Showcasing research from the groups of Dr Gustav Berggren and Professor Peter Lindblad at Uppsala University, Department of Chemistry - Ångström, Sweden.

*In vivo* activation of an [FeFe] hydrogenase using synthetic cofactors

[FeFe] hydrogenases catalyze the formation of hydrogen gas with remarkable efficiency and have attracted considerable attention due to their biotechnological potential. The reaction occurs at the H-cluster, which contains an organometallic [2Fe] subsite. The unique nature of the [2Fe] subsite makes it dependent on a specific set of maturation enzymes for its biosynthesis and incorporation into the apo-enzyme. Herein we report on how this can be circumvented, and the apo-enzyme activated inside living organisms by synthetic active site analogues spontaneously taken up by the cell.

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**In vivo** activation of an [FeFe] hydrogenase using synthetic cofactors†

N. Khanna,‡a C. Esmieu,‡b L. S. Mészáros,‡b P. Lindblad*‡b and G. Berggren*‡b

[FeFe] hydrogenases catalyze the reduction of protons, and oxidation of hydrogen gas, with remarkable efficiency. The reaction occurs at the H-cluster, which contains an organometallic [2Fe] subsite. The unique nature of the [2Fe] subsite makes it dependent on a specific set of maturation enzymes for its biosynthesis and incorporation into the apo-enzyme. Herein we report on how this can be circumvented, and the apo-enzyme activated *in vivo* by synthetic active site analogues taken up by the living cell.

Hydrogenases are ancient gas processing enzymes catalyzing the interconversion between H₂ gas and protons. In the case of [FeFe] hydrogenases (HydA), found in bacteria and certain eukaryotes, the reaction occurs at the H-cluster. This unique cofactor consists of a “classical” cuboidal [4Fe4S] cluster coupled to a dinuclear [2Fe] subsite, featuring diatomic CO and CN⁻ ligands, as well as a bridging aza-dithiolate ligand (Fig. 1a).²⁻⁵

The remarkable catalytic capacity of the hydrogenases makes them highly relevant for biological hydrogen production, and in technological applications as an alternative to Pt-based catalysts in e.g. (photo-)electrolysers and fuel cells.⁶⁻⁸ Indeed, even the highly active [FeFe] hydrogenases have been shown to be active for days on electrode surfaces, and have been successfully employed in a hydrogen oxidizing fuel cell.⁹,¹⁰ Consequently, these enzymes and their post-translational maturation have been intensively studied, and it is well established that the biosynthetic assembly of the H-cluster occurs via two separate processes. The [4Fe4S] cluster is first inserted by the standard iron–sulfur housekeeping machinery (Fig. 1b).¹¹ The subsequent formation and integration of the [2Fe] subsite into HydA follow a complex pathway that requires a minimum of three HydA specific accessory proteins, the maturases denoted HydE, HydF and HydG.¹²⁻¹⁴ It was recently shown how this Hyd-maturation machinery can be circumvented, and purified forms of apo-HydA activated, or matured, *in vitro* using synthetic analogues of the [2Fe] subsite.⁵,¹⁵⁻¹⁸ Remarkably, the dinuclear Fe complex [Fe₂(CN)₆(CO)₄(adt)]²⁻ (adt = SCH₂NHCH₂S⁻) (1) (Fig. 1c), featuring one CN⁻ and two CO ligands per iron ion as well as an aza-dithiolate bridge,¹⁹ spontaneously enters the apo-enzyme and rearranges inside the active site cavity to generate semi-synthetic enzymes indistinguishable from native HydA.¹⁵ The technique has since then been utilized to facilitate the preparation of HydA from a number of different organisms.²⁰⁻²² However, it is currently restricted to *in vitro* conditions with purified enzymes. In parallel, the concept of introducing metal complexes and other small synthetic molecules into living cells for the purpose of activating a specific protein has seen great progress during the last decade. Such gain-of-function approaches generally focus on either specific...
Hyd-maturation machinery (HydE, -F, -G) this results in an inactive low background activity of the strain’s native [NiFe] hydrogenases. Vector or lacking the vector (data not shown), and is attributed to a deficiencies.31,32 Accordingly, when cultures of this strain used has been previously found to not even trace amounts of H2 could be detected from these cultures in glucose enriched LB media assayed for hydrogen production as described above. As expected, not even trace amounts of H2 could be detected from these cultures in the absence of complex 1. Conversely, treating 100 mL cultures with 100 µg (156 nmoles) of 1 resulted in H2 production, close to what was observed for the BL21(DE3) strain under identical conditions (Fig. S2, ESF†).
It is noteworthy that no genetic modifications to cellular transporters were required for the activation to proceed in neither the BL21 nor the FTD147 strain. Whether the anionic complex 1 enters the cell by directly crossing the cytoplasmic membrane or via a transporter is still an open question. Still, the observation that it occurs spontaneously in two different E. coli strains suggests a general applicability of the technique.

