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# Monitoring the Activity of 2-Oxoglutarate Dependent Histone Demethylases by NMR Spectroscopy: Direct Observation of Formaldehyde

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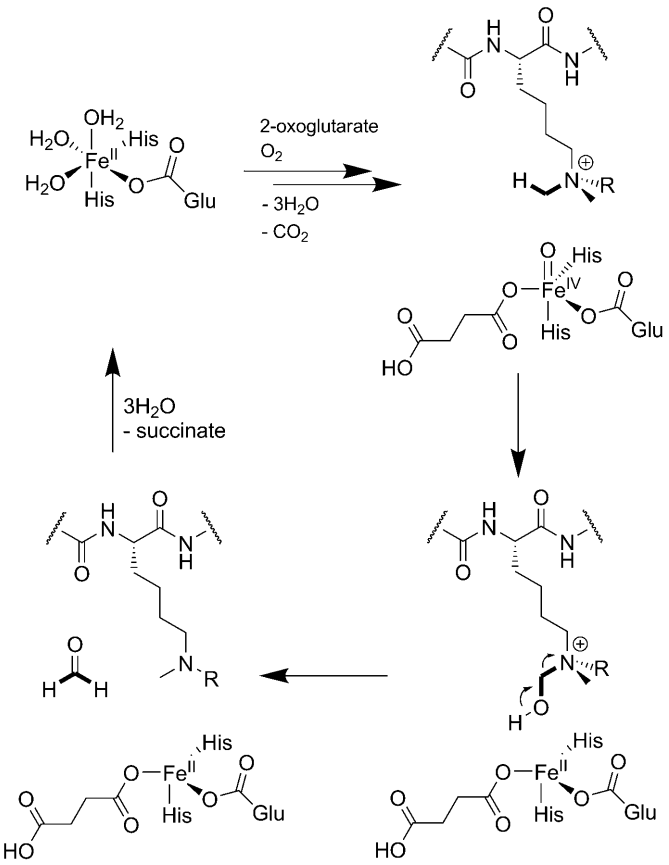
Ferrous iron and 2-oxoglutarate (2OG) dependent oxygenases are a diverse superfamily, with members involved in many important biological processes, including oxygen sensing, epigenetic regulation, and collagen, antibiotic, and fatty acid biosynthesis.[1,2] The 2OG oxygenase histone demethylase (HDM) subfamily catalyses the demethylation of $N^{\varepsilon}$-methylated lysine residues in the *N*-terminal tails of histones. Methylated histone tail lysines are involved in the establishment of different chromatin states, and contribute to both gene silencing and activation.[3] Various HDM subfamilies have been implicated in disease states, with the JMJD2 HDMs being linked to prostate and oesophageal cancers.[4]

$N^{\varepsilon}$-Methyllysine demethylation is proposed to occur via hydroxylation of the $N^{\varepsilon}$-methylated lysine (with concomitant oxidation of the cosubstrate 2OG, and decarboxylation to give carbon dioxide and succinate), followed by fragmentation of the $N^{\varepsilon}$-hydroxymethyllysine, to give formaldehyde and the demethylated lysine (Scheme 1).[5] The $Fe^{II}$/2OG-dependent oxygenases can be challenging to study in detail, with three substrates ("prime" substrate, 2OG and oxygen) and at least three products (in the case of the histone demethylases, four: demethylated peptide, succinate, formaldehyde and carbon dioxide). NMR spectroscopy is a potentially useful technique for studying 2OG oxygenase reactions in a single assay mixture. Previous reports regarding quantification of formaldehyde released from biocatalysed reactions or formaldehyde levels in biological systems have been based either on its oxidation to formic acid,[6] or on its derivatisation with reagents, such as dimedone or ampicillin.[7–10] To our knowledge, formaldehyde detection by NMR spectroscopy in enzyme-catalysed reactions has not been reported. Here, we report the use of NMR spectroscopy for monitoring *N*-demethylation by JMJD2E (a HDM that is sufficiently active for kinetic studies) by monitoring 2OG conversion to succinate, demethylation of $N^{\varepsilon}$-trimethyl and $N^{\varepsilon}$-dimethyllysine residues, and both direct and indirect detection of formaldehyde production in demethylation reactions.

