Studies on the reaction of glutathione and formaldehyde using NMR†

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Within cells it is proposed that a major mechanism for the metabolism of formaldehyde is *via* its reaction with glutathione (GSH) to form *S*-hydroxymethylglutathione (HMG), which undergoes subsequent oxidation. In addition to HMG and the previously reported (5*R*,10*S*)-5-(carboxymethylcarbamoyl)-7-oxo-3-thia-1,6-diazabicyclo[4.4.1]undecane-10-carboxylic acid ($BiGF_2$), NMR studies on the reaction of GSH with formaldehyde reveal two previously unassigned monocyclic structures. The results imply that the biologically relevant reactions between aldehydes and peptides/proteins may be more complex than presently perceived.

Introduction

Formaldehyde (HCHO), the simplest aldehyde, is widely distributed, being present in the intercellular medium and very likely in all cells.[1] Above threshold levels, HCHO is cytotoxic, though the precise mechanisms of this toxicity are unclear.[2] HCHO reacts rapidly with nucleophiles, forming adducts with DNA and proteins as well as cross-linking different biomacromolecules and small molecules. For example, HCHO derived cross-links have been reported in the binding of DNA to the anticancer agent Adriamycin and in the binding of DNA to glutathione.[3,4] Although quantitative data on the subcellular concentrations of HCHO are lacking, at least at a local level its concentration may be sufficiently high for 'non-specific' reactions to occur. Work in recent years has shown that HCHO is produced by histone lysine demethylases and DNA demethylases.[5,6] HCHO is proposed to be metabolised in an oxidative pathway employing the ubiquitous thiol reducing agent glutathione (Scheme 1).[7] In some organisms, there is evidence that the first step in this pathway (*i.e.* formation of *S*-hydroxymethylglutathione (HMG)), is enzyme catalysed (by Glutathione-Dependent Formaldehyde-Activating Enzyme (Gfa) in *Paracoccus denitrificans*).[8] Subsequent oxidation by an alcohol dehydrogenase (ADH5 in humans) yields an *S*-formyl derivative, which is subsequently hydrolysed to give formate.

Glutathione (GSH) is a biologically important reducing agent that is involved in a wide range of biochemical processes.[9,10] GSH is present in almost all, if not all, eukaryotes and also in both gram positive and gram negative bacteria. The nucleophilic and redox properties of GSH enable it to play a role in the detoxification of cellular electrophiles.[11–13] Several studies on the non-enzymatic reaction of GSH and HCHO have been reported.[14–16] In early work by NMR, evidence for the production of cyclic species was obtained.[15] Recently, the structure of one of the proposed cyclic products of the reaction of GSH and HCHO was revised as (5*R*,10*S*)-5-(carboxymethylcarbamoyl)-7-oxo-3-thia-1,6-diazabicyclo[4.4.1]undecane-10-carboxylic acid ($BiGF_2$) on the basis of both NMR studies in solution and from protein X-ray crystallographic data, where $BiGF_2$ was observed bound to human Carbonyl Reductase 1.[16]

We are interested in the biological reactivity of HCHO particularly within the context of chromatin biochemistry, and therefore wished to understand the reactivity of GSH with HCHO. The studies on $BiGF_2$[16] raise the question of how $BiGF_2$ is formed, and whether it or other products of the reaction of GSH and HCHO are of biological relevance. Here, we report NMR studies on the reaction of GSH and HCHO; the results provide insights into the formation and stability of $BiGF_2$ under different conditions and reveal evidence for the presence of two other cyclic GSH-HCHO adducts. More generally, they suggest that the reaction between aldehydes and peptides/proteins may be more complex than previously envisaged.

Results and discussion

As reported[15,16] we found that the reaction of GSH with HCHO gave a complex mixture of products. Initial experiments demonstrated that the mixture obtained was condition and time dependent. We then developed a procedure for the isolation of $BiGF_2$. Detailed analyses by ^1H, ^{13}C, COSY, HSQC, HMBC and NOESY NMR experiments were conducted on a sample of purified $BiGF_2$, which was obtained by reacting GSH with HCHO (4 molar equivalents) at pD 6.5 (deuterated Na_3PO_4 buffer solution) followed by HPLC purification with MS-guided fraction analysis. ^1H, ^{13}C and COSY data for $BiGF_2$ were in good correlation with those previously reported (structural characterisation by NMR is shown in the ESI†).[16] NOE correlations present in the NOESY spectrum of the sample (Fig. S8†) supported a solution conformation similar to that portrayed in the X-ray structure of $BiGF_2$ in complex with human Carbonyl Reductase 1.[16]

