Cofactor engineering for advancing chemical biotechnology.

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Abstract
Cofactors provide redox carriers for biosynthetic reactions, catabolic reactions and act as important agents in transfer of energy for the cell. Recent advances in manipulating cofactors include culture conditions or additive alterations, genetic modification of host pathways for increased availability of desired cofactor, changes in enzyme cofactor specificity, and introduction of novel redox partners to form effective circuits for biochemical processes and biocatalysts. Genetic strategies to employ ferredoxin, NADH and NADPH most effectively in natural or novel pathways have improved yield and efficiency of large-scale processes for fuels and chemicals and have been demonstrated with a variety of microbial organisms.

Introduction

Cofactors provide redox carriers for biosynthetic reactions, catabolic reactions and act as important agents in transfer of energy for the cell. Some examples illustrating the requirement for cofactor balance and availability include: the conversion of biomass feedstocks containing xylose to ethanol where the formation of xylitol is a problem [1-7]; as a driving force for more effective production of reduced compounds such as biofuels [8]; in using cytochrome P450s in specific oxidation reactions where the recycling of active enzyme is required [9-11]; and the production of chiral pharmaceutical intermediates where specific reductions require a certain cofactor [12,13]. Experimental studies along with more complete computational models have shown a global picture of the flow of reducing equivalents and its connection to cell physiology and allowed these insights to be considered for metabolic engineering purposes [14-17]. The synthetic biology revolution has allowed easier ways to construct combinations of redox complexes and connect them to give a focused controllable circuit that can be directed to a desired metabolite [18]. Advances in protein engineering also have helped direct cell redox metabolism toward desired ends [6,19-23]. These advances intertwine with the theme of generating effective bioprocesses for fuels and chemicals from biomass and more recently from H_2/CO_2 or electrodes [24,25]. The brief review will cover some recent developments in analyzing redox flow in microbial cells and its physiological consequences [26], engineering proteins and pathways for optimal performance and applications of cofactor engineering to commercial processes.

Studies connecting cofactor balance to cell physiology

The effect of extracellular additives on redox cofactor physiology has been observed in several reports. The addition of hydrogenase substrates/inhibitors can affect the availability of NADH and lead to higher butyrate formation in clostridium cultures [27]. Electron mediators such as methyl viologen or neutral red can also serve to increase butyrate productivity [28] and demonstrate potential for electrically driven acidogenesis. The addition of pyridine nucleotide precursors to the medium improved pools in *E. coli* and aided formation of a chiral pharmaceutical intermediate [29]. Experimental and modeling studies of the contribution of the pentose phosphate pathway in forming NADPH have been published. As demand for NADPH was increased, cells increased flux through the pentose phosphate and acetate pathways and employed a cytosolic transhydrogenase [15]. The group also developed a constraint-based model to analyze the contribution of pathways producing NADPH [14] supporting the findings and proposing, a glycerol-DHA futile cycle, could provide additional NADPH. These studies have shown a variety of effects of cofactor manipulation on cellular metabolism.

Recent studies of redox reactions in a variety of anaerobes has shown the importance of ferredoxins as electron carriers and the recent discovery of bifurcating systems coupling this cofactor to generating membrane gradients and regeneration of NADH/NADPH while catalyzing less energy requiring reductions has brought new appreciation to their role as major carriers of electron flow and allowed initial efforts to engineer such systems in a useful fashion. Ferredoxin is an electron transfer carrier with a low midpoint potential so the reduced form is a potent reducing agent. The analysis of the specificity of the partner and coupling mechanism have been active areas of research. The NADPH specificity of ferredoxin-NAD(P)H oxidoreductase (FNR) is often decided by the interaction at the 2'-phosphate group of NADPH. A Y308S mutation in the pea enzyme reduced its preference for NADPH [23]. Mutation Y258F in *Plasmodium falciparum* FNR deleted the hydroxyl group which interacts with the 2'-phosphate group of NADPH and abolished the enzyme's cofactor selectivity [30]. The crystal structure of a novel *Bacillus subtilis* BsFNR encoded by *yumC* complexed with $NADP^+$ [31] showed a binding mode

