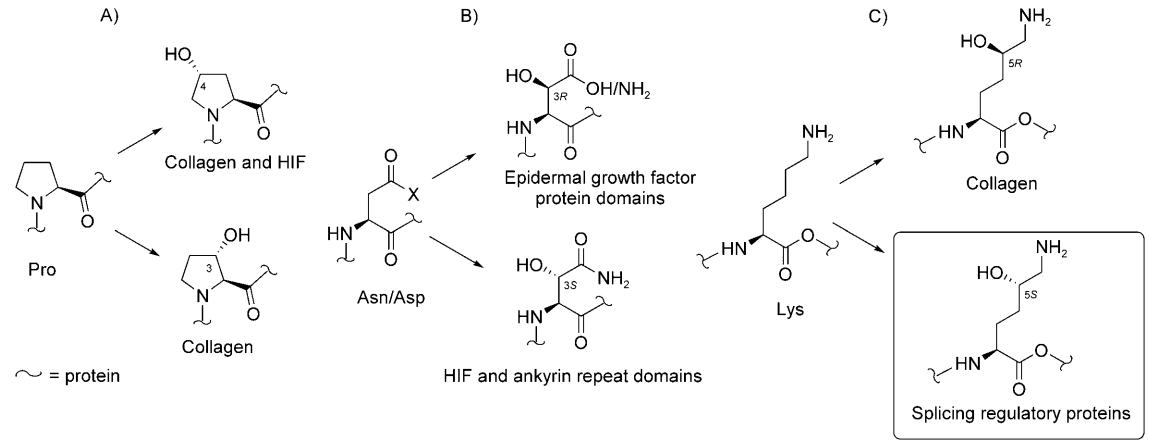
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000641.



Scheme 1. Different observed regio- and stereochemistries of the hydroxylation products of 2OG-dependent oxygenases in animals. Products of A) collagen and HIF prolylhydroxylation, B) epidermal growth factor-type domain and aspartyl and asparaginyl hydroxylations and C) collagen and splicing regulatory protein hydroxylation (this work).

