Hydrogenases catalyze the reversible reduction of protons to yield molecular hydrogen (H₂) and occur in three evolutionarily unrelated forms termed the [Fe]-, [FeFe]-, and [NiFe]-hydrogenases. [1,2] [FeFe]-hydrogenases all contain a complex active-site cofactor termed the H cluster (Scheme 1) that consists of a regular [4Fe4S] cluster bridged by a shared cysteine thiolate sulfur atom to a 2Fe subcluster with biologically unique carbon monoxide, cyanide, and dithiolate ligands.[3,4] The H cluster is biosynthesized in a stepwise process in which generalized host cell machinery[5] is directed towards the synthesis of a [4Fe4S] subcluster with subsequent synthesis and insertion of the 2Fe subcluster by specialized hyd encoded proteins, HydE, HydF, and HydG.[1,6] HydE and HydG are radical S-adenosylmethionine (AdoMet) enzymes thought to be responsible for the synthesis and proper incorporation of the nonprotein ligands.[1,7–9] HydF has been proposed to function as a scaffold for assembly of the H cluster 2Fe subcluster and also to mediate its subsequent insertion into HydA.[6,10] HydG has recently been shown to catalyze radical-mediated tyrosine cleavage and generate p-cresol,[11] which is a known fermentation product of several anaerobes.[12,13] The HydG-catalyzed reaction is proposed to be similar to that catalyzed by the thiamine biosynthetic enzyme ThiH and to yield dehydroglycine as an intermediate.[14–16] It was hypothesized by Pilet et al.[11] that two molecules of dehydroglycine condense at an FeS cluster on HydG and result in generation of the dithiolate ligand. Herein, we demonstrate that cyanide is a product of HydG-catalyzed tyrosine cleavage, a result which clarifies the role of HydG and indicates that tyrosine is the source of the cyanide ligands in the H cluster.

To investigate the reaction products formed by HydG, enzyme activity assays containing chemically reconstituted HydG (on average 5.1 ± 0.5 Fe per HydG), tyrosine, AdoMet, and sodium dithionite were prepared. At selected time points, assays were stopped by acidification, and the precipitated protein was removed by centrifugation. HPLC-based analysis methods were then used to measure the concentration of reaction products in the supernatant, thus confirming the turnover of AdoMet to yield deoxyadenosine (DOA), whilst tyrosine was cleaved to yield p-cresol.[15]

Cyanide was detected and quantified after derivatization by a modification of the method of Traquitt et al.[17] in which the cyanide anion reacts with naphthalene-2,3-dicarbaldheyde (NDA) 5 and a primary amine (either taurine (6) or N,N′-dimethylethane-1,2-diamine (7)), thus generating the fluorescent 1-cyanobenz[f]isoindole (CBI) derivatives 8 and 9 (Figure 1A). After derivatization, HPLC analysis showed a fluorescent peak which coeluted with a standard of the CBI derivative (Figure 1B). The components required for cyanide-forming activity were tyrosine, AdoMet, a reducing agent, and HydG. Omission of any of these components resulted in loss of activity, consistent with a HydG-mediated cleavage of tyrosine to form cyanide using radical AdoMet chemistry. In these experiments, 5′-methylthioadenosine/
S-adenosylhomocysteine nucleosidase (MTAN) was added to the reaction mixture to hydrolyze the DOA formed in the reaction, thus minimizing potential product inhibition\cite{18} and maximizing the formation of tyrosine-derived reaction products. Under these conditions, tyrosine cleavage reached 43\%, forming 430 mm \textit{p}-cresol (Figure S2 in the Supporting Information). To confirm that tyrosine is the source of cyanide, l-
\textit{-U}-[13C,15N]tyrosine was incubated with HydG, AdoMet, and dithionite. After removal of HydG, the assay supernatant was derivatized with NDA\textsuperscript{5} and amine\textsuperscript{7} and analyzed by LC-MS. Relative to the product obtained with an unlabeled cyanide standard, the product formed from labeled tyrosine resulted in a mass change of +2 Da, consistent with the formation of 13C,15N-labeled cyanide (Figure 2C,D).

ThiH and HydG have identical substrates and at least three products in common (methionine, DOA, and \textit{p}-cresol). As the fourth reaction product of ThiH is dehydroglycine (10, Scheme 2), we attempted to obtain evidence for the presence of dehydroglycine associated with HydG. Dehydroglycine is hydrolytically sensitive and has been detected during in vitro activity assays by the formation of the hydrolysis product, glyoxylate.\cite{15} Purified HydG protein was denatured, and approximately 10 mol\% per mole glyoxylate was detected in the solution. In optimized activity assays, the concentration of glyoxylate was below 10\% of the HydG concentration, consistent with it being an intermediate.