The time and pD dependence of the GSH-HCHO reaction was then investigated by analysing Na_3PO_4 buffered samples of GSH (13.3 mM) with four equivalents of HCHO (at pD 5.5, 6.5, 7.5, 8.5 and 9.5) by ^1H NMR. Formation rates for all the observed major GSH-HCHO adducts were determined by monitoring each sample every 293 s over 105 min of reaction, and then calculating the concentration of each species by integrating over characteristic ^1H resonances. In most cases, the time course experiments revealed the formation of multiple products; many of these species could not be characterised due to their relatively low abundance and

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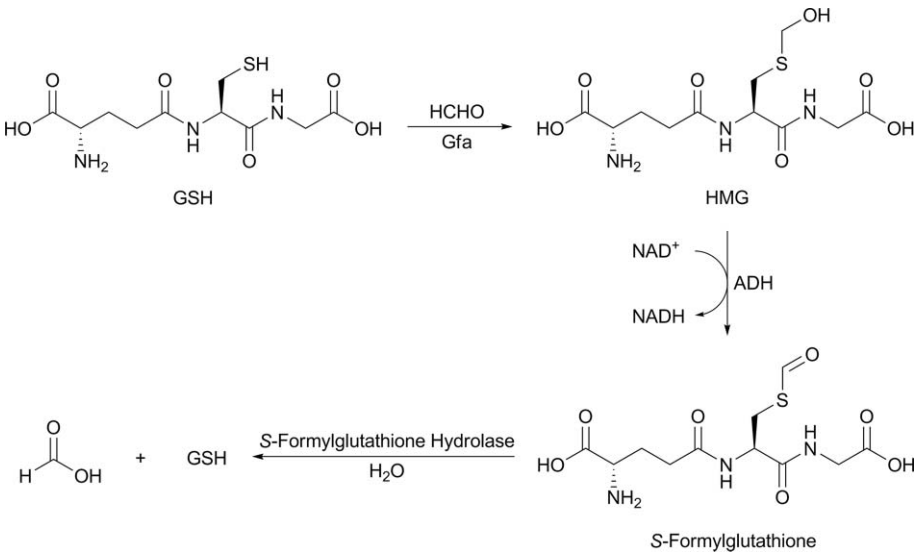


Scheme 1 The proposed mechanism for the metabolism/detoxification of formaldehyde.[7] ADH = Alcohol Dehydrogenase.

short life-times. It is likely that some of these signals arise from acyclic hemiaminals.[17–19] HMG was detected in all samples but was found to be most abundant in the more acidic media, being the major species at pD 5.5, 6.5 and 7.5, over a 105 min analysis period. At pD 7.5 and pD 8.5, the concentration of HMG dropped in an approximately linear fashion after the first NMR time point, suggesting that HMG may be an intermediate in the formation of other adducts under such conditions (Fig. 1d). HMG was only detected in trace amounts at pD 9.5. Conversely, $BiGF_2$ was not detected at pD 5.5 and was found to be most abundant at pD 9.5, reaching a maximum concentration of 8 mM after 105 min. Analysis after 3 days reaction revealed $BiGF_2$ to be present in all samples, and was found to be most prevalent at pD 9.5 (12.7 mM, Fig. 1c). HMG was detected in the samples at pD 5.5 and 6.5 after 3 days, but was not present in the 3 more basic samples (Fig. 1c).

In addition to HMG and $BiGF_2$, two previously unreported products were identified (Fig. 1a and b). The first of these was present in all samples after 3 days, but was found to be slow-forming compared to most other species in the reaction mixtures (Fig. 1c). Only the sample at pD 6.5 revealed the new species during the initial 105 min analysis period, with its concentration peaking at this pD after 3 days (4 mM, Fig. 1c). Ascribed a *m/z* value of 319 by LC-MS and HRMS, corresponding to a 1 : 1 GSH : HCHO adduct, ^{13}C NMR analysis revealed a relatively low ^{13}C resonance for the HCHO-derived methylene (42.8 ppm), suggesting that the environment around this carbon differed significantly from those around the corresponding carbons in HMG and $BiGF_2$. HMBC correlations from the protons attached to this carbon implied that the molecule had undergone a rearrangement of the GSH backbone during its formation, resulting in a pyroglutamate ring-containing structure (Fig. 1a). Purification of the new species was achieved by LC-MS; NMR analyses on the purified product led to the assignment of its structure as (*S*)-1-(((*R*)-2-amino-3-(carboxymethylamino)-3-oxopropylthio)methyl)-5-oxopyrrolidine-2-carboxylic acid (PGF, Fig. 2a and c). It is quite possible that PGF is the unidentified constituent reported by Bateman *et al.*,[16] seen at low levels under acidic conditions with an observed δ_C 44.1 ppm originating from ^{13}C-labelled HCHO. The signal at δ_C 44.1 ppm corresponds to the carbon linking the sulfur and nitrogen groups in PGF (observed by us at δ_C 42.8 ppm for PGF in D_2O, Fig. 2).

