VIP Cyanide Biosynthesis

DOI: 10.1002/anie.200907047

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

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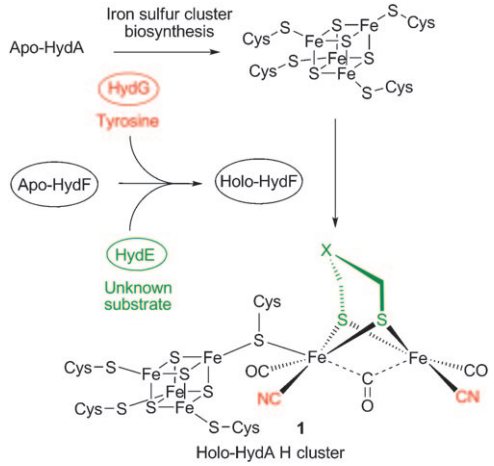
Hydrogenases catalyze the reversible reduction of protons to yield molecular hydrogen (H_2) and occur in three evolutionarily unrelated forms termed the [Fe]-, [FeFe]-, and [NiFe]-hydrogenases.[1,2] [FeFe]-hydrogenases all contain a complex active-site cofactor termed the H cluster (**1**, Scheme 1) 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.



Scheme 1. Proposed biosynthesis of the HydA H cluster. HydA contains a preformed [4Fe4S] cluster; HydE, HydG, and HydF then function to form the 2Fe subcluster and non-protein-derived ligands on HydF, which is then transferred to HydA. Recent spectroscopic results suggest that X is an amine.[4]

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.[11]

Cyanide was detected and quantified after derivatization by a modification of the method of Tracqui et al.[17] in which the cyanide anion reacts with naphthalene-2,3-dicarbaldehyde (NDA) **5** and a primary amine (either taurine (**6**) or N^1,N^1-dimethylethane-1,2-diamine (**7**)), thus generating the fluorescent 1-cyanobenz[f]isoindole (CBI) derivatives **8** and **9** (Figure 1 A). After derivatization, HPLC analysis showed a fluorescent peak which coeluted with a standard of the CBI derivative (Figure 1 B). 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/

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[**] This work was supported by the Biotechnology and Biological Sciences Research Council (M.R.C.), the Biochemical Society (R.C.D.), the NASA Astrobiology Institute (J.B.B. & J.W.P.) and the Airforce Office of Scientific Research (J.W.P.). S.E.M. is supported by an NSF IGERT Fellowship (DGE 0654336). E.S.B. was supported by NAI Postdoctoral Fellowship.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200907047.