Initially, the JMJD2E catalysed demethylation reaction was investigated by monitoring the demethylation of octapeptide fragments of the histone H3 N-terminal tail (residues Ala7 to Lys14, $N^{\varepsilon}$-methylated at residue Lys9). A peptide length of eight amino acids was selected (Ala-Arg-lys($Me_3$)-Ser-Thr-Gly-Gly-Lys) in order to allow sufficient substrate recognition by the enzyme whilst reducing signal overlap in the $^1$H NMR spectra. A standardised demethylation reaction protocol was developed, firstly by screening a variety of buffers suitable for $^1$H NMR spectroscopy (nondeuterated potassium phosphate and ammonium formate buffers, both at 50 mM, pH 7.5) and then by optimising concentrations of reagents to allow NMR detection. JMJD2E only displayed sufficient activity in ammonium formate buffer, and this was selected for further work. L-Ascorbate has been previously shown to increase activity of some $Fe^{II}$/2OG-dependent oxygenases and was therefore included in the assay mixture.[11] The presence of $Fe^{II}$ ions at 100 μM did not cause any noticeable loss of resolution. The demethylation reactions with tri- and dimethylated peptides (K9me3 and K9me2, respectively) under optimised conditions




**Scheme 1.** Proposed mechanism for JMJD2-catalysed demethylation. Oxidative decarboxylation of 2OG generates a $Fe^{IV}$=O species, which reacts with the methylated lysine residue. Hydroxymethyllysine then fragments to give formaldehyde and the demethylated product. The demethylated carbon is highlighted in bold; R = H or $CH_3$.

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were then monitored by $^1$H NMR spectroscopy (700 MHz) over 30 min, at 75 s intervals, and experiments were carried out with varying the concentration of peptide substrate, with an excess of 2OG. The relative concentrations of peptide substrate and demethylated products were calculated at each time point by integration of the corresponding $N^{\varepsilon}$-$CH_3$ $^1$H resonances, and normalising each value for the number of protons and initial substrate concentration. Kinetic parameters were then calculated by analysing the initial rates of demethylation at different substrate concentrations. The $K_M$ for the Lys9 trimethylated peptide K9me3 ($203 \pm 79$ μM) was found to be lower than that for the analogous dimethylated peptide K9me2 ($282 \pm 36$ μM). However, the $V_{max}$ ($0.180 \pm 0.018$ and $0.196 \pm 0.008$ μM s$^{-1}$ for K9me3 and K9me2, respectively) and $k_{cat}$ values ($0.018 \pm 0.002$ and $0.020 \pm 0.001$ s$^{-1}$ for K9me3 and K9me2, respectively) for both substrates had higher similarity. The $k_{cat}$ values that we obtained for JMJD2E are substantially higher than those previously reported for JMJD2A/D,[6] although this might reflect differences in assay conditions. Our results are consistent with prior studies[6,12] conducted with a formaldehyde dehydrogenase-coupled assay, which indicate that trimethylated H3K9 is preferred to the dimethylated form, and imply that this selectivity arises, predominantly, from a difference in $K_M$.

The NMR spectroscopy method was then used to investigate how succinate and demethylated peptide product concentrations varied with time. K9me3 and K9me2 were incubated with JMJD2E by using the described protocol and were analysed at 75 s intervals over a period of 15 min. The concentrations of demethylated products (summed in the case of K9me3 demethylation) and succinate were then compared at each time point. In both cases, concentrations grew in a linear relationship relative to each other over all time points (Figure 1C) with an excess of succinate to demethylated peptide, indicating that 2OG turnover is largely, but not absolutely, coupled to substrate demethylation. It is possible that the extent of coupling is condition or substrate dependent, as observed for other 2OG oxygenases.[1] The proportion of "uncoupled" turnover remained constant for each substrate over time ($24.8 \pm 1.3$ and $16.2 \pm 0.5$% for K9me3 and K9me2, respectively, relative to demethylated product concentration), implying that it did not arise at a time point prior to the first NMR analysis (150 s after mixing).