The stoichiometry of product formation in HydG reactions was investigated over a one-hour time course. Activity assays were initiated by the addition of dithionite and stopped at selected time points (between 1 and 60 min) by acidification to precipitate HydG and release any protein-bound products into solution. HPLC analysis with UV/Vis detection allowed the measurement of the products DOA and \textit{p}-cresol, whereas cyanide was detected as the CBI fluorescent derivative\textsuperscript{8}. Accurate measurement of cyanide required careful calibration using standard cyanide solutions that were subjected to conditions identical to the reaction and workup conditions of HydG activity assays. The time-course data was fitted to a first-order process (Figure 2 and Table 1) and demonstrated that cyanide and \textit{p}-cresol were formed in a 1:1 ratio, with each HydG producing 4.7 mole equivalents of cyanide. The initial turnover number for HydG (Table 1, $k_{\text{cat.}} = \frac{20 \pm 2 \times 10^{-4} \text{s}^{-1}}{C_6^2} / C_{148^10} / C_{0^4}^1$) is comparable to the observed activity of the closely related ThiH (for \textit{p}-cresol formation, $k_{\text{cat.}} = \frac{32 \pm 8 \times 10^{-4} \text{s}^{-1}}{C_{6^8}^8}$).\cite{16} The ratio of DOA to \textit{p}-cresol and cyanide was observed to be 1.3:1, indicating some uncoupled turnover.\cite{19} However, activity assays that
Table 1: Kinetic parameters of product formation.  

<table>
<thead>
<tr>
<th>Product</th>
<th>[Product]_{max} [μM]</th>
<th>k [× 10^{-4} s^{-1}]</th>
<th>k_{cat} [× 10^{-4} s^{-1}]</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOA</td>
<td>373 ± 11</td>
<td>5.2 ± 0.3</td>
<td>31 ± 3</td>
<td>0.99</td>
</tr>
<tr>
<td>p-cresol</td>
<td>281 ± 15</td>
<td>5.2 ± 0.5</td>
<td>24 ± 3</td>
<td>0.99</td>
</tr>
<tr>
<td>cyanide</td>
<td>280 ± 16</td>
<td>4.3 ± 0.4</td>
<td>20 ± 2</td>
<td>0.99</td>
</tr>
</tbody>
</table>

(a) Data from Figure 2 were fitted to a first-order process to determine the final concentration of each product, the first-order rate constant, and the initial turnover number. Values are shown with standard errors, and R² is a measure of the goodness of fit. (b) Rate constants (k) and turnover numbers (k_{cat}) were derived as described previously.  

In conclusion, we have demonstrated that HydG catalyzes radical AdoMet chemistry, cleaving tyrosine to form a 1:1 stoichiometric ratio of cyanide to p-cresol. A slight excess of DOA is generated, thus implying that a degree of uncoupled AdoMet turnover occurs. The tyrosine-derived cyanide is proposed to become associated with HydF, which functions as a scaffold for H-cluster assembly, and the cluster is finally transferred to HydA. Furthermore, this study provides the first experimental evidence that some hydrogenase ligands can be derived from the radical-mediated decomposition of an amino acid and may have interesting implications in the context of prebiotic chemistry. The groundwork has now been laid for further work focused on approaches to investigate the unresolved issues of the source of the dithiolate and carbon monoxide ligands.

Received: December 14, 2009
Published online: January 27, 2010

**Keywords:** bioinorganic chemistry · biosynthesis · cyanides · hydrogenases · metalloenzymes

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Supporting Information

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69451 Weinheim, Germany

[FeFe]-Hydrogenase Cyanide Ligands Derived From S-Adenosylmethionine-Dependent Cleavage of Tyrosine**

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anie_200907047_sm_miscellaneous_information.pdf
Supplementary material to the communication.

Experimental

S1. Materials and Methods. All protein purifications, reconstitutions and assays were maintained under nitrogen in an anaerobic glove box with less than 2 ppm of O₂ (Belle Technology, Portesham, UK) equipped with a Pharmacia Acta FPLC and VC130 sonicator (Somics and Materials, Newtown, Connecticut, USA). Cell lysates were cleared in 250 ml gas-tight polycarbonate centrifuge bottles (Beckman-Coulter, High Wycombe, UK). A Gilson 321 HPLC work center equipped with a dual wavelength UV-visible detector and a Shimadzu RF-10Axl fluorescence detector was used for analytical HPLC methods; chromatograms were collected and analyzed using the Gilson Unipoint software (Gilson). LC-MS analysis used a Gilson HPLC coupled to a Thermo Finnigan Surveyor MSQ single quadrupole mass spectrometer with electrospray ionization. The data were collected and processed using the XCalibur software system. UV-visible spectra were recorded with an Ocean Optics (Duiven, The Netherlands) USB2000 spectrophotometer using a Mini-D2-GS light source connected by P-400-2-UV/SR optical fibers to a cuvette holder inside the glove box. MTAN was purified as described previously[1]. Glyoxylate concentrations were analyzed as described previously[2].