The second new species was only detected in the samples at pD 7.5, 8.5 and 9.5 and was found to be most prevalent at early time points during the reaction (Fig. 1d). This product was shown by ^1H NMR to contain only one HCHO-derived methylene and its concentration was observed to decrease over time (from 3 mM to 1.5 mM over 105 min at pD 8.5), suggesting that it undergoes further reaction, possibly to give $BiGF_2$. This product was not detected in the reaction mixtures after 3 days. The apparent dissipation of this product appeared to contrast with the increase in concentration of $BiGF_2$ over time, which was observed to grow in a near linear fashion over the 105 min reaction period (Fig. 1d). Although attempts to purify the compound by LC-MS were unsuccessful, NMR analyses on the reaction mixtures enabled assignment of its structure as (4*S*,9*R*)-9-(carboxymethylcarbamoyl)-7-oxo-1,3,8-thiadiazecane-4-carboxylic acid (MGF, Fig. 2b and d). Purified $BiGF_2$ was observed to partially decompose to PGF and HMG demonstrating that its formation is reversible. This is consistent with the proposal that MGF is an intermediate in the formation of $BiGF_2$.

To investigate the relative abundance of the described GSH-HCHO adducts with lower relative HCHO concentrations, which may be more relevant to physiological conditions, ^1H NMR analyses were conducted on 3 samples containing GSH (13.3 mM, Na_3PO_4 buffered at pD 5.5, 7.5 and 9.5) and 0.25 equivalents of HCHO. Time course data over 105 min of reaction revealed that HMG was the dominant product in the samples at pD 5.5 and pD 7.5. HMG was observed in the sample at pD 9.5 but the major adduct in this sample was MGF, both during the 105 min period and after 3 days reaction. MGF was observed at relatively low levels in the sample at pD 7.5 during the initial 105 min analysis. PGF was present in all samples, being found at highest concentration at pD 7.5 after 3 days (2 mM). However, in contrast to the samples containing a 4-fold excess of HCHO, formation of $BiGF_2$ was only observed in the sample at pD 9.5 (in trace amounts) after 3 days. These results suggest that whilst $BiGF_2$