similar to *Escherichia coli* NADPH-thioredoxin reductase. The specificity was altered but it still had ~ 1/8 the activity as with NADPH, whereas the *Chlorobium tepidum* FNR shows a two-fold difference in activity between the two pyridine nucleotides [32]. In *Acetobacterium woodii* the Rnf complex can produce reduced ferredoxin from H_2 by coupling with a soluble four subunit (HydABCD) electron bifurcating hydrogenase [33] The trimeric [FeFe]-type hydrogenase of *Thermotoga maritima* [34] gives an overall reaction of Fd_{red} + NADH + H^+ -> $2H_2$ = Fd_{ox} + NAD^+. This sort of bifurcating enzyme mechanism has been documented in the coupled ferredoxin and crotonyl-coenzyme A reduction with NADH of *Clostridium kluyveri* [35] where a second bifurcating enzyme was found [36] that forms 2 NADPH molecules by reduction of 2 $NADP^+$ molecules with 1 NADH and 1 Fd_{red}.

The use of ferredoxin coupled reactions was creatively used in the formation of a synthetic hydrogen-producing electron transfer circuit in *E. coli* [18]. Heterologously expressed [Fe-Fe]-hydrogenase, ferredoxin, and pyruvate-ferredoxin oxidoreductase allowed production of hydrogen gas coupled to sugar metabolism. The synthetic pathway was "insulated" from normal host reactions by deletion of competing pathway genes and protein interactions were engineered by various protein fusions with Fd partners, and co-localization of pathway enzymes on heterologous protein scaffolds. Subsequently a novel use of RNA scaffolds in this system [37] showed a new way to localize and enhance cellular redox pathways and suggested additional opportunities for harnessing ferredoxin-mediated processes.

Cofactor considerations in metabolic engineering

While in general the reduced cofactor is the desired form, there are instances where it is useful to remove the reduced form and recycle NAD^+. The use of NADH oxidase to remove excess NADH and aid formation of oxidized products has been reported previously. NADH oxidase from *Lactobacillus brevis* was overexpressed in combination with a (2R,3R)-2,3-butanediol dehydrogenase to produce chiral (3R)-acetoin as an example of specific formation of a chiral product [13]. The characterization of a new NADH oxidase from *Streptococcus pyogenes* MGAS10394 revealed it had the highest activity among known NADH oxidases [38]. A library bearing different strength *noxE* promoters was constructed to alter *noxE* expression and NADH/NAD^+ ratios in the lactic acid bacteria, which altered the distribution of lactate and diacetyl [39].
In a number of pathways NADPH is the reducing cofactor, so there have been several successful efforts to increase the availability of this cofactor in the host (Figure 1). Sources of NADPH include the pentose phosphate pathway, isocitrate dehydrogenase and malic enzyme. To increase NADPH, overexpression of a NAD kinase gene has been studied. In yeast a number of studies have been performed. The mitochrondrial NADH kinase Pos5p is important in generating the active FeS centers of a number of metabolic proteins [40] needed for respiration [41]. These studies showed significant physiological effects of altering the mitochondrial or cytoplasmic pool of NADPH. Overexpressing NADH kinase in the cytosol had varying effects on cell products depending on the conditions of growth on glucose, e.g. more to ethanol during aerobic growth and more to ethanol and acetate during anaerobic growth [42]. The strategy did not reduce the formation of xylitol in xylose cultures. In this regard, decreasing cytosolic NADH lowered glycerol production, while changes in product profile were linked to the ATP requirement for biomass synthesis and the efficiency of oxidative phosphorylation [43]. The yeast NAD kinase was expressed in *E. coli* **t**o aid production of guanosine diphosphate (GDP)-L-fucose and epsilon-caprolactone with a 51 % and 96% enhancement observed, respectively [44]. The authors suggested the enhanced availability of NADPH through more efficient regeneration of NADPH and that it could be applied to other products. NAD kinase was also overexpressed generating a higher yield of lysine in *Corynebacterium glutamicum* [45].