S2. HydG Expression and Purification. The plasmid encoding HydGΔEF[3] was transformed into E. coli BL21(DE3). An overnight culture (100 mL of 2YT medium) was prepared from a glycerol freeze stock of this derivative strain and used as 1% inocula into 4 x 1.25 L of 2YT media. The resulting culture was incubated at 37 ºC in an orbital shaker (180 rpm) until the OD600nm reached ~0.6. Protein expression was induced by the addition of IPTG (1 mM) and the culture was supplemented with ferrous ammonium sulphate (FAS, 0.075 g/L). After incubation at 27 ºC for an additional 2 h, FAS (0.075 g/L) was added and the cultures transferred to sealed 1 L Schott bottles and stirred at 4 ºC overnight before being harvested by centrifugation (SLC6000, 7500 rpm, 30 min, 4 ºC), yielding 20 - 25 g cell paste which was stored at - 80 ºC until further use.

For protein purification, the cell paste was resuspended in anaerobic buffer A (20 mM HEPES pH 7.4, 0.5 M NaCl, 5% glycerol, 20 mM imidazole) and stirred for 30 min in a cooled water bath before lysozyme (0.1 mg/ml) and PMSF (1 mM) was added. Two further PMSF aliquots were added over a 1 h period. Lysis was achieved by sonication (3 x 10 min, 1 s bursts, 20W) and the lysate cleared by centrifugation (SLA1500, 13000 rpm, 40 min, 4 ºC). The extract was applied to a chelating Sepharose fast flow column (2.6 x 20 cm, charged with NiSO₄ and pre-equilibrated with anaerobic buffer A). The column was then washed with buffer A (300 mL) before the protein was eluted by applying a linear gradient to 250 mM imidazole over 50 mL using buffer B (20 mM HEPES pH 7.4, 0.5 M NaCl, 5% glycerol, 500 mM imidazole). 5 mL fractions were collected and the most concentrated (up to a total of 30 mL) applied to a Superdex S-75 column (50 mL), previously equilibrated with buffer C (20 mM HEPES pH 7.4, 0.5 M NaCl, 5% glycerol, 5 mM DTT). The protein eluted with buffer C and the protein containing fractions were concentrated by ultrafiltration (10 kDa MWCO) to ~20 mg/mL and stored at - 80 ºC as 1 mL aliquots.

S3. Reconstitution of HydG. An aliquot of HydG was incubated anaerobically at 20 ºC with 5 mM DTT for 25 min and then 6.5 equivalents (w.r.t. HydG) of FeCl₃ were added dropwise from a freshly prepared anaerobic 10 mM stock solution and the protein solution stirred for 25 min. 6.5 equivalents of Na₂S.9H₂O were added in similar fashion and the solution stirred for further 70 min. Precipitated iron sulfide was removed by centrifugation and the protein desalted into 100 mM phosphate buffer pH 7.5 via a NAP-10 or PD-10 column (GE Healthcare). The efficiency of chemical reconstitution was assessed by UV-visible spectroscopy (Figure S1) and the iron content of isolated and chemically reconstituted samples of HydG was determined by the method of Fish[4] (Table S1).

Table S1. Number of equivalents of iron in as isolated and chemically reconstituted samples of HydG. Each sample is determined from a different reconstitution of HydG.