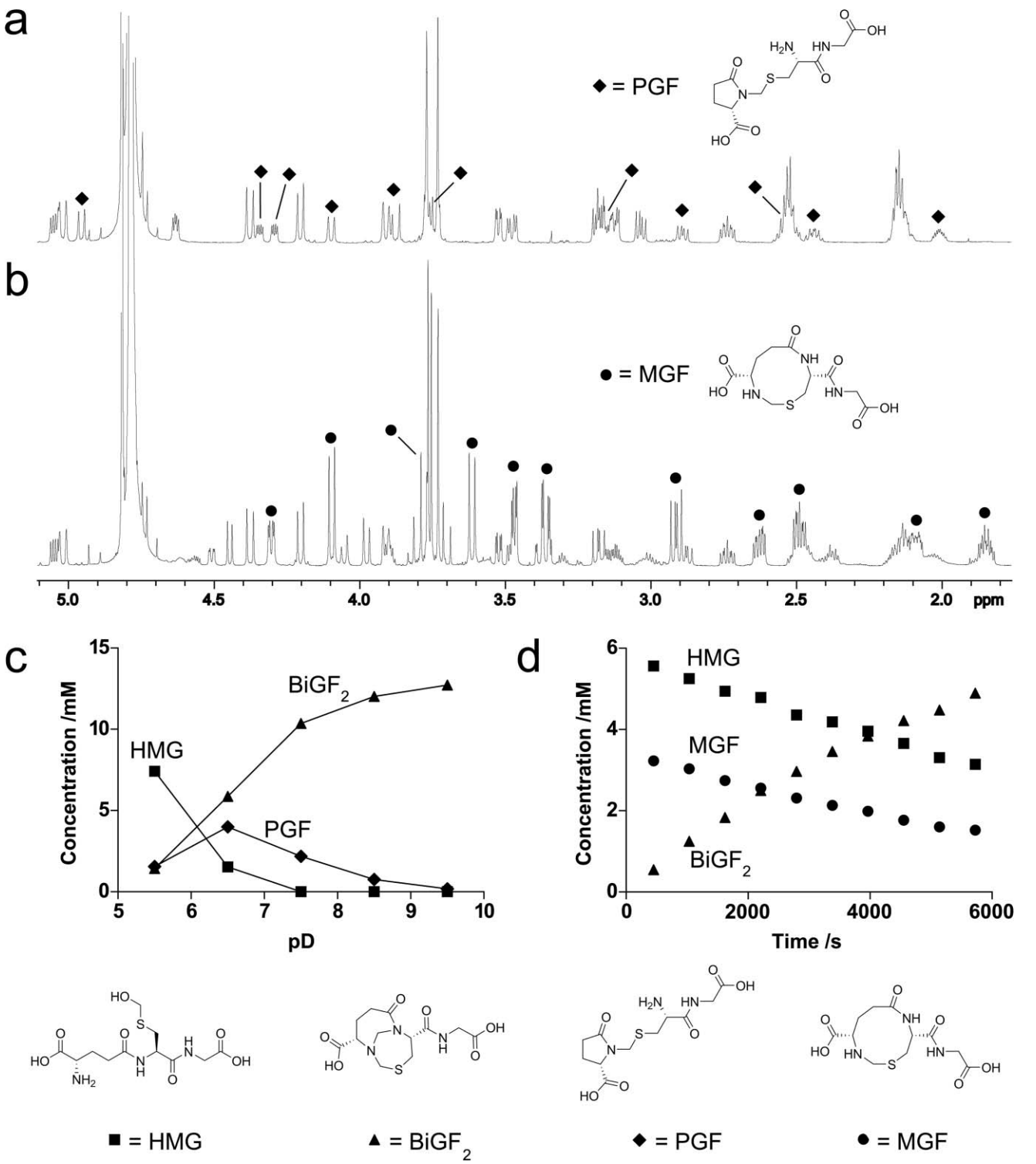
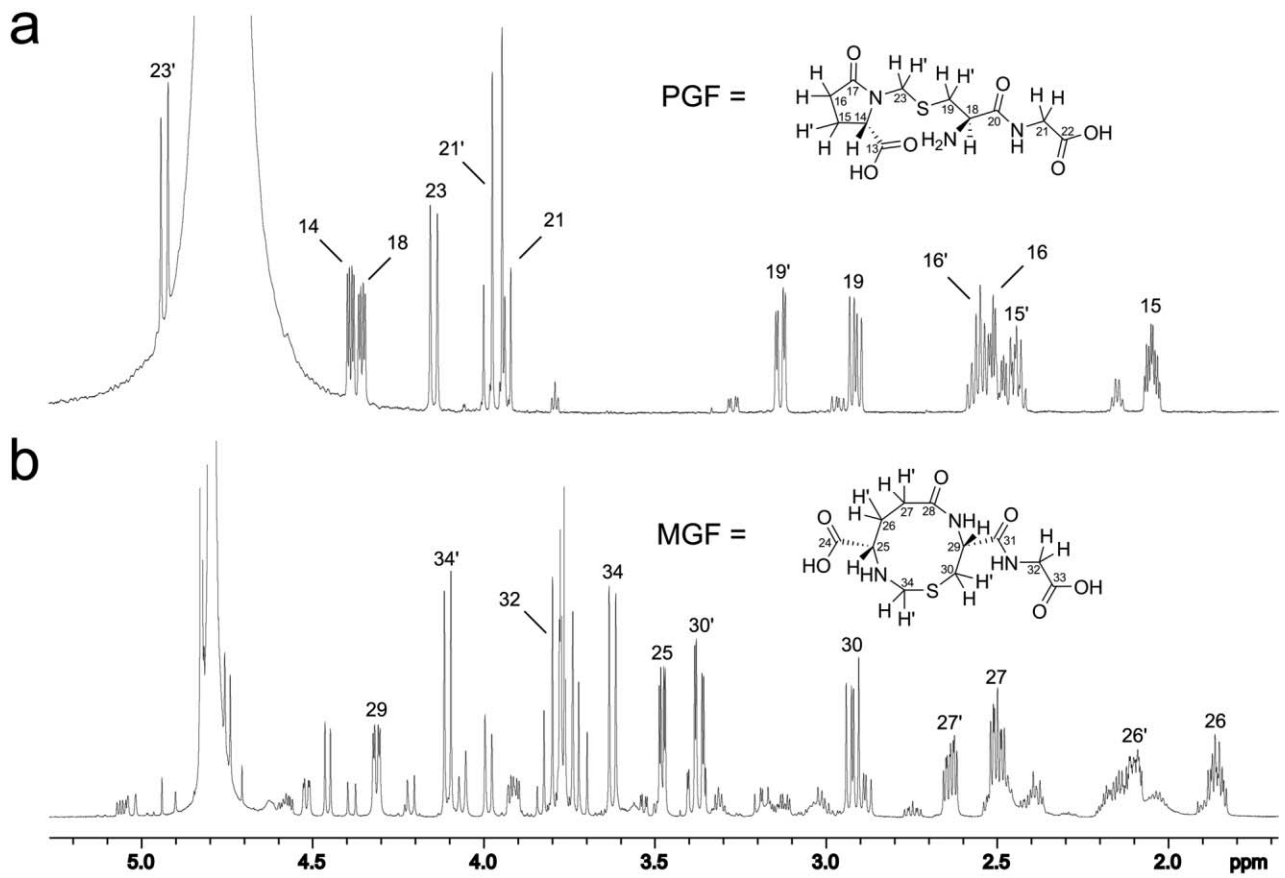


