Constructing and testing the thermodynamic limits of synthetic NAD(P)H:H₂ pathways

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Summary
NAD(P)H:H₂ pathways are theoretically predicted to reach equilibrium at very low partial headspace H₂ pressure. An evaluation of the directionality of such near-equilibrium pathways in vivo, using a defined experimental system, is therefore important in order to determine its potential for application. Many anaerobic microorganisms have evolved NAD(P)H:H₂ pathways; however, they are either not genetically tractable, and/or contain multiple H₂ synthesis/consumption pathways linked with other more thermodynamically favourable substrates, such as pyruvate. We therefore constructed a synthetic ferredoxin-dependent NAD(P)H:H₂ pathway model system in Escherichia coli BL21(DE3) and experimentally evaluated the thermodynamic limitations of nucleotide pyridine-dependent H₂ synthesis under closed batch conditions. NADPH-dependent H₂ accumulation was observed with a maximum partial H₂ pressure equivalent to a biochemically effective intracellular NADPH/NAD⁺ ratio of 13:1. The molar yield of the NADPH:H₂ pathway was restricted by thermodynamic limitations as it was strongly dependent on the headspace:liquid ratio of the culture vessels. When the substrate specificity was extended to NADH, only the reverse pathway directionality, H₂ consumption, was observed above a partial H₂ pressure of 40 Pa. Substitution of NADH with NADPH or other intermediates, as the main electron acceptor/donor of glucose catabolism and precursor of H₂, is more likely to be applicable for H₂ production.

Introduction
Molecular hydrogen is a promising energy carrier for both mobile and stationary applications in the future (Cho, 2004). Many bacteria and archaeabacteria, with large variation in metabolic pathway diversity, are capable of producing and consuming H₂. In sugar-fermenting species, the most common glycolytic intermediate node for H₂ production is pyruvate, with either formate (for example Enterobacter spp.) (Wu, Wu et al., 1986; Sawers, 2005) or ferredoxin (formerly Clostridium spp.) (Thauer et al., 1971; Angenent et al., 2004) as intermediate electron acceptor/donors. Both reactions are thermodynamically favourable and will therefore proceed even at high partial pressure of H₂ with a maximum yield of 2 mol H₂ (mol glucose)⁻¹ (Angenent et al., 2004). The glyceraldehyde-3-phosphate (GAP) node is another important intermediate for which microorganisms have evolved both carbon-dependent (ethanol, lactate, butanol) and -independent (H₂) pathways to remove unwanted electrons generated through oxidation (Gottschalk, 1986). In most organisms, the nucleotide pyridine couple NADH/NAD⁺ serves as the main electron acceptor/donor of GAP oxidation. Due to its central role in a large number of metabolically important reactions, the relative ratio of NADH/NAD⁺ has important implications for overall metabolism and is maintained at measurable so-called ‘steady-state’ levels (Alexeeva et al., 2003). In theory, another 2 mol H₂ (mol glucose)⁻¹ could potentially be obtained from the GAP node by channelling all of the NADH generated in the GAPDH-dependent reaction towards H₂ formation by the following reaction (1):

\[
\text{NADH} + \text{H}^+ \leftrightarrow \text{NAD}^+ + \text{H}_2
\] (1)

NADH:H₂ pathways can potentially be catalysed either by multimeric NADH-dependent hydrogenases (Verhagen et al., 1999; Soboh et al., 2004; Vignais and Colbeau, 2004) or by the combined activities of three distinct proteins: NAD(P)H:ferredoxin oxidoreductase (NFOR), ferredoxin and hydrogenase (Jungermann et al., 1971; 1973; Thauer et al., 1971). Although extracts of several Clostridium spp. were earlier shown to catalyse NADH-dependent H₂ synthesis in vitro (Jungermann et al., 1971; 1973), not a single NFOR operating in a NADH:H₂ pathway has yet been identified at the gene sequence level.

There remains some question of whether NADH-dependent H₂ synthesis is metabolically relevant or not under conditions where H₂ is allowed to accumulate in the headspace (Jungermann et al., 1973). NADH-dependent...
H₂ synthesis at reported cellular NADH/NAD⁺ ratios is unlikely to be utilizable in a biotechnological process if the reaction reaches equilibrium at the very low partial pressure of H₂ (60 Pa) that has been theoretically predicted (Angenent et al., 2004). Furthermore, it has been reported that the solubility of H₂ in microbial cultures can exceed the theoretical limit derived from Henry’s Law by up to 80 times (Lamed et al., 1988; Pauss et al., 1990), potentially lowering the theoretical partial H₂ pressure limit for H₂ synthesis substantially further. In contrast, there are several convincing reports (Lamed et al., 1988; Kumar et al., 2001) suggesting that GAP-dependent H₂ synthesis indeed is operational in several microorganisms, even at partial H₂ pressure levels substantially above the limit suggested by Angenent and colleagues (2004). For example, based on fermentation balance analysis of mutant strains in which NADH-consuming reactions had been deleted, it was deduced that Enterobacter aerogenes most likely does catalyse NADH-dependent H₂ synthesis (Nakashimada et al., 2002) even under closed batch conditions. Also, cultures of the extreme thermophile Caldicellulosiruptor saccharolyticus have been reported to generate H₂ yields above 3.0 mol H₂ (mol glucose)⁻¹ (de Vrije et al., 2007) although H₂ productivity was unaffected by product accumulation until the partial H₂ pressure level reached above 5000 Pa (van Niel et al., 2003). The main caveat with the preceding studies, however, is that the full complement of native H₂-metabolizing pathways, and their respective pathway directionality, remain unknown. Furthermore, as the pathways are not investigated in isolation, it is difficult to discriminate between them. Nevertheless, NADH-dependent H₂ synthesis reactions have been demonstrated in crude lysates of E. aerogenes (Nakashimada et al., 2002) and it is difficult to believe that yields above 2.0 can be reached in any non-phototrophic species without oxidation of GAP. The question of whether NADH-dependent H₂ synthesis is thermodynamically limited or not therefore remains an open question until it can be verified using a defined experimental system. As utilization of GAP-dependent H₂ synthesis is critical in order to obtain reasonable H₂ yields, it is important to address this question to guide further research and development efforts towards enhancing microbial H₂ yield.