The glucose dependent hydrogen production strongly supports the notion that artificial HydA activation can be achieved also under in vivo conditions. However, it does not allow for a direct measurement of the extent of activation. In order to address this, in vitro enzymatic assays were performed. To first quantify the amount of enzyme available for activation we performed in vitro maturation assays with lysed cells. E. coli cultures containing the C. r. hydA1 vector were lysed under anaerobic conditions upon reaching the early log phase, to generate crude cell extracts containing apo-HydA1. These crude extracts were then incubated with varying amounts of complex 1 for 10 minutes before the hydrogen evolution capacity of the preparations was evaluated by a standard in vitro hydrogenase assay protocol, using reduced methyl viologen (MV) as an electron donor.34 Under these conditions, the hydrogen production was substantially higher than under in vivo conditions, as expected in such a reducing environment, and a plateau was reached with regards to activity at around 5.5 ± 2 nmoles H2 min⁻¹ mL⁻¹ O.D.⁻¹ (Fig. 3a).

**In vitro** maturation of apo-HydA1 using complex 1 results in fully active enzyme,15 and the specific activity of C. r. HydA1 is well established under these assay conditions (730 ± 20% μM H2 min⁻¹ mg⁻¹).15,15 In combination, this allows an approximation of the total amount of C. r. HydA1 available for activation in our preparations to 0.15 μg or 3 pmoles ± 35%/100 mL culture. The plateau was approached for cell extracts treated with only 1 μg (1.56 nmoles) of complex 1 per 100 mL cell culture, i.e. ≈ a 500-fold excess. Moreover, we observe approximately 30% of the maximum activity (1.7 ± 0.9 nmoles H2 min⁻¹ mL⁻¹ O.D.⁻¹) already with the addition of 10 ng (15.6 pmoles, ≈ 5-fold excess) of complex 1.

In order to estimate the efficiency of the in vivo artificial maturation protocol the activation was performed with whole cells, after which the cells were lysed and in vitro assays were performed. More specifically, anaerobic early log-phase cultures were incubated for 1 h in the presence of complex 1 at 37 °C, in order to ensure that the complex had entered the cells and H2 evolution could be observed from the cultures. The cells were then spun down and washed three times with aqueous buffer (Tris 100 mM, 150 mM NaCl, pH 7.5) to remove residual complex 1 attached to the exterior of the cells prior to cell lysis. The extent of washing was monitored by checking the capacity of the supernatant from the separate wash steps to activate the apo-enzyme in fresh cells. When the cells were activated with 10 μg of 1 already the second wash step supernatant had lost the capacity to activate the enzyme. In experiments using 100 μg of 1 the washing was less efficient, indicating that a significant fraction of 1 indeed attached to the cells via unknown binding modes. Nevertheless, following the wash steps the activation capacity of the supernatant had dropped significantly (< 30%) compared to the original LB media used for activation (for a detailed overview see Fig. S3 and S4, ESI†). After the wash protocol the in vivo activated samples were lysed under anaerobic conditions and assayed analogously to the in vitro activated samples, using reduced MV. Following in vivo activation the maximum activity observed was very similar to the in vitro activated samples, and a plateau was again observed close to 5.5 nmoles H2 min⁻¹ mL⁻¹ O.D.⁻¹ (Fig. 3b). However, activation of the enzyme inside the cells required a larger excess of complex 1 as compared to in vitro activation, and 1 μg (1.56 nmoles) of 1 resulted in only trace amounts of H2 produced. Partial activation of the enzyme was observed with 10 μg (15.6 nmoles) of 1, and in agreement with the H2 evolution profiles of the E. coli cultures maximum activity was not observed until 100 μg (156 nmoles) was added per 100 mL cell culture. Despite the lower activation efficiency, the results clearly show that regardless of whether the artificial maturation is performed in vivo or in vitro we activate close to the same amount of apo-HydA1. The differences in experimental conditions between the in vivo and in vitro assays complicate a direct comparison, and spectroscopic studies of the process are currently on-going. Still, the results strongly support the notion that complete activation is achieved also under in vivo conditions.