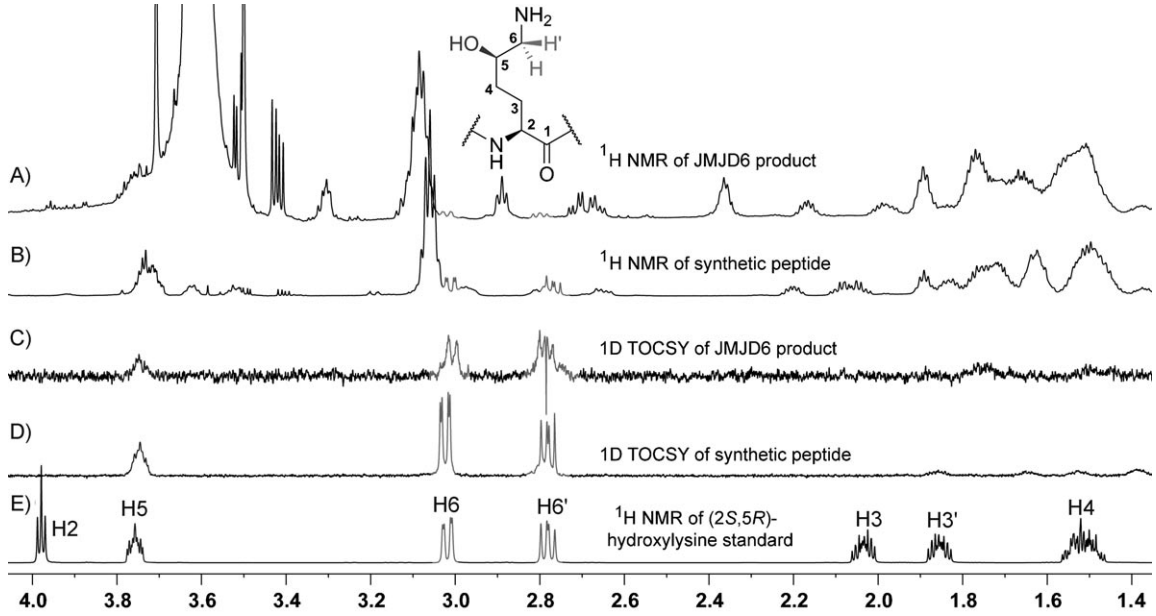


Figure 1. ^{1}H NMR spectra of A) JMJD6-catalysed hydroxylation of $LUC7L2_{267-278}$ peptide and B) synthetic peptide $LUC7L2_{267-278}$ containing a 2*S*,5*R*-hydroxylysine residue at position 269. 1D TOCSY spectra of C) the JMJD6-catalysed $LUC7L2_{267-278}$ product and D) synthetic peptide $LUC7L2_{267-278}$ containing 2*S*,5*R* hydroxylysine; E) ^{1}H NMR spectrum of a 2*S*,5*R*-hydroxylysine standard.

ison, modern cow-bone collagen (Femur, type-1) extracted by standard laboratory procedure[18] was also analysed. Initial experiments in tris(hydroxymethyl)aminomethane (Tris) buffer were hampered by its reaction with AQC to give derivatised buffer. We therefore tested other buffers—(3-(*N*-morpholino)-propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and phosphate buffers)—MOPS gave the highest yields and was used in subsequent analyses.

LC-MS analyses after hydrochloric acid-catalysed hydrolysis and derivatisation of both the JMJD6 and collagen products revealed peaks at 8.54 min (corresponding to 2*S*,5*S*- or 2*R*,5*R*-hydroxylysine) and 8.74 min (corresponding to 2*S*,5*R*- or 2*R*,5*S*-hydroxylysine, Figure 2). Although for the collagen sample, the 2*S*,5*R*/2*R*,5*S* peak was larger, these results could not be used for stereochemical assignment. This is probably because, under the acidic hydrolysis conditions, C-2 epimerisation of C-5 hydroxylysine occurs through the formation of a six-membered lactone involving stereochemically favoured 6-*exo-trig* cyclisation of the C-5 hydroxy group onto the protected C-2 carboxylic acid (Scheme 2); lactone formation lowers the pK_a of the C-2 hydrogen, thereby enabling epimerisation by enolisation.[19] To minimise C-2 epimerisation, we developed an enzyme-catalysed hydrolysis procedure involving initial treatment with trypsin to cleave the amide bond C-terminal to the hydroxylysine residue, followed by treatment with carboxypeptidase-Y to release the C-5 hydroxylated lysine (Scheme 2). When treated in this manner, collagen gave a single peak corresponding to the retention time of derivatised 2*S*,5*R*-hydroxy-