NADPH:H₂ pathways also exist in several microorganisms (Thauer et al., 1971; Malik et al., 1995; Silva et al., 2000) and could theoretically be utilized to enhance H₂ production substantially, as demonstrated by Woodward and colleagues (2000) through in vitro reconstitution of pentose phosphate pathway-dependent H₂ production. However, the possibility of extending such a concept to in vivo production of H₂ is dependent on the outcome of a number of hitherto untested questions, including the directionality of NADP⁻:H₂ pathways. Several reports suggest that the pathway operates in the direction of NADP⁻ reduction (Malki et al., 1995; Steuber et al., 1999), although this is yet to be convincingly demonstrated.

We engineered a synthetic NAD(P):H₂ pathway using Escherichia coli BL21(DE3) as a host. This strain differs substantially in H₂ metabolism from that of E. coli K-12 strains (Yoshida et al., 2005; 2006; Maeda et al., 2008), as it is effectively void of native H₂ metabolism (Akhtar and Jones, 2008a). The synthetic pathway consisted of a ferredoxin-dependent hydrogenase (King et al., 2006; Akhtar and Jones, 2008b), a [4Fe4S] ferredoxin as intermediate electron acceptor/donor, and NFORs with specificity for either NADPH or NADH and NADPH (Fig. 1). This system was then used to experimentally determine to what degree NAD(P):H₂ pathways are thermodynamically limited in vivo, with ultimate consequences for pathway directionality.

Results

Construction of ferredoxin-dependent H₂ synthesis in E. coli BL21(DE3)

Prior to constructing the model system, we evaluated the H₂ metabolism of BL21(DE3) as a potential host. The accumulation and consumption of H₂ in closed cultures (headspace : liquid ratio of 2:2:1) of BL21(DE3) wild type,
in which the headspace contained either N₂ or 2% (v/v) H₂ (remainder N₂), was monitored over 52 h. BL21(DE3) pCDF-Duet (empty) accumulated H₂ (33 ± 6 Pa) to a level just above the limit of detection (~20 Pa) after 22 h, a level also observed using BL21(DE3) wild type (40 ± 6 Pa) even after 52 h of incubation. To cultures in which 2% (v/v) H₂ was added at the start of incubation, there was no change in the H₂ level even after 52 h. Together, this suggests that the native pyruvate formate lyase- and formate hydrogenlyase-dependent pathway (Sawers, 2005) effectively does not function in BL21(DE3) under the present experimental conditions, in contrast to E. coli K-12 strains (Yoshida et al., 2005; 2006; Maeda et al., 2008). Furthermore, as cultures of BL21(DE3) grown in MOPS minimal media glucose under anaerobic conditions do not consume added H₂, BL21(DE3) is therefore an ideal host for studying introduced or constructed H₂ metabolism, as it is effectively void of any form of H₂ metabolism.

As E. coli is not known to express a [4Fe4S] ferredoxin, the most likely native electron acceptor/donor partners of both Clostridium acetobutylicum HydA (Gorwa et al., 1996) and the thioredoxin reductase like NFORs (Seo and Sakurai, 1996) and the thioredoxin reductase like NFORs (Seo and Sakurai, 2002; Seo et al., 2004), construction of NAD(P)H:H₂ pathways followed the two-step process of first introducing a recombinant [4Fe4S] ferredoxin, followed by the addition of NFORs. Successful synthesis of recombinant FeFe hydrogenase in E. coli BL21(DE3) was recently reported (Posewitz et al., 2004; King et al., 2006). Following on from this work, we constructed the plasmid pCDOPFEGA carrying a synthetic operon with genes encoding the three required FeFe hydrogenase maturation factors (Posewitz et al., 2004), and the FeFe hydrogenase HydA from C. acetobutylicum (Akhtar and Jones, 2008b). Strains carrying pCDOPFEGA and a separate plasmid allowing expression of either the gene encoding [4Fe4S] ferredoxin from Clostridium pasteurianum (CpfD, plasmid p4Fd) (Graves et al., 1985), or the gene encoding [2Fe2S] ferredoxin from C. pasteurianum (plasmid p2Fd) (Meyer et al., 1986), were prepared. Following overnight anaerobic expression, hydrogenase assays were initiated by the addition of dithionite to crude lysates. Reactions conducted with lysates expressing recombinant [4Fe4S] ferredoxin displayed three- to fourfold greater hydrogenase activity (9.5 ± 1 nmol H₂ min⁻¹ mg⁻¹) compared with strains expressing [2Fe2S] ferredoxin (2.5 ± 1 nmol H₂ min⁻¹ mg⁻¹) or no recombinant ferredoxin (2 ± 1 nmol H₂ min⁻¹ mg⁻¹). Consequently, CpfD was chosen as a suitable candidate for further engineering and the gene encoding CpfD was added to pCDOPFEGA in order to generate the plasmid pCDOPFEGAFdx (Fig. 1).

Using small headspace cultures (headspace : liquid ratio of 0.6:1), the accumulation of H₂ in BL21(DE3) harbouring (i) pCDOPFEG (carrying only the hydrogenase maturation factors, negative control), (ii) pCDOPFEGA or (iii) pCDOPFEGAFdx was monitored for 22 h (Table 1). The addition of recombinant HydA to BL21(DE3) expressing the three hydrogenase maturation factors resulted in an increase in molar H₂ yield, an increase that was further enhanced in the dual presence of both HydA and CpfD (Table 1). This indicated that one or several native E. coli enzymes were capable of either directly or indirectly reducing both HydA and recombinant CpfD. We were interested in determining the unknown factor(s) responsible for H₂ synthesis in BL21(DE3) pCDOPFEGAFdx and therefore prepared deletion mutants of the two most likely candidates: fpr (annotated as the NADPH:flavodoxin ferredoxin oxidoreductase Fpr) and ydbK (annotated as a putative pyruvate:flavodoxin/ferredoxin oxidoreductase, YdbK) (Blaschkowski et al., 1982), as well as the double mutant thereof. All three deletion strains accumulated H₂ with lower molar yield (Table 1), although Δtpr deletion strains displayed poor growth under aerobic conditions (data not shown). BL21(DE3)ydbK was therefore chosen as a host strain for further characterization of NFOR-dependent accumulation of H₂ in vivo, in order to limit H₂ metabolism by undefined pathways involving native enzyme(s). The fact that E. coli BL21(DE3) is capable of reducing the recombinant [4Fe4S] ferredoxin and HydA is surprising given that no [4Fe4S] ferredoxin, nor FeFe hydrogenase, has been reported in E. coli. The partial but incomplete reduction in CpfD- and HydA-dependent H₂ accumulation in response to the deletion of both ydbK and fpr suggests that other unknown proteins of E. coli with the capability of reducing CpfD and HydA must still remain. No obvious candidates exist in the genome, however (Serres et al., 2001).