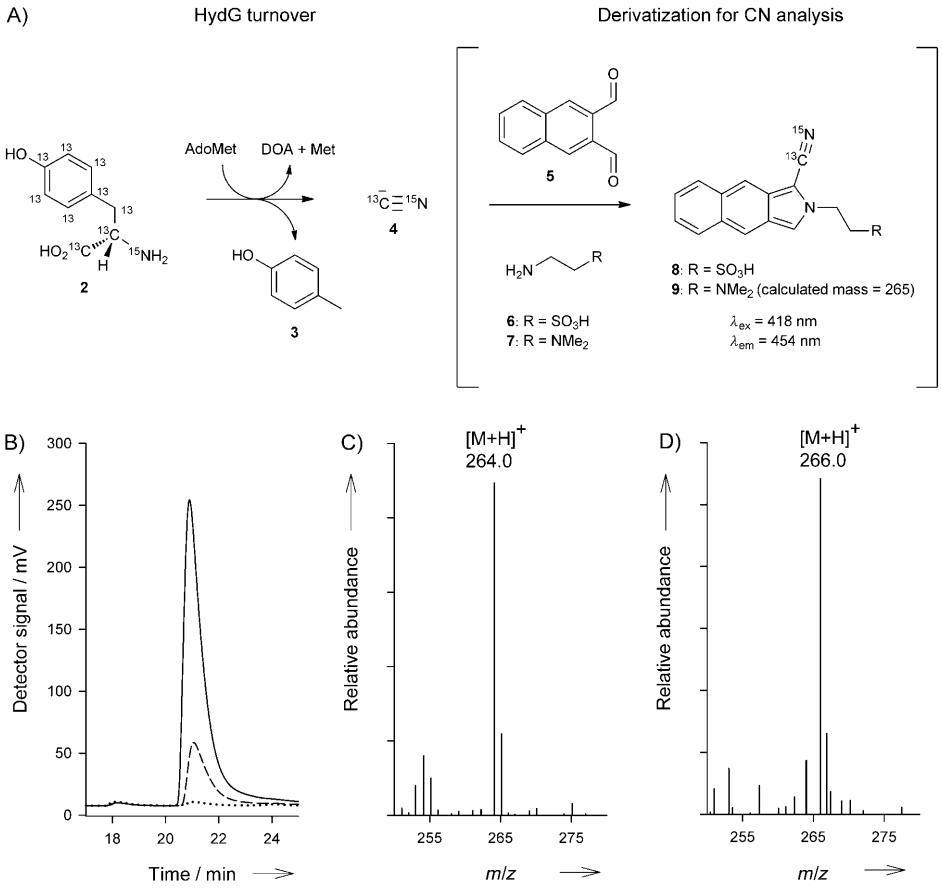


Figure 1. Characterization of cyanide as a product of HydG catalyzed tyrosine cleavage. A) Formation of cyanide from tyrosine by HydG and subsequent precolumn derivatization with amine **6** for fluorescent HPLC or amine **7** for LC-MS analysis. B) Fluorescent HPLC analysis of CBI **8** from a negative control HydG assay lacking tyrosine (•••••), the full assay (——), and a 50 μM synthetic standard of cyanide (– – –). C) Mass spectrum of CBI derivative **9** prepared from an unlabeled cyanide standard. D) Mass spectrum of CBI derivative **9** from an assay mixture incubated with L-*U*-[^{13}C,^{15}N]tyrosine.

S-adenosylhomocysteine nucleosidase (MTAN) was added to the reaction mixture to hydrolyze the DOA formed in the reaction, thus minimizing potential product inhibition[18] and maximizing the formation of tyrosine-derived reaction products. Under these conditions, tyrosine cleavage reached 43%, forming 430 μM *p*-cresol (Figure S2 in the Supporting Information). To confirm that tyrosine is the source of cyanide, L-*U*-[^{13}C,^{15}N]tyrosine was incubated with HydG, AdoMet, and dithionite. After removal of HydG, the assay supernatant was derivatized with NDA **5** and amine **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 ^{13}C,^{15}N-labeled cyanide (Figure 2C,D).

ThiH and HydG have identical substrates and at least three products in common (methionine, DOA, and *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.[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 *p*-cresol, whereas cyanide was detected as the CBI fluorescent derivative **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 *p*-cresol were formed in a 1:1 ratio, with each HydG producing

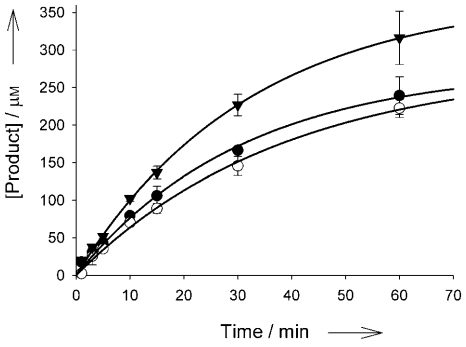
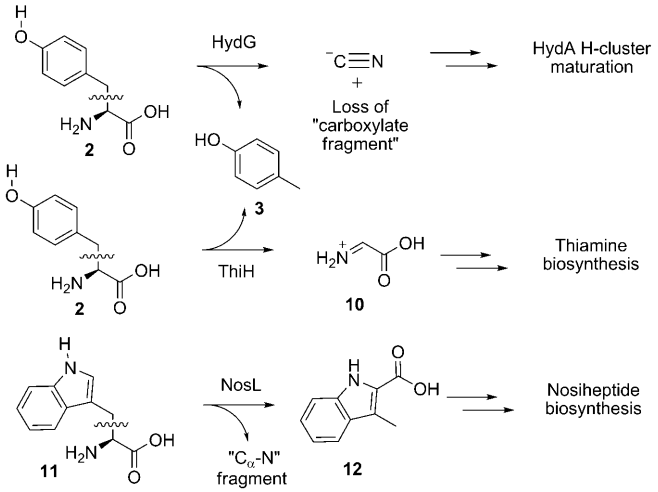


Figure 2. Time course of DOA (▼), *p*-cresol (●), and cyanide (○) formation from a HydG assay containing HydG (62 μM), tyrosine (1 mM), AdoMet (1 mM), and sodium dithionite (1 mM).