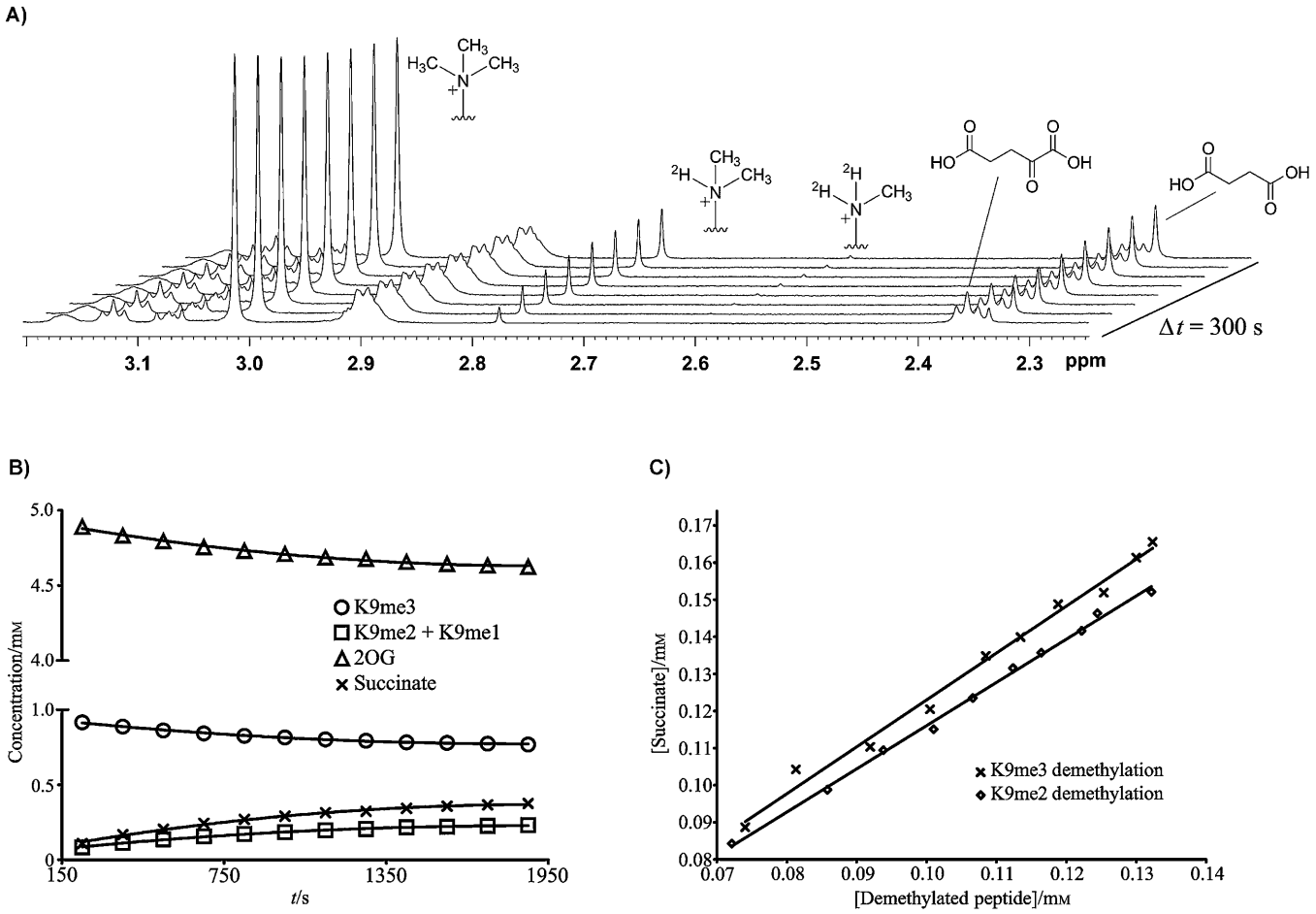


**Figure 1.** Monitoring JMJD2E-catalysed demethylation over time. A) $^1$H NMR spectra of a time course monitoring the JMJD2E-catalysed demethylation of K9me3. Spectra are shown at 300 s intervals (acquisition time for each spectrum was 75 s). The first acquisition was started 150 s after mixing the assay components. The initial peptide concentration was 1 mM. B) Concentrations of peptide substrate, 2OG, demethylated products and succinate during a time course monitoring the JMJD2E-catalysed demethylation of K9me3. C) Demethylated peptide product concentration plotted against succinate concentration at time points during JMJD2E-catalysed demethylation of K9me3 and K9me2. The initial peptide concentrations were 0.75 mM.