**Table 1.** H₂ accumulation in BL21(DE3) in response to the expression of recombinant HydA and CpfD and to the deletion of ydbK and fpr.

| Strains            | H₂ yield [mmol (mol glucose)⁻¹] |
|--------------------|---------------------------------|
| BL21(DE3) pFEG     | 2.1 ± 0.3                       |
| BL21(DE3) pFEGA    | 3.5 ± 0.3                       |
| BL21(DE3) pFEGAFdx| 5.2 ± 0.2                       |
| BL21(DE3)ydbK pFEGAFdx | 4.3 ± 0.1             |
| BL21(DE3)tpr pFEGAFdx | 4.6 ± 0.3             |
| BL21(DE3)ydbKfpr pFEGAFdx | 4.2 ± 0.5             |

The molar yield of H₂ per mol of glucose was determined after 22 h of IPTG induction (0.05 mM) in MOPS minimal media using vessels with a headspace : liquid ratio of 0.6. Error bars indicate standard deviation (n = 2).

Construction of NAD(P)H:H₂ pathways in E. coli – NADPH is a more suitable intermediate for H₂ production than NADH in E. coli BL21

Recently, a NADPH:[4Fe4S] ferredoxin oxidoreductase from Bacillus subtilis (Seo et al., 2004) (BsNFOR), and a
NADPH and NADH: [4Fe–4S] ferredoxin oxidoreductase from Chlorobium tepidum (Seo and Sakurai, 2002) (CtNFOR) were described. The former enzyme, which has an unknown physiological role, is unlikely to be involved in H2 metabolism as there are no reports that B. subtilis either consumes or produces H2, while the latter enzyme is more likely to serve a role in photosynthesis similar to that of plant NADPH: [2Fe–2S] ferredoxin oxidoreductases (Carrillo and Ceccarelli, 2003). The heterologous expression, catalytic function and substrate specificity of recombinant BsNFOR and CtNFOR were confirmed by SDS-PAGE and activity assays (Appendix S1). No NADH-dependent reduction of methyl viologen (MV) was detected with crude extracts containing recombinant BsNFOR.

To construct NADPH and NADPH/NADH:H2 pathways, the two NADH- and/or NADPH-dependent NFORs were coexpressed (in vessels with a headspace : liquid ratio of 0.6:1) along with CpFd and HydA. BsNFOR-dependent H2 accumulation varied over time (Fig. 2). The maximum yield and partial H2 pressure was achieved in strains coexpressing HydA, CpFd and BsNFOR, with partial H2 pressure reaching a maximum of 770 Pa over 48 h in the small headspace vessels (Fig. 2). When expression of the NADPH-specific BsNFOR instead was substituted with the NADH- and NADPH-specific CtNFOR, H2 accumulation was strongly reduced in comparison with strains in which no recombinant NFOR was expressed, and remained below 40 Pa throughout the whole cultivation period (Fig. 2). This suggests that the enhanced substrate specificity towards NADH displayed by CtNFOR results in a reversal in the directionality of the introduced pathway, i.e. consumption of H2.

A comparison between strains with and without BsNFOR in small headspace vessels (headspace : liquid ratio of 0.6:1) showed that the introduced NADPH-dependent H2 synthesis pathway only was effective after the cells had entered the stationary phase of growth (Fig. 2), a point at which the unknown native capability to reduce recombinant CpFd effectively stalled. In fact, strains expressing BsNFOR even displayed lower H2 accumulation relative to strains expressing no NFOR at
the end of exponential phase, suggesting that NADPH was generated rather than consumed. After 28 h of cultivation, the yield of H₂ in cultures of BsNFOR-expressing strains, with and without subtraction of the yield obtained for cultures with strains where no recombinant NFOR was expressed, was 6.5 and 10.5 (± 0.7) mmol H₂ (mol glucose)⁻¹ respectively. H₂ yields obtained from several independent experiments, although strongly dependent on the headspace : liquid ratio (as discussed below), varied between 10 and 40 mmol H₂ (mol glucose)⁻¹ at most. Additional overexpression of *E. coli* glucose 6-phosphate dehydrogenase (G6PDH; encoded by zwf, under the same conditions was 26 mmol H₂ (mol glucose)⁻¹.

In order to verify the existence of introduced functioning pathways, cells were collected and lysed under anaerobic conditions, following 24–48 h of recombinant protein expression, and tested for NADH- and NADPH-dependent *in vitro* H₂ synthesis. The addition of cofactor regeneration systems was necessary in order to observe any product formation. In contrast to the *in vivo* results, extracts from hydrogenase- and ferredoxin-producing strains heterologously expressing CtNFOR displayed greater ability to catalyse NAD(P)H-dependent H₂ formation [110 ± 64 and 33 ± 13 nmol H₂ min⁻¹ (mg protein)⁻¹ with NADPH and NADH respectively] than extracts prepared from cells expressing BsNFOR [70 ± 14 nmol H₂ min⁻¹ (mg protein)⁻¹, NADPH only]. No H₂ was observed in the headspace with either cofactor, using crude extracts of negative controls, i.e. without expression of either recombinant NFOR or CpFd. Addition of 250 μM MV to *in vitro* reactions generally enhanced the rate of H₂ synthesis by a factor of 10–20. As increased H₂ evolution activities also were observed for purified *C. pasteurianum* HydA using a high concentration of MV relative to purified CpFdx (Fitzgerald *et al.*, 1980), an insufficient quantity of functional recombinant CpFdx is the most likely explanation for the large response to the addition of MV.