The dependence on intracellular HydA1 concentration was determined by chemically inducing expression of the enzyme.
Cultures were grown as described above, but with IPTG added at the time of inoculation. This resulted in a significant increase in the amount of HydA1 expressed by the cells, readily observable by Western blots (Fig. S5, ESI†) and increased H₂ production rates in the in vitro activation assay (Fig. S6, ESI†). Importantly, the presence of IPTG had negligible effect on cell growth, which facilitated comparisons to the non-induced cultures. The increased expression of HydA1 resulted in a 2–3 fold increase in H₂ production from the cell cultures following addition of 1 (Fig. 4a). A similar increase in activity was observed also in the in vivo assays, using crude cell extracts containing HydA1 activated in vivo as described above (Fig. 4b). This latter observation under-scores that the increase observed in H₂ production under in vivo conditions is indeed attributable to an increase in the amount of HydA1 available for activation following IPTG induction. Moreover, the increased intracellular concentration of HydA1 improves the efficiency of the in vivo activation protocol. H₂ production is readily observable already following addition of 100 ng and 1 μg of 1 per 100 mL cell culture (Fig. 4).

One benefit of the artificial maturation technique is the possible preparation of artificial HydAs via the incorporation of modified synthetic cofactors. A notable example of this is the monocyanide complex [Fe₅(CN)(CO)₃(adt)]⁻ (2),₁⁶,₃⁶ in which one cyanide ligand is replaced by a CO ligand, resulting in a decreased overall negative charge (Fig. 1c). This complex has been successfully introduced into purified enzymes to generate 2-HydA, as demonstrated by both spectroscopy and enzymatic assays.₁⁶ Under in vitro conditions 2-HydA has been shown to have activities of nearly 50% of the enzyme’s native activity. Remarkably, the activity of the artificial enzyme (2-HydA) was retained also under in vivo conditions (Fig. 5). Treating E. coli with complex 2 resulted in H₂ evolution with rates of approximately 30% during first hour, as compared to cell cultures treated with equimolar amounts of complex 1 under otherwise identical conditions (Fig. 2 and 5). Thus, the method does not only allow for artificial maturation of the apo-hydrogenase, but also the preparation of new artificial hydrogenases under in vivo conditions.

Fig. 5  In vivo hydrogen production observed from E. coli cultures in glucose enriched LB media; (a) in vivo H₂ production of apo-HydA1 in early log-phase E. coli cultures (O.D. = 0.20 ± 0.02) using varying amounts of complex 1. (b) In vivo H₂ production of E. coli cultures in glucose enriched LB media; (c) in vitro H₂ production rates observed using crude cell extracts of cultures incubated for 1 h with complex 1 under in vivo conditions, rates calculated at the 45 min time point ([MV] = 10 mM, [dithionite] = 20 mM, [kPi] = 60 mM, pH 6.8). All data points represent means of at least two independent repeats with duplicate samples from each culture, error bars ± S.D.

Conclusions

In summary, our data clearly demonstrate that the concept of artificial maturation of hydrogenases can be extended to in vivo conditions. To the best of our knowledge this represents the first example of intracellular activation of a natural apo-enzyme using a synthetic metallo-cofactor without relying on enhanced cellular import functions.

More specifically for hydrogenase research, the technique can facilitate in vivo spectroscopic studies, via the incorporation of designed synthetic cofactors, and provides a powerful tool for gain-of-function studies, allowing e.g. detailed studies of the effect of an active hydrogenase on the metabolic state of the host organism. Moreover, it provides a simple and straightforward tool for assaying the suitability of different host organisms under in vivo conditions, without a requirement for co-expression of the still incompletely characterized maturation system.

Finally, it opens up for the possibility of modifying the hydrogenase enzyme not only via synthetic biology techniques, but also using synthetically modified cofactors, as exemplified using the monocyanide complex 2. The in vivo activity of the enzyme incorporating synthetically modified co-factors is currently under further investigation, with the aim of obtaining...
the most efficient, and robust, hydrogenases for cellular H₂ producing factories.

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