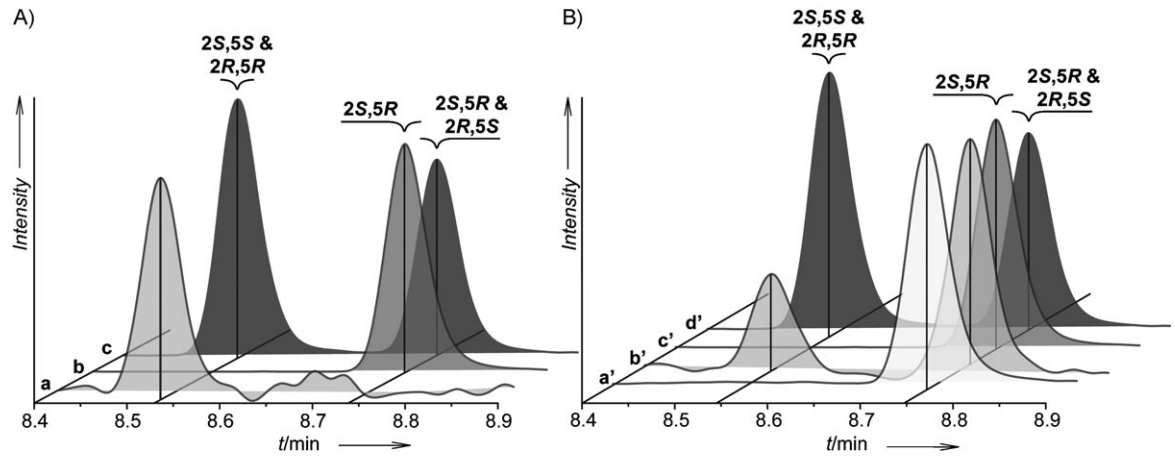
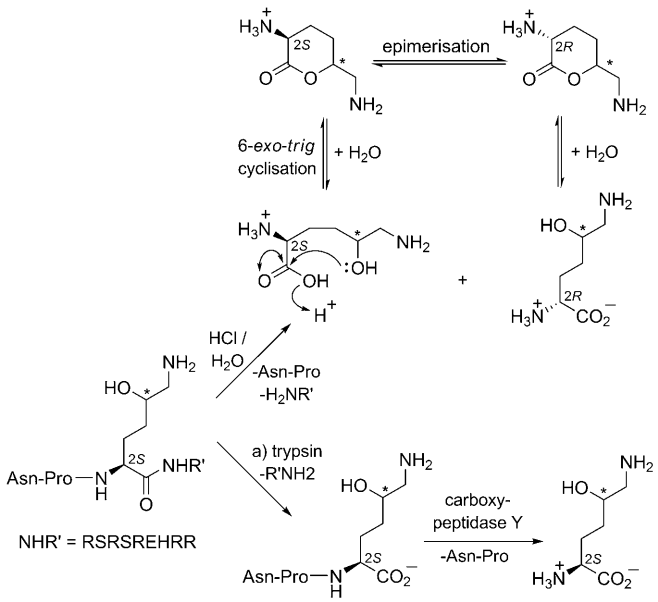


Figure 2. Amino acid analyses comparing the hydroxylysine products of JMJD6 catalysis with that in collagen. A) a) Hydroxylysine product of JMJD6-catalysed LUC7L2$_{267-278}$ after treatment with trypsin and carboxypeptidase Y; b) 2*S*,5*R*-hydroxylysine; c) mixture of 2*R*,5*R*/2*S*,5*S*- and 2*R*,5*S*/2*S*,5*R*-hydroxylysine (58:42, respectively); B) a′) Hydroxylysine product after treatment of collagen with trypsin and carboxypeptidase Y; b′) Collagen standard after HCl-catalysed hydrolysis; c′) 2*S*,5*R*-hydroxylysine; d′) mixture of 2*R*,5*R*/2*S*,5*S*- and 2*R*,5*S*/2*S*,5*R*-hydroxylysine (58:42, respectively).



Scheme 2. Outcomes of acid- and enzyme-catalysed hydrolyses of hydroxylysine-containing peptides. Note the formation of 2*S*- and 2*R*-hydroxylysine products through epimerisation at C-2 in HCl-catalysed hydrolysis[19] and formation of the product with retained C-2*S* stereochemistry when using the enzyme-catalysed hydrolysis procedure.

lysine (it is subsequently assumed that the 2*S* (L) stereochemistry is maintained). This result demonstrates that C-5 hydroxylation does not ablate trypsin activity. In contrast, and unexpectedly, when the hydroxylated LUC7L2$_{267-278}$ peptide was subjected to the same enzyme-catalysed hydrolysis protocol, a single peak corresponding to 2*S*,5*S*-hydroxylysine (1.4 ± 0.4 pmol μL^{-1}; $n = 4$) was observed (the limit of detection is 1 pmol μL^{-1}; Figure 2).

In conclusion, we have found that JMJD6 catalyses hydroxylation of lysyl residues to give products with the 2*S*,5*S*-stereochemistry, a clear difference from the collagen lysyl hydroxylases, which give products with the 2*S*,5*R* stereochemistry. Bioinformatic studies imply that JMJD6 is more closely related to the JmjC family of N^{ε}-methyl lysine demethylases and hydroxylases (FIH) than it is to the collagen hydroxylases.[20] Thus, whilst it seems likely that most, if not all, animal 2OG oxygenases evolved from a common ancestor, at least two different subfamilies of lysyl hydroxylases might have evolved with different stereochemical selectivities. Given the proximity of the C-5 and N^{ε}-amino group of lysine, it is possible that an N^{ε}-methyl demethylases have evolved from a C-5 hydroxylase, or vice versa.