**Testing the thermodynamic limitations of NAD(P)H-dependent H₂ synthesis**

The partial hydrogen pressure at the equilibrium of reaction (1), in a closed space with a given molar cofactor ratio [NAD(P)H/NAD(P)+], was estimated to be 35 and 142 Pa, for NADH and NADPH respectively, using Eqn A1 in Appendix S2. The estimation assumes that the cofactor ratio is fixed, that Henry’s Law for the distribution of H₂ between the gaseous and liquid phase of only water applies, and uses previously reported values for nicotinamide cofactor content in *E. coli* under anaerobic conditions [0.75–0.88 for NADH/NAD⁺ and 2.6 for NADPH/NADP⁺ (Alexeeva *et al.*, 2003; Brumaghim *et al.*, 2003)] (Fig. 3A). The theoretical equilibrium of each H₂ pathway, depicted in Fig. 3A, is linearly related to the biochemically effective intracellular cofactor ratio, as the redox potentials of the two nucleotide couples are highly similar (Alberty, 2001). In order to experimentally verify that the NAD(P)H/H₂ pathway is thermodynamically limited in isolation, we affinity-purified recombinant HydA and CtNFOR under anaerobic conditions, and reconstituted the pathway *in vitro* with and without the addition of *Saccharomyces cerevisiae* G6PDH and glucose 6-phosphate (G6P) (Fig. 3B). As the addition of cofactor is finite the ratio of reduced to oxidized nicotinamide cofactor will vary during the reaction. The maximum possible partial H₂ pressure levels of the *in vitro* reaction without G6PDH were estimated to be 332 and 214 Pa for 5.5 and 12 ml headspace volume respectively, by determining the positive cofactor ratio at which the pressure derived from the ideal gas law (as given by Eqn A2 in Appendix S2) equals the pressure estimated at the equilibrium of reaction (1) given by Eqn A1. The experimentally determined partial H₂ pressure level reached approximately 30% and 45% [experimentally determined/theoretical predicted partial H₂ pressure (Pa)] for 5.5 and 12 ml headspace volume respectively, of the calculated theoretical maxima. The addition of G6PDH raised the maximum accumulated H₂ more than twofold. Yeast G6PDH was not able to reduce HydA directly or via any intermediate electron acceptor/donor as omission of BsNFOR abolished H₂ formation. The most plausible interpretation, of the effect of G6PDH on *in vitro* NADPH-dependent H₂ synthesis using purified components, is that the G6PDH maintained a low level of [NADP⁺].

To test the effect of thermodynamic limitations on product accumulation *in vivo*, the headspace volume was varied while all other cultivation parameters were fixed. When the headspace volume of culture vessels was increased [headspace : liquid ratios of 5:1 and 31:1 (8 and 51 times larger than the small headspace cultures shown in Fig. 2 respectively)], the accumulation profile was significantly altered and the molar yield of H₂ increased (Fig. 4A). After 28 h, average BsNFOR-dependent H₂ yields (calculated by subtracting the yield observed with cultures using negative control strains, i.e. without expression of BsNFOR) were 4.9 (±0.7), 16.5 (±0.6) and 36.4 (±0.5) mmol H₂ (mol glucose)⁻¹ for cultures with a headspace : liquid ratio of 0.6:1, 5:1 and 31:1 respectively (Fig. 4C). The change in headspace affected both BsNFOR-independent and BsNFOR-dependent H₂ accumulation, as the molar yield of BsNFOR-independent H₂ accumulation over 28 h [7.0, 20.0 and 31.7 mmol H₂ (mol glucose)⁻¹].
glucose) in cultures with a headspace : liquid ratio of 0.6:1, 5:1 and 31:1 respectively] also was greater in the large headspace vessels. The only other parameter that is expected to change in response to variation in headspace volume is the partial pressure of CO2. However, the addition of 2% (v/v) CO2, a concentration we typically observed only after >24 h of growth in small headspace vessels, to large headspace vessel cultures at the start of induction did not affect H2 accumulation or growth (data not shown).

To obtain an approximation of the in vivo equilibrium point for reaction (1) that was not influenced by growth stage-related changes to metabolism, H2 was instead added at different levels to closed batch cultures (headspace : liquid ratio 2.2) after the cells had entered stationary phase. While cultures expressing BsNFOR accumulated hydrogen to partial H2 pressure levels above 400 Pa, CtNFOR expressing cultures rapidly consumed H2 down to a partial H2 pressure level below 50 Pa (Fig. 4B). Rapid consumption of H2 by CtNFOR-expressing cells supports the fact that the introduced pathway is functioning, although the directionality of the catalysed reaction is in the opposite direction of interest in cultures to which H2 was added, but not in cultures to which H2 was not added, as the latter cultures accumulated H2 overall.

Based on the partial H2 pressure levels that were obtained (as shown in Fig. 2B), we calculated the minimum intracellular NAD(P)(H) cofactor ratios using Eqn A1: 0.68 ± 0.13 (NADH/NAD+, end of exponential phase), 0.91 ± 0.28 (NADH/NAD+, late stationary phase), 4.3 ± 0.6 (NADPH/NADP+, end of exponential phase), 13.1 ± 1.0 (NADPH/NADP+, late stationary phase). We propose that these values are more likely to reflect biochemically effective intracellular cofactor ratios than those obtained by analysis using extracts, although accuracy will be improved further if all BsNFOR- and CtNFOR-independent H2 synthesis can be completely removed and if an NFOR with strict specificity for only NADH can be obtained.

**Discussion**

In the present study we describe construction and verification of NAD(P)H:H2 pathways and then use them to gain understanding regarding a fundamental issue which may pose a major stumbling block towards engineering for improved H2 production, i.e. thermodynamic limita-
In addition, we also verify functional construction of NADPH-dependent H₂ synthesis, a pathway directionality which to date has not been demonstrated in any organism under growing conditions as far as we are aware. The turnover of NADP(H) in wild-type *E. coli* BL21 is expected to be lower than that of NAD(H) given that only ~5% of the total glycolytic flux will pass through the pentose phosphate pathway under anaerobic conditions (Fischer and Sauer, 2003). A proportion of NADH may also contribute towards NADPH under anaerobic conditions (Varma et al., 1993) via the transhydrogenase PntAB, although the contribution by this route so far only has been quantified for *E. coli* K-12 strains under aerobic conditions (Sauer et al., 2004). Consequently, the yield and rate of BsNFOR-dependent H₂ accumulation will ultimately be limited by a low turnover of NADP(H) under anaerobic conditions, and can therefore not be properly evaluated until that turnover has been enhanced. The use of defined controls in the present study, differing only in respect to whether a particular NFOR is present or absent, allowed the directionality and yield of the NFOR-dependent pathways to be evaluated, even though strains lacking BsNFOR also accumulated H₂.