  

The NMR assay was then used to investigate the effect of L-ascorbate on 2OG oxidation/decarboxylation and peptide demethylation. The level of substrate-uncoupled 2OG turnover is known to vary significantly between members of the $Fe^{II}$/2OG-dependent oxygenase family,[1] and in some cases to vary in the presence of ascorbate.[11] Our experiments were carried out with K9me3, K9me2, $N^{\varepsilon}$-monomethylated K9me1, unmethylated K9me0, and in the absence of any peptide substrate. Experiments were carried out with saturating concentrations of peptide and 2OG (JMJD2E:K9me0/1/2/3:2OG, 10 μM:750 μM:5 mM); initial rates were measured for peptide demethylation (where applicable) and 2OG conversion to succinate, in the presence (1 mM) or in the absence of L-ascorbate (Figure 2). In the presence of K9me2/me3 peptides, ascorbate stimulated activity of both peptide demethylation and 2OG turnover. Ascorbate also accelerated 2OG turnover in assays with K9me1. In the absence of peptide and in the presence of K9me0, the differences with and without ascorbate were within overall experimental error. The levels of stimulation of K9me3 and K9me2 demethylation were significant, but less than observed for some other 2OG oxygenases (e.g., collagen prolyl 4-hydroxylase).[11] While there is evidence to suggest that longer peptide fragments of K9-monomethylated H3 are substrates for JMJD2E (N. R. Rose, unpublished data) we did not observe demethylation of K9me1 by our NMR assay, and the extent of 2OG turnover was not above that observed in the absence of peptide.

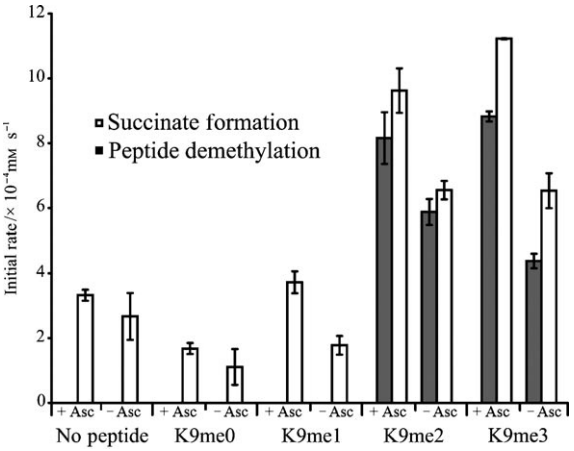


**Figure 2.** Rates of peptide demethylation (where applicable) and 2OG turnover (succinate formation) in the presence (+Asc) and absence (−Asc) of ascorbate. Error bars are displayed as standard deviations.

To directly detect free formaldehyde formed by demethylation of methylated lysine, $^{13}$C-labelled K9me2 (K9$^{13}$Cme2) was synthesised and its reaction with JMJD2E was analysed by 1D $^{13}$C heteronuclear single quantum coherence (HSQC) NMR spectroscopy (Figure 3). The HSQC experiment benefits from the greater sensitivity of proton observation and also provides selectivity by editing the 1D $^{1}$H spectrum to retain responses

We then investigated the detection of formaldehyde by NMR spectroscopy. Formaldehyde was not unambiguously observed in the $^{1}$H NMR spectra, probably due partly to its low concentration relative to the other reagents in solution, and also because of overlap of the signal with the solvent resonance (HDO). Thus, dimedone (5,5-dimethyl-cyclohexane-1,3-dione) was added to the reaction mixture to capture formaldehyde and allow its detection by $^{1}$H NMR spectroscopy. Dimedone reacts with formaldehyde (Scheme S2 in the Supporting Information) to form two different adducts, both of which have distinctive proton NMR chemical shifts (Figure S8 in the Supporting Information). Both of these adducts were observed for formaldehyde produced by JMJD2E catalysis (Figure S9 in the Supporting Information) with the "mono" adduct (2-hydroxymethyldimedone) being the major product observed.