<table>
<thead>
<tr>
<th>Sample</th>
<th>As isolated</th>
<th>Reconstituted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9 ± 0.04</td>
<td>3.3 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.9 ± 0.1</td>
<td>6.9 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>1.4 ± 0.1</td>
<td>4.0 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.5 ± 0.02</td>
<td>4.7 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>1.2 ± 0.08</td>
<td>5.7 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>1.3 ± 0.02</td>
<td>5.4 ± 0.05</td>
</tr>
</tbody>
</table>

S4. Reactions with HydG. All manipulations of HydG were conducted under strictly anaerobic conditions using an anaerobic glove box (Belle Technology, Portesham, UK). Activity assays were prepared by adding the substrates AdoMet and tyrosine (both to final concentrations of 1 mM) to chemically reconstituted HydG (60 – 200 µM). The solution was equilibrated at 37 ºC and the HydG catalyzed reaction initiated by addition of sodium dithionite (1 mM). Reactions were stopped by precipitating the protein by the addition of acid (20% perchloric acid (15 µL)) followed by centrifugation (10 min, Eppendorf 5415D microcentrifuge, maximum speed). Modifications of this assay specific for different experiments are described below.
Improving the activity with MTAN. To test for product inhibition, DOA (1 mM), methionine (1 mM) and DOA plus methionine (1 mM of each) were added to standard assays. The effects of MTAN were investigated by adding MTAN (10 μM) and/or DOA plus methionine (1 mM of each) to assays.

Figure S1. UV-Visible spectroscopy of HydG.

Figure S2. Effect of adding MTAN to HydG activity assays. Sample (1), standard assay; sample (2), as (1) plus 1 mM Met; sample (3), as (1) plus 1 mM DOA; sample (4), as (1) plus 1 mM DOA and 1 mM Met; sample (5), as (1) plus 10 μM MTAN; sample (6), as (1) plus 1 mM DOA, 1 mM Met and 10 μM MTAN.

S5. Characterisation of Tyrosine Derived Cyanide. Assays designed for the detection of cyanide contained MTAN (10 μM) to maximize turnover efficiency and L-U-[13C, 15N] tyrosine (500 μM). The assay was incubated at 37 °C for 3 h and after precipitation of the proteins by the addition of 20% perchloric acid (15 μL), the solution was immediately buffered to pH 7.5 by the addition of 1 M potassium phosphate, pH7.5 (20 μL) and 1 M NaOH (10 μL), prior to centrifugation. For each activity assay, 10 μL of the supernatant was derivatized by adding to a pre-mixed solution of NDA (2.5 mM), NH₃ (1% v/v), dimethylaminoethylamine 7 (12.5 mM) in 70% MeOH/H₂O. After 5 min incubation at room temperature the solution was diluted 1:1 with water prior to LC-MS analysis. Chromatographic separation was achieved by injecting 75 μL of derivatized supernatant onto a Hypersil C18 reverse phase column (4.6 x 150 mm). The chromatography buffers were 2 mM ammonium formate, pH 3 (solvent A) and methanol (solvent B) and the initial mobile phase contained 20% solvent B for 5 min, followed by a gradient to 65% solvent B over 20 min and then 100% solvent B over the next 2 min, where it was held isocratically for 10 min. The CBI derivative 9 eluted with a retention time of ~28 min. Mass spectra were recorded employing a 1:4 (MS:UV) post column split and a make up buffer of 3% formic acid in 50% MeOH/H₂O. Spectra were recorded using ES+ ionisation mode and data was processed using Xcalibur software.
S6. Kinetics of Product Formation. Fourteen assays containing 62 µM of chemically reconstituted HydG were prepared as described above, initiated in parallel and incubated at 37 °C. Duplicate assays at selected time points of 1, 3, 5, 10, 15, 30 and 60 minutes were achieved by stopping the reaction with addition of 20% perchloric acid (15 µl). The solution was neutralized by immediate addition of 1 M phosphate buffer pH 7.5 (20 µl) and 1 M NaOH (30 µl) before removal of the precipitated protein by centrifugation (10 min, Eppendorf 5415D microcentrifuge, maximum speed). To establish a cyanide calibration plot (Figure S4), potassium cyanide standards at a range of concentrations (25 - 400 µM) in 100 mM phosphate pH 7.5 were prepared and subjected to the same conditions as HydG assays. Particular care was taken in preparing these cyanide standards, so that they lacked tyrosine but contained HydG and dithionite. They were prepared in triplicate, incubated for reaction times of 3 min, 12.5 min and 45 min and subjected to the same precipitation and neutralization conditions as assay samples. A calibration curve for cyanide concentration was constructed from average values obtained from these three sets of cyanide standards.

Figure S3. HPLC chromatograms of HydG assays with detection at 280 nm. Peaks were identified as (a) AdoMet, (b) tyrosine, (c) DOA, (d) p-cresol by comparison with authentic standards.