4.7 mole equivalents of cyanide. The initial turnover number for HydG (Table 1, $k_{cat.}^{0} = (20 \pm 2) \times 10^{-4}\ s^{-1}$) is comparable to the observed activity of the closely related ThiH (for *p*-cresol formation, $k_{cat.}^{0} = (32 \pm 8) \times 10^{-4}\ s^{-1}$).[16] The ratio of DOA to *p*-cresol and cyanide was observed to be 1.3:1, indicating some uncoupled turnover.[19] However, activity assays that



Scheme 2. The C_α–C_β lyase subfamily with currently characterized biochemical functions.

Table 1: Kinetic parameters of product formation.[a]

Product	$[Product]_{max}$ [μM]	k [$\times 10^{-4}$ s^{-1}][b]	$k_{cat.}^0$ [$\times 10^{-4}$ s^{-1}][b]	R^2
DOA	373 ± 11	5.2 ± 0.3	31 ± 3	0.99
p-cresol	281 ± 15	5.2 ± 0.5	24 ± 3	0.99
cyanide	280 ± 16	4.3 ± 0.4	20 ± 2	0.99

[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 R^2 is a measure of the goodness of fit. [b] Rate constants (k) and turnover numbers ($k_{cat.}^0$) were derived as described previously.[16]

contained AdoMet and the reductant but lacked tyrosine showed very little uncoupled turnover (Figure S3 in the Supporting Information), implying that, like ThiH, uncoupled turnover likely proceeds by futile cycling involving a tyrosinyl radical.[16]

Sequence analysis of several radical AdoMet proteins showed that HydG forms a small subfamily with two other proteins, ThiH and NosL (Figure S5 in the Supporting Information). NosL is a radical AdoMet enzyme required for the rearrangement of tryptophan (**11**) to provide the 3-methylindolyl moiety **12** during the biosynthesis of the antibiotic nosiheptide.[20] This rearrangement requires a C_α–C_β bond cleavage and the extrusion of the C_α–N fragment of tryptophan (Scheme 2). In anaerobic bacteria, ThiH cleaves tyrosine to form *p*-cresol as an aromatic byproduct and dehydroglycine (**10**), which is used for the biosynthesis of thiamine. The reaction mechanism of ThiH is proposed to involve a phenolic radical, which permits C_α–C_β bond cleavage.[15,16] ThiH, HydG, and NosL share the common feature of cleaving the C_α–C_β bond of aromatic amino acids (Scheme 2), and it is likely that these transformations proceed by related mechanisms, potentially resulting in a benzylic radical intermediate.

Cyanide is biosynthesized by a number of metabolic pathways. In plants, cyanide is formed from cyanogenic glycosides and as a byproduct of the ethylene-forming enzyme aminocyclopropanecarboxylic acid oxidase (ACC oxidase).[21] In bacteria, it has been shown that for the [NiFe]-hydrogenase, cyanide ligands are generated from carbamoyl phosphate through the initial formation of thiocyanate.[22] This mechanism does not involve free cyanide as an intermediate in the formation of the [NiFe]-hydrogenase cofactor. For [FeFe]-hydrogenases, it is unclear whether cyanide is released by HydG into free solution or is directly transferred to another protein such as HydF. Several *Pseudomonas* species express a hydrogen cyanide synthase,[23] which is proposed to oxidize glycine to generate dehydroglycine as an intermediate[24] that upon oxidative decarboxylation yields cyanide.[25] Several potential mechanisms can account for the formation of cyanide from tyrosine by HydG. If the reaction proceeds via the postulated intermediate dehydroglycine (**10**), oxidative decarboxylation would yield cyanide and carbon dioxide, but a decarbonylation mechanism, which has chemical precedent,[26] would yield both the cyanide and carbon monoxide ligands in a single step ($NHCHCO_2H \rightarrow HCN + CO + H_2O$).

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.[27] 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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