Fig. 1 Monitoring the time and pD dependence of the reaction of GSH and HCHO (1 : 4 ratio). (a) ^{1}H NMR spectrum of a reaction of GSH and HCHO (4 equivalents) at pD 6.5 after 3 days. Peaks notated with a diamond correspond to the GSH-HCHO adduct PGF. (b) ^{1}H NMR spectrum of a reaction of GSH and HCHO (4 equivalents) at pD 9.5 after 29 min. Resonances corresponding to MGF are highlighted with circles. (c) Concentrations of HMG, $BiGF_2$ and PGF formed after 3 days reaction time at different pD values. (d) Variation in the observed concentrations of HMG, $BiGF_2$ and MGF over time at pD 8.5.



c PGF

Position:	^{13}C/ ppm:	^{1}H/ ppm:	Multiplicity:	Coupling constant/ Hz:	HMBC Correlations (Carbon Atom Number):
13	178.1				
14	60.8	4.40	dd	4.0, 9.0	15, 17
15	22.8	2.06	m		13, 16
15'		2.47	m		13, 14, 16, 17
16	29.9	2.52	m		14, 15, 17
16'		2.55	m		14, 15, 17
17	179.8				
18	52.0	4.37	dd	4.0, 9.5	19, 20
19	31.1	2.93	dd	9.5, 15.0	18, 20, 23
19'		3.14	dd	4.0, 15.0	20, 23
20	168.5				
21	42.1	3.94	d	17.5	20, 22
21'		4.00	d	17.5	20, 22
22	174.0				
23	42.8	4.16	d	14.5	14, 17, 19
23'		4.94	d	14.5	14, 17, 19