Quantification of Reaction Products. The amount of DOA and p-cresol present in assay supernatants was measured by HPLC using the method of Challand et al\textsuperscript{[5]}, Cyanide was analyzed using the method of Tracqui et al\textsuperscript{[6]} with slight modifications as follows: 10 µL of the supernatants were derivatized by adding to a pre-mixed solution of NDA (2.5 mM), NH\textsubscript{3} (1%), taurine 6 (12.5 mM) in 70% MeOH/H\textsubscript{2}O. After 5 min incubation at room temperature the solution was diluted 1:1 with H\textsubscript{2}O and analyzed by HPLC. Chromatographic analysis was carried out under conditions identical to the LC-MS analysis. The CBI derivative 8 eluted with a retention time of 21 min (main paper, Figure 2) and was detected using fluorescence detection (λ\textsubscript{ex} 418 nm, λ\textsubscript{em} 454 nm).
Figure S4. Calibration plot of cyanide standards.

Table S2. Time course of DOA formation.

<table>
<thead>
<tr>
<th>Time / min</th>
<th>Sample 1 / µM</th>
<th>Sample 2 / µM</th>
<th>Average / µM</th>
<th>Standard deviation</th>
</tr>
</thead>
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<td>60</td>
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<td>341</td>
<td>316</td>
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</table>

Table S3. Time course of p-cresol formation.

<table>
<thead>
<tr>
<th>Time / min</th>
<th>Sample 1 / µM</th>
<th>Sample 2 / µM</th>
<th>Average / µM</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
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<td>60</td>
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<td>257</td>
<td>239</td>
<td>25</td>
</tr>
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</table>
S7. Phylogenetic Analysis of Representative Radical AdoMet Proteins. Representative protein sequences from several members of the radical AdoMet superfamily with known biochemical functions (HydE, HydG, PflA, BchE, NirJ, LAM, NiB, MoaA, LipA, ThiH, BioB, NosL) were compiled using query sequences outlined in Sofia et al.\textsuperscript{[7]}, and Yu et al.\textsuperscript{[8]}, with the DOE-IMG BLASTp server. Individual sets of compiled sequences were aligned with ClustalX (version 2.0.8)\textsuperscript{[9]} employing the Gonnet protein weight matrix and default alignment parameters. Sequences representing the primary lineages for each locus were identified and compiled. Compiled AdoMet sequences were aligned as described above. The best fit protein evolutionary model was determined with ProtTest\textsuperscript{[10]}. The phylogenetic position of HydG in relation to representative members of the AdoMet superfamily of proteins was evaluated using a Bayesian likelihood approach (MrBayes version 3.1.2\textsuperscript{[11,12]} with the default settings and the WAG model with gamma-distributed rate variation across sites (I+G in evolutionary model). A total of 2.63e7 generations were calculated for the data set and a phylogram with mean branch lengths was obtained with a parameter burn-in of 1.25e7 (standard deviation of split trees was <0.03). Phylograms were projected from a consensus of ~5200 trees sampled at stationarity with FigTree. Accession numbers for the proteins used in this analysis are BchE: ZP_01386524, YP_002016635, YP_0019955726, YP_002461698, YP_002571652, YP_001770400, YP_001003191, YP_001179319, YP_461123, YP_001211533, YP_001673638, YP_357021, YP_001389301, YP_877565, ZP_02074795, ZP_02087032, ZP_02417503, ZP_03168009, ZP_01962185, ZP_03015922, YP_101169, ZP_02428409, ZP_03054246, ZP_001421419, ZP_01090100, ZP_00378055, YP_143873, YP_863969, ZP_02275539, YP_002305219, YP_303287, YP_877356, YP_910606; HydG: NP_03941120, YP_002302007, YP_356874, CAJ73158, YP_001179319, YP_461123, YP_001188029, YP_03149965, YP_002482262, YP_001158413, YP_002644658, YP_938502, YP_189896, YP_252184, YP_647165, ZP_03565789, YP_04280510, YP_002531621, YP_980477, ZP_02875815, YP_001487995, ZP_03054246, YP_001421419, ZP_01090100, ZP_00378055, YP_143873, YP_863969, ZP_02275539, YP_002305219, YP_303287, YP_877356, YP_910606; HydG: NP_03941120, YP_002302007, YP_356874, CAJ73158, YP_001179319, YP_461123, YP_001188029, YP_03149965, YP_002482262, YP_001158413, YP_002644658, YP_938502, YP_189896, YP_252184, YP_647165, ZP_03565789, YP_04280510, YP_002531621, YP_980477, ZP_02875815, YP_001487995, YP_001047376, YP_001548375, ZP_031537724.
**Figure S5.** Phylogenetic reconstruction of representative radical AdoMet proteins. Closed circles denote 90-100% posterior probability (PP); gray circles denote 80-89% PP; open circles denote 70-79% PP; no symbol indicates <69% PP.

**References for Supporting Information**