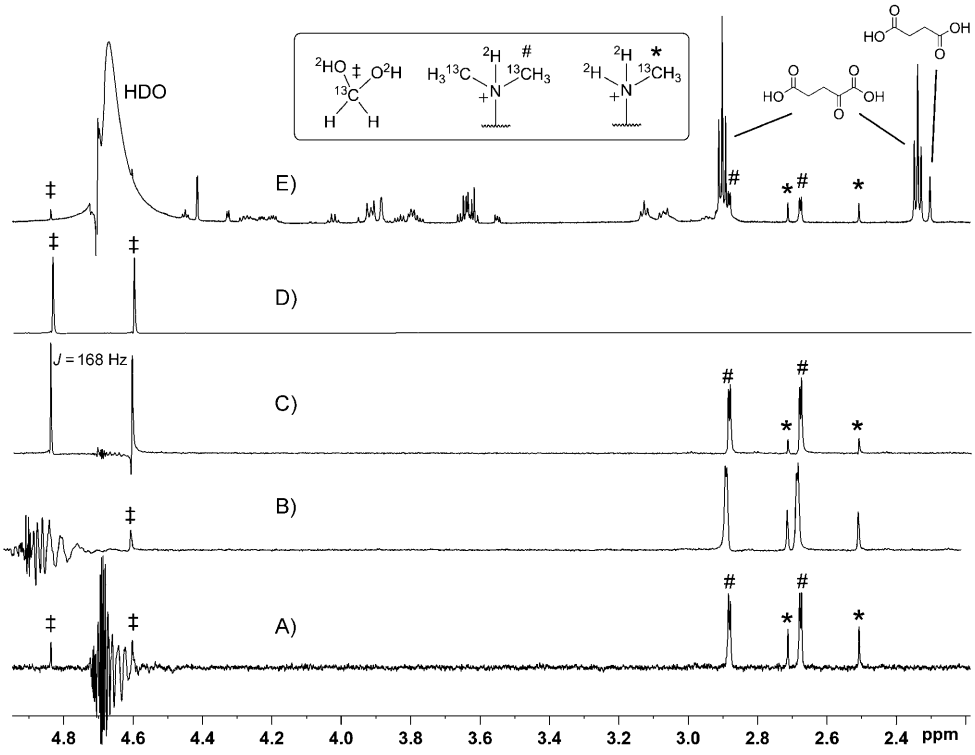


**Figure 3.** Direct observation of formaldehyde production by JMJD2E catalysis. A)–D) 1D $^{13}$C HSQC, and E) $^{1}$H NMR spectra show production of $^{13}$C-formaldehyde ($^{1}J_{CH}$ = 168 Hz) from the demethylation of K9$^{13}$Cme2. A) and B) Demethylation and $^{13}$C-formaldehyde production at 25 and 4 °C, respectively. C) Reaction mixture enriched with authentic $^{13}$C-formaldehyde. D) Authentic $^{13}$C-formaldehyde. E) $^{1}$H NMR spectrum of JMJD2E-catalysed demethylation of K9$^{13}$Cme2. Note: The small coupling observed in the K9$^{13}$Cme2 methyl resonance arises from a three-bond $^{1}$H–$^{13}$C coupling to the adjacent methyl carbon ($^{3}J_{CH}$ = 4 Hz).

  

only from those protons bound directly to $^{13}C$. Thus, only species containing the $^{13}C$ label originating from the labelled methyl groups were observed, with all other resonances being eliminated (down to the level of natural $^{13}C$ abundance, 1.1%). Owing to the removal of $^{13}C$ decoupling in the 1D HSQC sequence employed, each resonance displays a distinctive doublet structure arising from the one-bond $^{1}H–^{13}C$ coupling ($^{1}J_{CH}$). $^{13}C$-Formaldehyde was observed in its hydrated form $CH_2(OH)_2$ ($\delta_H = 4.72$ ppm, $^{1}J_{CH} = 168$ Hz) as expected under these solution conditions (Figure 3, spectra A, B and E). Varying the temperature at which the NMR spectroscopy experiments were carried out caused a relative shift in the HDO resonance, allowing both resonances for the $^{13}C$-formaldehyde to be observed (Figure 3, spectrum B). A 2D HSQC experiment performed on the final reaction mixture (Figure S10 in the Supporting Information) gave the $^{13}C$ shift of the formaldehyde as 82 ppm, which was consistent with an authentic sample. Enriching the reaction mixture with an authentic sample of $^{13}C$-formaldehyde confirmed that the chemical shifts observed in the enzymatic reaction mixture corresponded to formaldehyde (Figure 3, spectrum C). The putative hydroxymethyllysine intermediate (Scheme 1) was not observed, implying that this intermediate is either enzyme-bound or too short-lived to be observed on the NMR timescales used here.