The partial H₂ pressure level and H₂ yield of BL21(DE3)ΔydbK pCDOPFEGA pCpFd pBsNFOR cultured in small headspace vessels (headspace : liquid ratio of 0.6) was at most twofold greater than the control strain that only differed by the absence of BsNFOR (Fig. 2).
Furthermore, the BsNFOR-dependent yield, at best (31:1 headspace : liquid ratio), did not exceed 40 mmol H₂ (mol glucose)⁻¹ (Fig. 4C). The relatively small response in H₂ accumulation to BsNFOR expression may be due to (i) a low turnover of NADP(H), (ii) thermodynamic limitations, affected by the headspace : liquid ratio and the intracellular NADPH/NAD⁺ ratio and/or (iii) an insufficient catalytic capability by the introduced BsNFOR:CpFd:HydA pathway. The two- to fivefold response in H₂ yield from the BsNFOR-dependent pathway, imparted by coexpression of E. coli Zwf, suggests that poor catalytic capability is not limiting BsNFOR-dependent H₂ accumulation. This could also explain why trials with greater isopropyl-β-D-thiogalactopyranoside (IPTG) concentration did not enhance BsNFOR-dependent H₂ accumulation (data not shown). However, it does not distinguish between low turnover and thermodynamic limitations, as the presumed enhanced flux through the pentose phosphate pathway could potentially both increase NADP(H) turnover and the NADPH/NAD⁺ ratio. The three- and sevenfold response in BsNFOR-dependent H₂ yield to increased headspace : liquid ratio (Fig. 4C), however, does suggest that the thermodynamic limitation constitutes the primary limitation that restricts BsNFOR-dependent H₂ accumulation in closed cultures with small headspace : liquid ratios. Nevertheless, as the response in H₂ yield to increased headspace : liquid ratio was lower when shifting from 5:1 to 31:1 than when shifting from 0.6:1 to 5:1 (Fig. 4C), we cannot fully exclude that also the other two limitations [(i) and/or (iii)] are affecting final H₂ yield.

While NADPH-dependent H₂ synthesis was functionally implemented with BsNFOR, only the apparent reverse pathway was observed with CsNFOR. When H₂ was absent in the headspace, oxidation of either NADPH, NADH and/or an unknown source of native reductant acted as a source of electrons to allow H₂ to accumulate just above the limit of detection (~30–40 Pa) (Fig. 2B). Once the partial H₂ pressure level reached the equilibrium point of both (NADPH:H₂ and NADH:H₂) pathways, no net flux occurred through the CsNFOR-dependent pathway in either direction. However, CpFd would still most likely be reduced by the unknown native source of reductant responsible for ‘background’ ferredoxin- and HydA-dependent H₂ production in the absence of any recombinant NFOR. The lack of increase in H₂ accumulation in such a case is most likely attributed to the oxidation of CpFd along with concomitant reduction of NAD⁺ by CsNFOR, thus preventing any further delivery of electrons from CpFd to HydA. There is no doubt that the introduced CsNFOR-dependent pathway was operational, as crude lysates of CsNFOR-expressing cells catalysed both NADH- and NADPH-dependent H₂ synthesis with equal or greater rates than lysates of BsNFOR-expressing cells. Most convincingly, CsNFOR-expressing cells consumed added H₂ rapidly and to a lower final partial H₂ pressure value than strains expressing BsNFOR, while H₂ accumulation was observed in cultures to which no H₂ was added (Fig. 4B). Assuming that only one net directionality is possible by any given pathway, inhibition of CsNFOR by any unknown molecular effectors can thus be ruled out as a reason for near-zero H₂ accumulation. The strongly reduced equilibrium point, in CsNFOR- compared with BsNFOR-expressing strains, is therefore most likely a direct result of differences in the biochemically effective intracellular ratio of reduced to oxidized cofactor between the NAD(H) and NADP(H) couples. The differences in reported ratios obtained from the analysis of cellular extracts of E. coli [NADH/NAD⁺ and NADPH/NADP⁺ ratios: 0.88 versus 2.6 (Brumaghim et al., 2003), 0.13 versus 1.3 (Andersen and von Meyenburg, 1977) respectively] support this conclusion. Pathway directionality for near-equilibrium reactions is therefore obviously more important than molar yield of precursor as the turnover of NADH per glucose most likely is >10-fold greater than that of NADPH in E. coli, as argued above.

In native organisms, there is only evidence of NADH-dependent H₂ synthesis (Jungermann et al., 1971; Nakashimada et al., 2002) and H₂-dependent NAD⁺ reduction (Malki et al., 1995; Steuber et al., 1999). It has been suggested that NADPH and NADH has net cellular benefit and cost respectively (Varma et al., 1993). The suggested directionality for NADH:H₂ (H₂ synthesis) and NADPH:H₂ (H₂ consumption) pathways in native organisms would therefore theoretically make sense. However, there are issues with such a concept. First, physiologically unfavourable pathway directionality would need to be restricted through regulatory mechanisms if external partial H₂ pressure levels do not favour the directionality of interest. This may explain why H₂-dependent NAD⁺ reduction in Clostridium spp. was demonstrated to be restricted by sensitive feedback inhibition of the NFOR by NADH (Jungermann et al., 1971; Thauer et al., 1971), and why NADH-dependent hydrogenase activity of crude extracts of Thermoanaerobacter tengcongensis was markedly reduced in cells grown under closed, as opposed to open, headspace conditions (Soboh et al., 2004). Second, how can GAP-dependent H₂ synthesis be physiologically relevant in native species, given that NADH-dependent H₂ synthesis is highly sensitive to end-product accumulation, and that the sensitivity most likely will be present regardless of whether a pathway is constructed or native? One possibility is that it is not metabolically relevant except in the special circumstance of very low partial H₂ pressure, as for example seen in syntrophic co-cultures shared with H₂-consuming methanogens (Lovley, 1985). Third, if H₂-dependent NAD⁺ reduction is physiologically more favourable than the reverse directionality, as suggested by both theoretical (Varma et al., 1993) and biochemical
analyses (Malki et al., 1995; Steuber et al., 1999), why then do we observe the reverse pathway directionality with the constructed pathway presented in the present study? This is most easily explained by the low partial H₂ pressure levels that were used in this study and a lack of evolved transcriptional regulation. Native NADPH-generating H₂ pathways may therefore only be metabolically relevant in environments with a high partial H₂ pressure level.