Experimental Section

Synthesis and purification of peptides: Peptides were synthesised by using standard methodology.[21] In the case of the peptide containing 5-hydroxylysine, Fmoc hydroxylysine (Boc-oxazolidine) was synthesised as described by Spetzer.[15] Peptides were purified on a Vydac C18 Peptide column and a Waters Quattro micro LC-MS system.

Sample preparation for NMR analysis: Hydroxylation reactions were initiated by mixing LUC7L2$_{267-278}$ peptide (1 mM final concentration), 2OG (5 mM) and Fe^{II} (4 mM) in ammonium acetate (10 mM, pH 7.5) and JMJD6 (200 μM), followed by incubation at 37 °C. For large-scale hydroxylation 10 mg of peptide was used. After 2 h, hydroxylation of the peptide was analysed by MALDI-TOF MS. The assay mixture was further incubated (at room temperature) until MS analysis showed substantial hydroxylation (> 90 %). Methanol (200 μL) was then added to each Eppendorf tube to precipitate protein. The mixture was centrifuged (10 min, 14 000 rpm, 14 462 *g*), and the decanted solution was freeze-dried, dissolved in 0.1 % aqueous formic acid and purified by LC-MS.

Sample preparation for amino acid analysis: For amino acid analysis, the hydroxylation reaction was initiated by mixing LUC7L2$_{267-278}$ (100 μM), 2OG (500 μM), Fe^{II} (400 μM) and ascorbic acid (100 μM) in MOPS buffer (50 mM, pH 7.5) and JMJD6 (20 μM), followed by incubation at 37 °C (1 h). Recombinant JMJD6 was purified as reported.[13] After it had been ensured by MALDI-TOF MS that the reac-

tion was complete, protein was precipitated by adding methanol to the mixture. The sample was centrifuged (10 min, 14000 rpm, 14462 *g*), then freeze dried. The assay mixtures were resuspended in 0.1% aq. formic acid and purified by HPLC on a Phenomenex Synergi Hydro RP C18 column. The purified peptide was then suspended in MOPS (50 mM) and incubated with trypsin (2 μL, 100 μg mL^{-1}; Promega) for 2 h at 37 °C. The trypsin was removed by centrifugation (10 min, 14000 rpm, 14462 *g*). Carboxypeptidase Y (1 μL, 5 mg mL^{-1}, Roche) was then added to the supernatant, and the mixture incubated (37 °C, overnight). Carboxypeptidase Y was then removed by centrifugation (14000 rpm, 14462 *g*, 10 min). For the acid hydrolysis, an excess of HCl (6 M) was added to each sample, which was then incubated for 24 h at 110 °C under an inert atmosphere. All the samples were concentrated in a vacuum centrifuge (Speed-Vac, Heto).

Cow-bone collagen was extracted from a femur according to the demineralisation and gelatinisation method of Longin.[18] Modern cow-bone collagen digests were carried out by using either hydrochloric acid or the trypsin-carboxypetidase Y method. For the acid-catalysed hydrolysis, 400 mL aqueous collagen solution (3 g L^{-1}) was dried in a Pyrex tube, which was then placed in an air-tight vial with HCl (200 μL, 6 N) at 100 mbar and 110 °C for 24 h. For the enzyme protocol, 50 μL of aqueous collagen solution (3 g L^{-1}) was used, and the enzyme-catalysed hydrolysis procedure was carried out as above. Hydrolysed peptides were derivatised by 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) zin acetonitrile according to the AccQTag Solution Protocol (Waters) and analysed by LC-MS as described in the Supporting Information.

See the Supporting Information for other methods.

Acknowledgements

The authors thank Dr. Celia J. Webby, Dr. Alexander Wolf and Prof. Angelika Böttger for support and advice. This work was funded by the Biotechnology and Biological Sciences Research Council, the Wellcome Trust and a Scatcherd European scholarship.

Keywords: collagen · conformation analysis · enzyme catalysis · JMJD6 · lysyl hydroxylation

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Received: October 24, 2010
Published online on January 18, 2011