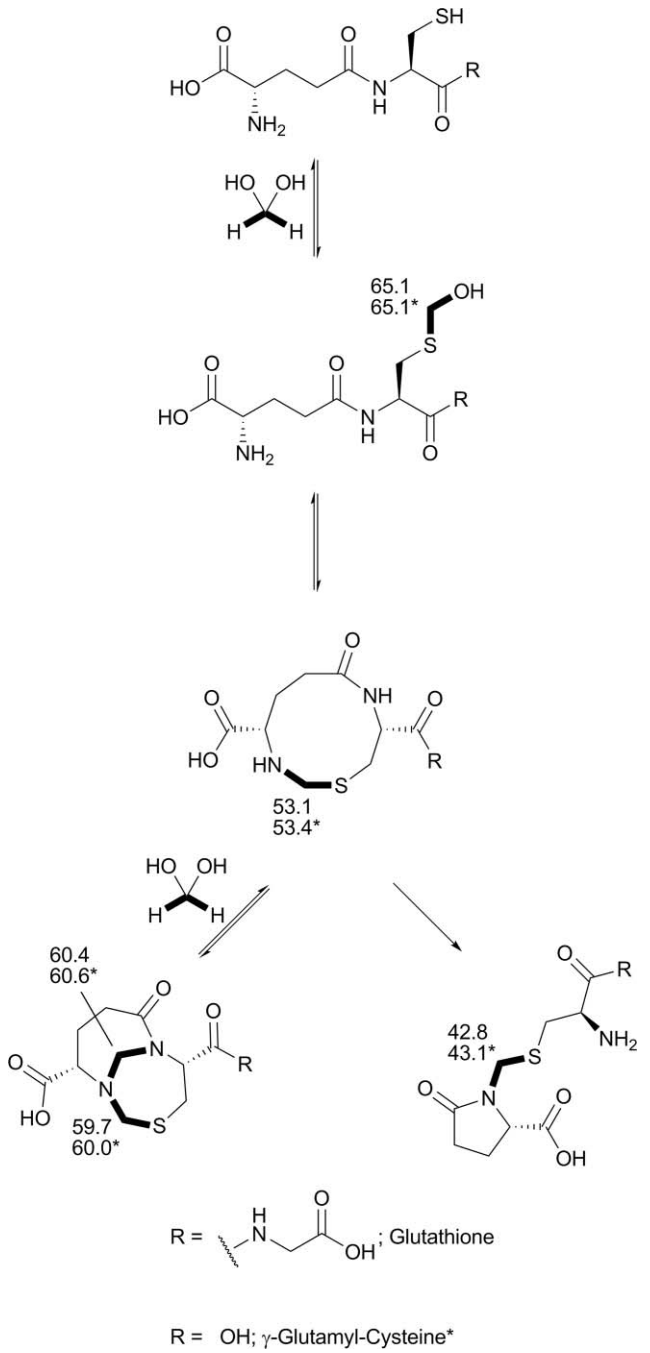
d MGF

Position:	^{13}C/ ppm:	^{1}H/ ppm:	Multiplicity:	Coupling constant/ Hz:	HMBC Correlations (Carbon Atom Number):
24	181.6				
25	58.3	3.49	dd	3.0, 9.0	24, 26, 27, 34
26	27.3	1.87	m		25, 27, 28
26'		2.10	m		27, 28
27	34.5	2.50	m		25, 26*, 28
27'		2.64	m		25, 26*, 28
28	177.4				
29	55.1	4.33	dd	3.5, 10.5	31
30	34.8	2.93	dd	10.5, 15.0	29, 31, 34
30'		3.38	dd	3.5, 15.0	34
31	172.3				
32	43.2	3.78	m		31, 33*
33	176.4*				
34	53.1	3.64	d	13.5	25, 30
34'		4.12	d	13.5	25, 30

Fig. 2 NMR characterisation of PGF and MGF. (a) ^{1}H NMR spectrum of PGF in D_2O. (b) ^{1}H NMR spectrum of a reaction mixture consisting of GSH and 4 equivalents of HCHO at pD 9.5 after 4 min. MGF is the major species. (c) Spectral assignments for PGF. (d) Spectral assignments for MGF. HMBC correlations marked with asterisks coincide with equivalent correlations from GSH. The δ_C of carbon 33 coincides with the analogous carbon of GSH.

may be the most prevalent species when HCHO is present in excess, HMG, MGF and PGF are likely more abundant adducts at lower HCHO : GSH ratios.

Experiments on the GSH-HCHO reaction prompted us to investigate reactions of HCHO with γ-glutamyl-cysteine. γ-Glutamyl-cysteine (GC) is a biosynthetic precursor of GSH[9] and contains both the glutamyl amine and thiol group involved in the formation of the GSH-HCHO adducts. GC, buffered in Na_3PO_4 solution, was reacted with a 4-fold excess of HCHO at pD 5.5 and pD 9.5, and analysed by ^{1}H NMR over 105 min of reaction. As expected, GC-containing analogues of HMG, MGF, PGF and $BiGF_2$ were all detected in the samples (Scheme 2). At pD 5.5, *S*-(hydroxymethyl)-γ-glutamylcysteine (HMGC) was identified as the major product after 105 min of analysis, however the PGF analogue PGCF was also present in trace amounts. As in the case of PGF, the formation of PGCF was very slow at this pD, but after 3 days reaction time a significant quantity of PGCF was observed. The $BiGF_2$ analogue $BiGCF_2$ was present in all 3 experiments and



Scheme 2 Possible reactions leading to the formation of $BiGF_2$, PGF and MGF. ^{13}C chemical shifts for the HCHO-derived carbons are given. Values highlighted with asterisks correspond to resonances from the analogous adduct from γ-glutamyl-cysteine.

was identified as the major product at pD 9.5 after 3 days. MGCF, the analogue of MGF, was present in the samples at pD 7.5 and pD 9.5 during the 105 min analysis period but was not detected after 3 days reaction. Overall the results obtained with GC were very similar to those obtained with GSH. The observations with GC help to verify the structural predictions (by demonstrating that the glycine of GSH is not involved in any of the proposed cyclisations) and demonstrate that the observed reactions are not limited to GSH.

We then investigated whether the reactions observed with GSH and HCHO could occur with other biologically relevant aldehydes. Acetaldehyde, an intermediate of ethanol metabolism, was mixed with GSH (13.3 mM, buffered in Na_3PO_4 solution) in a four fold excess at pD 5.5, pD 7.5 and pD 9.5 and the samples were analysed by ^1H NMR. At pD 5.5, both *R* and *S* diastereomers of the HMG analogue (2*S*)-2-amino-5-((2*R*)-1-(carboxymethylamino)-3-(1-hydroxyethylthio)-1-oxopropan-2-ylamino)-5-oxopentanoic acid (HEG) were observed in equal amounts, but the formation of these compounds was noticeably slower than in the reactions of GSH with HCHO (Fig. S29a†). In fact, only ~60% of the GSH in the sample had been converted to HEG after 3 days reaction, whereas almost all of the GSH was observed to react with HCHO over the same period. At pD 7.5 and pD 9.5, formation of HEG was found to be more efficient than at pD 5.5, as evidenced by the observation of high HEG concentrations in both samples at the first NMR analysis (~500 s after mixing). A number of species could be identified in the sample at pD 9.5, but the low concentrations of these molecules hindered their structural characterisation.