Theoretical prediction of pathway limitation is based on the assumption that ‘fixed’ cofactor ratios derived from post-extraction analysis can be used to predict pathway directionality for near-equilibrium reactions. Although it may be possible to measure a particular cofactor ratio in cellular extracts, is this exactly the ratio that influences >100 NAD(P)(H)-related reactions in vivo? Reported NADPH/NADP⁺ cofactor ratios for E. coli vary greatly: 6.2 under aerobic conditions (Bautista et al., 1979), 1.0 under aerobic conditions in exponential phase and 0.1 in stationary phase (Lee, Kim, Park and Lee et al., 1996), 2.6 under anaerobic conditions and 0.11 under aerobic conditions (Brumaghim et al., 2003), 0.5 under both anaerobic and aerobic conditions (Andersen and von Meyenburg, 1977), and 0.4 under aerobic conditions of both exponential and stationary phase (Walton and Stewart, 2004). A part of the variation is most likely due to the instability of NAD(P)H under conditions employed using existing extraction methods (Kimball and Rabinowitz, 2004). A part of the variation is most likely due to the instability of NAD(P)H under conditions employed using existing extraction methods (Kimball and Rabinowitz, 2004; Pollak et al., 2007; Rabinowiz and Kimball, 2007). In addition, the majority of intracellular NAD(P)H has been reported to be bound to enzymes (Patterson et al., 2000) and it is not clear whether cofactors in the bound state could influence biochemical reactions differently from that of freely soluble cofactors (Pollak et al., 2007). The ratio that determines the equilibrium point for NAD(P)H:H₂ pathways, the biochemically effective intracellular cofactor ratio, is therefore potentially influenced by at least two different parameters: (i) the total molar quantity of each cofactor form and (ii) the relative proportion of bound to free cofactor form. We determined the biochemically effective intracellular cofactor ratios using Eqn A1 and the experimentally determined partial H₂ pressure limits shown in Fig. 4. The differences between the ratios of the NADPH/NAD⁺ and the NADH/NAD⁺ couples were surprisingly large, a factor of 6 and 14 at the end of exponential and late stationary phase respectively. The estimated ratios for NADH/NAD⁺ are within the range that is commonly reported 0.1–1.0, while the highest estimated ratio of NADPH/NAD⁺ based on partial H₂ pressure levels is more than twofold above previously reported ratios (Andersen and von Meyenburg, 1977; Alexeeva et al., 2003; Brumaghim et al., 2003). The difference between partial H₂ pressure- and extraction-based ratios is most likely explained by the chemical lability of NAD(P)H, although it cannot be ruled out that the relative proportion of free to bound cofactor also could influence the equilibrium point.

The consistently large differences observed between the two cofactor couples are in line with conclusions derived from whole-cell thermodynamic in silico analysis, from which it was predicted that cofactor ratio estimates obtained from extraction-based analysis were close to the maximum (NADH/NAD⁺) and minimum (NADPH/NAD⁺) thermodynamically feasible ratios that would allow cellular metabolism and growth to proceed (Henry et al., 2007). Interestingly, this suggests that there is potential for further increasing the NADPH/NAD⁺ ratio, before reaching the point where whole-cell metabolism is predicted to be thermodynamically unfeasible. This is worth keeping in mind given that also NADPH-dependent H₂ synthesis is highly sensitive to end-product inhibition as shown in Fig. 4A, a degree of sensitivity that still may be too high for industrial application (van Groenestijn et al., 2002), and that further studies are needed to explore the possibility of enhancing the biochemically effective intracellular NADPH/NAD⁺ ratio.

In conclusion, we have constructed and verified two NAD(P)H:H₂ model pathways and demonstrated that both pathways are severely inhibited by end-product accumulation, although the equilibrium of the NADPH:H₂ pathway favoured H₂ accumulation to a greater extent than that of the NADH:H₂ pathway. Basing any future engineering on NAD(H) as a combined central carbon catabolite intermediate and H₂ precursor therefore appears impractical. However, some promise does exist for NAD(P)H, although low NAD(P)H turnover is a major bottleneck that would need to be increased substantially to allow for any eventual application.

**Experimental procedures**

**Chemicals and other reagents**

All chemical reagents were obtained from SIGMA-Aldrich (Tokyo, Japan) except where stated. Restriction enzymes were from New England Biolabs (Hitchin, Hertfordshire, England). Genomic DNA for PCR amplification was either obtained directly from ATCC (C. acetobutylicum 824D-5, C. tepidum 49652D) or isolated from reference strains obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (B. subtilis DSM 402; C. pasteurianum DSM 525). Isolation of DNA was performed using the DNAeasy kit (Qiagen Sciences, MD, USA). SDS-PAGE was conducted using pre-cast 12% gels (TEFCO, Japan), gels were stained using SYPRO Orange (Invitrogen, Carlsbad, CA) and visualized using a Fujifilm FLA-3000 scanner according to manufacturer’s instructions.