Overall, in situ NMR analyses of the JMJD2E reaction with a model substrate have provided information on the stoichiometry of its reactions and kinetic data. Formaldehyde was detected as a reaction product, both through its derivatisation with dimedone, and as free formaldehyde in solution. To our knowledge, this is the first reported instance of direct detection by NMR spectroscopy of enzymatically produced formaldehyde. The NMR methods described could be useful in investigating the stoichiometry and mechanisms of other $Fe^{II}$/2OG-dependent oxygenases; when appropriate spectrometers are available they provide a useful alternative to assays based on labelled 2OG, or chromatography, which have been widely used in the field. In the field of histone modifying and related enzymes, functional assignments are commonly made by mass spectrometric analyses, which can be difficult and, at the biochemical level, are complicated by redundancy issues. We believe that, whenever possible, NMR spectroscopy should be used in such assignments, as reported recently for JMJD6.[13]

## Experimental Section

**Expression and purification of the histone demethylase, JMJD2E:** The catalytic domain of human JMJD2E (residues 1–337) was produced as an N-terminally $His_6$-tagged protein in *E. coli*, and purified by Ni-affinity and size-exclusion chromatography, and stored at a concentration of 60 mg mL$^{-1}$ in HEPES (50 mM), NaCl (500 mM), pH 7.5, as reported.[14]

**Synthesis of peptides:** Synthesis of peptide substrates was carried out by using standard Fmoc-based solid phase peptide synthesis (SPPS) with a CSPep336X peptide synthesiser (CSBio, California, USA). Peptides were synthesised on a PL-AMS (aminomethylpolystyrene) resin (Polymer Labs) by using a Rink amide linker, and cleaved from the resin by using $CF_3COOH$/triisopropylsilane (97.5%/2.5%, *w/v*) and purified to >95% purity by HPLC. Details on the synthesis of $^{13}C$-labelled $N^{\varepsilon}$-dimethyllysine and full characterisation of peptides by $^{1}H$ NMR spectroscopy are described in the Supporting Information.

**NMR spectroscopy:** NMR analyses employed a Bruker AVIII 700 spectrometer equipped with an inverse TCI cryoprobe optimised for $^{1}H$ observation and installed with TOPSPIN 2 software. Chemical shifts are reported in ppm relative to $D_2O$ ($\delta_H = 4.72$ ppm); the deuterium signal was used as an internal lock signal and the HDO signal was reduced by presaturation where necessary. The spectrometer conditions were optimised by using a control sample with all the components of the reaction except JMJD2E. The experiments were performed on an identical sample; the enzyme was added to the assay mixture directly prior to transfer to a 2 mm NMR tube. The NMR spectroscopy tube was centrifuged for a few seconds in a hand centrifuge. The sample was introduced to the magnet and data acquisition was started after a brief optimisation (total time lapse between adding the enzyme and the start of data acquisition was 150 s). The time course data were collected by using an automated routine. Twenty four analyses were performed on each sample, each accumulating 16 transients corresponding to 75 s of total acquisition time and providing a single spectrum. The delay time between analyses was 0 s. The sample temperature was maintained at 298 K throughout the run. Data were processed by using automated routines and spectra integrated with absolute intensity scaling to monitor changes in intensity of signals of interest. $^{1}H$ NMR spectra of the substrate peptides gave signal intensities consistent with their predicted relative values.

**1D $^{13}C$ HSQC NMR spectroscopy:** Demethylation of the $^{13}C$-labelled peptide (K9$^{13}$Cme2) was followed by using a gradient-selected 1D $^{13}C$ HSQC method in which the standard 2D HSQC sequence was modified by removal of both the variable $t_1$ period and $^{13}C$ decoupling during data acquisition. The $1/2J_{CH}$ delays were optimised for $^{1}J_{CH}$ of 145 Hz. For each 1D experiment, eight transients were accumulated corresponding to 39 s of total acquisition time. The experiments were performed at two different temperatures (298 and 277 K) in order to clearly see the two $^{13}C$-coupled signals for formaldehyde and avoid the interference from the HDO signal.

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