Methylglyoxal (MG) is a reactive cellular aldehyde that has been linked to human disease states including diabetes, hypertension and atherosclerosis.[20,21] MG is known to react non-enzymatically with GSH, forming the hemithioacetal (2*S*)-2-amino-5-((2*R*)-1-(carboxymethylamino)-3-(1-hydroxy-2-oxopropylthio)-1-oxopropan-2-ylamino)-5-oxopentanoic acid (MGG). This species is thought to facilitate MG metabolism by acting as a substrate for glyoxalase I, an isomerase which converts MGG to *S*-lactoylglutathione by converting the hemithioacetal group to a thioester.[22,23] Hydrolysis of the thioester bond of *S*-lactoylglutathione by glyoxalase II results in the formation of D-lactate and returns the catalytic GSH. The ^1H NMR spectrum of MGG has been previously reported from a solution of GSH and MG under acidic conditions (pH 4.4, unadjusted pH meter reading),[22] but the products from the reaction in more basic media have not been elucidated. We analysed samples of GSH (13.3 mM) that had been treated with a 4-fold excess of MG (40 wt.% in H_2O, diluted in D_2O) at pD 5.5, pD 7.5 and pD 9.5. Notably, all 3 samples revealed the formation of MGG as the only significant product (Fig. S33a†). In fact, formation of MGG appeared to be completed before the first time point of NMR analysis (~ 500 s), indicating that the reaction of GSH and MG to form the hemithioacetal is very fast across a wide pD range. Analysis of the sample at pD 9.5 after 3 days revealed a number of low level species, although MGG was still the major product in the reaction mixture. PGF and $BiGF_2$ were also observed at low levels (<10%) in the sample, although their formation is likely due to the presence in trace amounts of HCHO in the commercial source of MG (as observed by NMR). Although we cannot rule out the possibility that variations in conditions may lead to different outcomes, the experiments with acetaldehyde and methylglyoxal imply that the formation of cyclic structures is more likely with formaldehyde than other aldehydes.

Conclusions

Overall, the combined NMR studies have revealed that GSH and γ-glutamyl-cysteine can react with HCHO to produce a mixture of adducts, the composition of which is time and pD dependent.

The observed products include not only the well-established *S*-hydroxymethyl species, but also $BiGF_2$[16] and at least two further cyclic products. The results with acetaldehyde and methylglyoxal do not rule out the formation of cyclic products, but suggest that the *S*-hydroxymethyl derivatives are likely to predominate.

The question arises as to whether the observed cyclic products have any biological relevance. At early time points, HMG and MGF predominate, with $BiGF_2$ only being observed at relatively high pD values. However, even at neutral pD values, HMG, $BiGF_2$ and PGF were observed to coexist. It is possible that within cells, HMG is reacted before it has time to further react with HCHO ($BiGF_2$ was not observed at low HCHO : GSH ratios). However, under conditions when HMG metabolism is impaired, when HCHO levels are elevated, or the pH of cells becomes alkaline, it seems quite possible that the cyclic products (MGF, $BiGF_2$ and PGF) will be formed. In this regard, it is interesting that either depletion of, or mutation at the proposed active site of CENP-V (a human homologue of Glutathione-Dependent Formaldehyde-Activating Enzyme (Gfa) from *Paracoccus denitrificans*) has a significant impact upon chromatin organisation.[24] Although a biochemical function for CENP-V has not been identified, it is possible that this enzyme is involved in HCHO metabolism. Finally, we note that if the reaction of HCHO with a single tripeptide can lead to at least three cyclic products plus acyclic products, formaldehyde likely has the potential to react with proteins (and other biomacromolecules including nucleic acids)[3] to give stable adducts, including 'stable' cyclic structures with N–CH_2–N, N–CH_2–S and S–CH_2–S linkages.

Materials and methods

Preparation of NMR samples

GSH (or GC) (40 mM) was dissolved in 150 mM deuterated Na_3PO_4 solution and buffered to the appropriate pD. A portion of this solution (25 μL) was then mixed with 50 μL of the appropriate aldehyde solution (80 mM) in D_2O. Immediately after mixing, 74 μL of the sample was removed and 1 μL of 1 mg mL^{-1} deuterated (3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid solution in D_2O was added. The sample was then transferred to a 2 mm NMR tube and analysed directly by NMR. HCHO solutions were prepared by heating paraformaldehyde powder in D_2O. MG solutions were prepared from diluting 40 wt.% stock solution in H_2O with D_2O. For samples containing 0.25 equivalents of HCHO, 25 μL of deuterated Na_3PO_4 buffered 40 mM GSH stock solution was mixed with 6.25 μL of 40 mM paraformaldehyde solution in D_2O. Then, 42.75 μL of D_2O was added, followed by 1 μL of the previously described (3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid solution. The mixture was then transferred entirely into a 2 mm tube for NMR analysis.