**Bacterial strains, plasmids and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 2 and all primers are listed in Table 3. Deletion
of the ydbK gene in E. coli MG1655 was performed according to the method of Datsenko and Wanner (2000). The FRT flanked kanamycin-resistance cassette of plasmid pKD13 was amplified by PCR using primers ydbK-for and ydbK-rev. After treatment with DpnI and purification, the deletion cassette was transformed to MG1655 WT carrying pKD46. Kanamycin-resistant clones were selected and the deletion was verified by PCR using primers K-ydbK-for and K-ydbK-rev. The resulting strain was designated MG1655 \( D_{\text{ydbK}} \) and used as donor strain to generate BL21(DE3) \( D_{\text{ydbK}} \) by P1 transduction (Miller, 1972) using BL21(DE3) (Novagen, Merck KGaA, Darmstadt, Germany) as recipient. After P1 transduction, the kanamycin-resistance gene was removed using the pCP20-encoded yeast recombinase (Datsenko and Wanner, 2000). Likewise the \( D_{\text{fpr}} \) and \( D_{\text{ydbK}} \ D_{\text{fpr}} \) mutant of BL21(DE3) was obtained by P1 transduction using an MG1655 \( D_{\text{fpr}} \) strain (University of Wisconsin, USA) as the donor and BL21(DE3) and BL21(DE3) \( D_{\text{ydbK}} \) as the respective recipient strains. The deletion of \( fpr \) was verified by PCR using primers K-fpr-for and K-fpr-rev.

### Table 2. Strains and plasmids used in the present study.

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| MG1655            | Wild type               | CGSC                |
| MG1655::ydbK::kan | MG1655 with ydbK deleted, kan’ | This study |
| MG1655::fpr::kan  | MG1655 with fpr deleted, kan’ | This study |
| BL21(DE3)         | lon’, repT              | Novagen             |
| BL21(DE3)::fpr    | BL21(DE3) with fpr deleted | This study |
| BL21(DE3)::ydbK   | BL21(DE3) with ydbK deleted | This study |
| BL21(DE3)::ydbK::fpr | BL21(DE3) with ydbK and fpr deleted | This study |
| **Plasmids**      |                         |                     |
| pCDF-Duet         | Expression vector, spec’ | Novagen             |
| pCOLA-Duet        | Expression vector, kan’  | Novagen             |
| pET-Duet          | Expression vector, carb’ | Novagen             |
| pET46-Ek/LIC      | Expression vector, carb’ | Novagen             |
| pCDOPFEG          | pCDF-Duet with C. acetobutylicum hydF (CAC1651), hydE (CAC1356) and hydG (CAC1356) in MCS1 with MCS2 deleted | Akhtar and Jones (2008b) |
| pCDOPFEGAfdx      | pCDOPFEG with addition of C. acetobutylicum hydA (U15277) | Akhtar and Jones (2008b) |
| pBSNFOR           | pET46-Ek/LIC with yumC of B. subtilis (CAB15201) | This study |
| pCtNFOR           | pET46-Ek/LIC with gene encoding CtNFOR (CT1512) of C. tepidum (NP_662397) | This study |
| pCpFd             | pCOLA-Duet with gene encoding [4Fe4S] ferredoxin of C. pasteurianum (M11214) in MCS1 | This study |
| p4Fd              | As above, except pET-Duet | This study |
| p2Fd              | pET-Duet with gene encoding [2Fe2S] ferredoxin of C. pasteurianum (JH0804) in MCS1 with MCS2 deleted | This study |
| pCOLAzwf          | pCOLA-Duet with E. coli zwf (b1852) in MCS1 with MCS2 deleted | M.O. Park and P.R. Jones (unpublished) |
| pKD13             | kan’, oriR              | Datsenko and Wanner (2000) |
| pCP20             | amp’, yeast FLP recombinase | Datsenko and Wanner (2000) |
| pKD46             | amp’, repA101(ts), araBP-gam-bet-exo | Datsenko and Wanner (2000) |

### Table 3. Oligonucleotides used in the present study.

| Primer name | Sequence (5’ → 3’) |
|-------------|--------------------|
| ydbK-for    | CCCTATTGCGGCAATGTAAGGTGTCAATATGACTAATTGTAGCAGGGTGATGGCTGAGCTGCTTCC |
| ydbK-rev    | GCCGAATTGCAATGGAATTGTACATATGACTAATTGTAGCAGGGTGATGGCTGAGCTGCTTCC |
| K-ydbK-for  | GCGCAAMGTAAGGGTTCG |
| K-ydbK-rev  | GTGCCGAAGAGCTCATAGCCG |
| K-fpr-for   | GTCCATCCCTATCTGATGTC |
| K-fpr-rev   | GTGATCGATAGTACAGTACCG |
| BsNFOR-for  | GACGAGCAAAATGCCTGAGCTGAGAATAAGGTTTATG |
| BsNFOR-rev  | GACGGAGAAGCCGGTTATATATCATATAAAAGACCCCTGTTGAGT |
| CttNFOR-for | GACGGAGCAAAATGCCTGAGCTGAGAATAAGGTTTATG |
| CttNFOR-rev | GACGGAGAAGCCGGTTATATATCATATAAAAGACCCCTGTTGAGT |
| Cp4Fdx-for  | ATCCATGGCATATAAACCTGATCTGATGTTAAGC |
| Cp4Fdx-rev  | TTTCGTCAGCTATTTTCTTGACCTGTCACAGT |
| Cp2Fdx-for  | TACCATGGCTAATATGACCTGATCCTGTTGAGT |
| Cp2Fdx-rev  | TACCATGGCTAATATGACCTGATCCTGTTGAGT |
| FEGAFdx-for | ATAGGGATCTGACGACTTATAATAATGACCTGATCCTGTTGAGT |
| FEGAFdx-rev | TACCTAGGAGGCGCTTATATGACCTGATCCTGTTGAGT |

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Plasmids (Table 2), except for pCDOPFEGAFdx, were constructed using Accuprime PFX Polymerase (Invitrogen), primers (Table 3) and genomic DNA template as listed in Table 2 and vectors obtained from Novagen according to manufacturer’s instructions. The insertion site for Ek/LIC vectors (pBsNFOR and pCNFOR) is predetermined as described in manufacturer’s instructions and all Ek/LIC-encoded gene products contained a vector-encoded N-terminal His-tag. All ferredoxin-encoding genes were cloned into MCS1 of pET-Duet or pCOLA-Duet using restriction sites Ncol and Sall (pCpFd and p4Fd) or AvrII (p2Fd), insertion sites that eliminate the native vector-encoded His-tag, using primers Cp4Fdx-for and Cp4Fdx-rev (pCpFd and p4Fd) and Cp2Fdx-for and Cp2Fdx-rev respectively. The plasmid pCDOPFEGAFdx was constructed by addition of the CpFd-encoding gene (M11214) at the 3’ end of hydA in pCDOPFEGA using primers FEGAFdx-for and FEGAFdx-rev (Akhtar and Jones, 2008b). The identity of each plasmid gene construct was verified by sequencing and function was verified by SDS-PAGE and/or enzyme activity assay of crude lysates after test expression. The plasmids were used to transform strains by electroporation.