NMR spectroscopy

Experiments were run on a Bruker Avance AVIII 700 MHz spectrometer with an inverse TCI cryoprobe, optimised for ^1H observation, and installed with TOPSPIN 2 software. Chemical shifts are reported in ppm relative to (3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid and the solvent deuterium signal was used as an internal lock signal. The spectrometer conditions were optimised before each experiment (total time lapse between mixing and the start of acquisition was between 300 and 800 s). Time course data on the GSH-HCHO reaction were collected using an automated routine; 20 analyses were performed, each accumulating 8 transients corresponding to 235 s of total acquisition time. The delay time between analyses was 58 s. All samples were kept at 298 K throughout analysis. Data were processed using automated routines and spectra were integrated with absolute intensity scaling to monitor changes in signals of interest. Coupling constants (*J*) are given to the nearest 0.5 Hz.

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Notes and references

1 H. Kalasz, *Mini-Rev. Med. Chem.*, 2003, **3**, 175–192.
2 C. C. Conaway, J. Whysner, L. K. Verna and G. M. Williams, *Pharmacol. Ther.*, 1996, **71**, 29–55.
3 S. M. Cutts, L. P. Swift, A. Rephaeli, A. Nudelman and D. R. Phillips, *Mol. Cancer Ther.*, 2003, **2**, 661–670.
4 K. Lu, W. Ye, A. Gold, L. M. Ball and J. A. Swenberg, *J. Am. Chem. Soc.*, 2009, **131**, 3414–3415.
5 R. J. Hopkinson, R. B. Hamed, N. R. Rose, T. D. W. Claridge and C. J. Schofield, *ChemBioChem*, 2010, **11**, 506–510.
6 S. Hamm, G. Just, N. Lacoste, N. Moitessier, M. Szyf and O. Mamer, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 1046–1049.
7 W. G. Gutheil, E Kasimoglu and P. C. Nicholson, *Biochem. Biophys. Res. Commun.*, 1997, **238**, 693–696.
8 M. Goenrich, S. Bartoschek, C. H. Hagemeier, C. Griesinger and J. A. Vorholt, *J. Biol. Chem.*, 2002, **277**, 3069–3072.
9 A. Meister and M. E. Anderson, *Annu. Rev. Biochem.*, 1983, **52**, 711–760.
10 A. Pompella, A. Visvikis, A. Paolicchi, V. De Tata and A. F. Cassini, *Biochem. Pharmacol.*, 2003, **66**, 1499–1503.
11 N. S. Kosower and E. M. Kosower, *Int. Rev. Cytol.*, 1978, **54**, 109–160.
12 R. C. Fahey, W. C. Brown, W. B. Adams and M. B. Worsham, *J. Bacteriol.*, 1978, **133**, 1126–1129.
13 M. Tedeschi, S. Bohm, F. Di Re, S. Oriana, G. B. Spatti, S. Tognella and F. Zunino, *Cancer Treat. Rev.*, 1990, **17**, 203–208.
14 R. P. Mason, S. Naylor, D. H. Williams, J. K. M. Sanders and G. Moneti, *Biochem. Soc. Trans.*, 1987, **15**, 230–231.
15 S. Naylor, R. P. Mason, J. K. M. Sanders, D. H. Williams and G. Moneti, *Biochem. J.*, 1988, **249**, 573–579.
16 R. Bateman, D. Rauh and K. M. Shokat, *Org. Biomol. Chem.*, 2007, **5**, 3363–3367.
17 E. H. Cordes and W. P. Jencks, *J. Am. Chem. Soc.*, 1962, **84**, 832–837.
18 R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, 1966, **241**, 5864–5878.
19 W. R. Abrams and R. G. Kallen, *J. Am. Chem. Soc.*, 1976, **98**, 7777–7789.
20 M. P. Kalapos, *Toxicol. Lett.*, 1999, **110**, 145–175.
21 K. M. Desai, T. Chang, H. Wang, A. Banigesh, A. Dhar, J. Liu, A. Untereiner and L. Wu, *Can. J. Physiol. Pharmacol.*, 2010, **88**, 273–284.
22 C. Rae, S. I. O'Donoghue, W. A. Bubb and P. W. Kuchel, *Biochemistry*, 1994, **33**, 3548–3559.
23 P. J. Thornally, *Biochem. Soc. Trans.*, 2003, **31**, 1343–1348.
24 A. M. B. Tadeu, S. Ribeiro, J. Johnston, I. Goldberg, D. Gerloff and W. C. Earnshaw, *EMBO J.*, 2008, **27**, 2510–2522.