Cultivations were performed in 4–50 ml of MOPS minimal medium (Teknova, Hollister, CA) containing 1.5% glucose as carbon source, supplemented with kanamycin (50 µg ml\(^{-1}\)), carbenicillin (50 µg ml\(^{-1}\)) or spectinomycin (50 µg ml\(^{-1}\)), where appropriate. Pre-cultures were inoculated from a fresh Luria–Bertani (LB) plate and grown at 30°C to an OD\(_{600}\) of 0.1–0.5. Pre-cultures were then used to inoculate main cultures (same media with addition of 0.05 mM IPTG) at an approximate OD\(_{600}\) of 0.02. All cultures were grown at 30°C in serum vessels (35 or 125 mL) capped with butyl rubber septum and sparged with 99.9995% N\(_2\) for >5 min following inoculation. Different levels of IPTG concentration were tested and 0.05 mM was found to be optimal for BsNFOR-dependent H\(_2\) accumulation.

Leakage of H\(_2\) from the serum bottles was assessed as follows. Four serum bottles (two containing 50 ml of MQ H\(_2\)O and the other two without any liquid) were capped with butyl rubber stoppers and exhaustively sparged (>5 min) with N\(_2\) using one input needle and one outlet needle to retain atmospheric pressure. All bottles were then transferred into the anaerobic hood that had an atmosphere of ~5% H\(_2\) and the butyl rubber stoppers removed. After 2 h of headspace gas equilibration, the same pierced butyl rubber stoppers were once again capped onto all four serum bottles. The H\(_2\) content of all four bottles were measured by gas chromatography, and two of the serum bottles were again transferred into the anaerobic chamber (one with liquid, one without) while the other two were placed in an incubator (30°C, 175 r.p.m., 48 h). After 48 h, the H\(_2\) content of all four bottles was once again determined. No detectable loss in H\(_2\) was observed.

Heterologous gene expression and protein purification

All cell cultures expressing the FeFe hydrogenase were prepared exclusively under anaerobic conditions using buffers that were N\(_2\)-sparged prior to transfer into the anaerobic hood. Strains were grown at 30°C in terrific broth or LB media (Sambrook et al., 1989) containing 0.2–0.5 mM isopropyl β-D-1-thiogalactopyranoside for 18–36 h and then harvested by centrifugation (5000 g, 10 min, 4°C). Pelleted cells were re-suspended in 1/50th culture volume of lysis buffer [100 mM Tris–HCl (pH 7.5), 0.2 % Triton X-100, 2 mM dithiothreitol, 0.5–1.0 mg ml\(^{-1}\) chicken lysozyme (Wako Pure Chemical Industries, Osaka, Japan) and 50–200 U recombinant DNase I (Roche Diagnostics, Mannheim, Germany)] followed by freeze/thaw and incubation (10 min, 25°C). After the removal of cell debris by centrifugation (17 000 g, 10 min, 4°C), purification of HydA and BsNFOR was carried out using His SpinTrap columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to manufacturer’s instructions. Purified proteins were stored on ice until enzyme assays were performed.

In vitro reconstitution

Crude lysates (~1 µg of total protein) or His-tag purified protein preparations (0.024 U of hydrogenase, 0.6 U of NFOR), stored on ice inside an anaerobic hood, were added to 2 mL of N\(_2\)-sparged reaction mixtures [100 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM dithiothreitol, 1.25–2.5 mM NAD(P)H, 1 µM FAD, 250 µM MV]. Where indicated, 4.5 U ml\(^{-1}\) S. cerevisiae G6PDH and 0.5 mM G6P was added to regenerate NADPH, or 3 U ml\(^{-1}\) Candida boidinii formate dehydrogenase and 0.5 mM sodium formate was added to regenerate NADH. One U of enzyme activity corresponds to 1 µmol of product formed per min.

Enzyme assays

All assays were conducted under anaerobic conditions using quartz cuvettes fitted with open top screw cap (GL Sciences, Tokyo, Japan) and butyl rubber stopper (Voigt Global Distribution, Kansas City, USA). All reaction buffers and other additives were N\(_2\)-sparged prior to use. NAD(P)H-dependent reduction of MV was measured by following the reduction of MV at 600 nm (absorbance). Between 1 and 10 µg of crude protein or 0.5–1.0 µg of purified protein was added to N\(_2\)-sparged reaction mixture [50 mM Tris-HCl (pH 7.5), 10 µM flavine adenine dinucleotide, 0.5–1.0 mM NAD(P)H, 1 mM MV] to start the reaction. Dithionite- and hydrogenase-dependent synthesis of H\(_2\) was measured by analysing the composition of the gaseous headspace of septum-closed serum bottles containing reaction mixtures (crude lysed cell extracts as described above, 0–0.5 mM MV, 10 mM Na\(_2\)SO\(_4\)) after incubation for up to 1–2 h at 30°C.

Analysis

Gaseous samples were analysed using a 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) fitted with a Carbosieve\textsuperscript{TM} (1010) PLOT capillary column (30 m × 0.32 mm) (Supelco, Bellefonte, USA). A 200 µl gas sample was withdrawn from the headspace of serum bottle cultures using a samplelock syringe (Hamilton, Reno, NV) and injected directly into the split/splitless inlet (ambient temperature, 5:1 split ratio). Samples were eluted isocratically (35°C, 4–5 min). The carrier gas was N\(_2\) (17.6 ml min\(^{-1}\)) and a thermal conductivity detector (230°C, 2.0 ml min\(^{-1}\)) was used to detect samples. H\(_2\) eluted at ~3.3 min and the partial headspace of H\(_2\) (partial H\(_2\) pressure) was quantified by comparison with calibration curves prepared with 0.25% and 5%
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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Verification of the expression and activity of recombinant NFORs.

Appendix S2. Estimating the equilibrium point of reaction (1) and maximum partial H2 pressure level for in vitro reaction employing finite starting substrate.