The native *C. glutamicum* NAD^+ dependent glyceraldehyde-3-phosphate dehydrogenase was replaced by a non-phosphorylating $NADP^+$ dependent GAPDH from *Streptococcus mutans* and an improved growth variant exhibited increased lysine production [46]. In an engineered xylitol producing *E. coli* strain, double deletions of phosphofructokinase *pfk* and transhydrogenase *sthA* or phosphoglucose isomerase *pgi* and *sthA* genes were made and both combinations showed increased xylitol yield [47]. The NADPH supply in an engineered *E. coli* strain was improved by overexpression of fructose 1,6-bisphosphatase II *glpX* or co-expression of *glpX* and glucose-6-phosphate 1-dehydrogenase *zwf* [48]. We have shown that replacement of the *E. coli* native NAD^+ dependent GAPDH *gapA* by $NADP^+$ dependent GAPDH *gapB* from *Bacillus subtilis* increased product formation and the strain showed further increased NADPH dependent biosynthesis when NAD kinase was co-expressed (unpublished results). In another strategy, phosphofructokinase has been deleted from *E. coli* [49-51] and studied in various combinations with other alterations such as GAPDH overexpression, NAD kinase, glucose uptake systems and transhydrogenase alterations. The altered glycolysis in *pfk* mutants routed glucose through pentose phosphate pathway and led to higher NADPH dependent biosynthesis in systems sensitive to NADPH availability reported in [49-51] and found by our group [52]. Xylitol formation using NADPH-dependent xylose reductase from *Candida boidinii* was improved in an *E. coli pfkA* and *sthA* mutant [51]. Deletion of *pgi* and *sthA* also showed increased glucose utilization and lowered acetate formation.

One of the options is to increase the activity of transhydrogenases that can convert excess NADH into NADPH. In *E. coli* two systems exist, membrane associated PntAB and a soluble Udh or Sth system. Our group (Jan et al, unpublished) and other researchers have combined overexpression or deletion of these genes with other host strain modifications to improve NADPH-dependent pathway performance where NADPH availability is measured in the presence of a NADPH utilizing reaction or "sink". In *E. coli* engineered for xylitol [53] or platform chemical 3-hydroxypropionic acid production [54], PntAB increased production. Studies of *Corynebacterium glutamicum* engineered for isobutanol formation indicated a cycle consisting of pyruvate and/or phosphoenolpyruvate carboxylase, malate dehydrogenase, and malic enzyme provided conversion of $NADH + H^+$ to $NADPH + H^+$, aided synthesis [55]. Engineering *C. glutamicum* for valine production by removing pyruvate dehydrogenase activity enhanced flux through the pentose phosphate pathway providing additional NADPH while adding *E. coli* PntAB decreased the pentose phosphate pathway flux [56].

Another way to address the NADH/NADPH balance problem in an engineered organism is to convert an enzyme's cofactor specificity to the desired characteristic. Production of 1,3-propanediol by1,3-propanediol oxidoreductase is limited by NADPH. To broaden cofactor specificity, the Asp41 interacting with the phosphate group of NADPH was changed to Gly allowing use of both cofactors [57]. In producing isobutanol, NADPH dependent keto-acid reductoisomerase and alcohol dehydrogenase were engineered into NADH dependent variants. The reconstructed isobutanol biosynthetic pathway resulted in 100% theoretical yield [19,21]. Formate dehydrogenase, which oxidizes formate into carbon dioxide, is widely used to regenerate reduced cofactors. The FDH from *Mycobacterium vaccae* N10 was mutated on the conserved NAD^+ binding motif to give a NADPH dependent activity [58] more resistant to haloketones.

Cofactor engineering in large-scale processes helps achieve optimal performance.

A number of articles have focused on limiting glycerol byproduct formation during yeast ethanol fermentation. A limitation in yeasts engineered to consume xylose is the formation of xylitol and the cofactor imbalance due to the usual NADPH-preferring xylose reductase and the NAD-dependent xylitol dehydrogenase. Routes to overcome these limitations have used deletion of the

NAD^+-dependent glycerol-3-phosphate dehydrogenase gene while introducing a non-phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase [2,3,5]. Overexpression of a *Kluyveromyces lactis* NADPH-forming GAPDH reduced xylitol production [1]. Protein engineering of xylose reductase to a NADH-preferring enzyme gave high ethanol productivity [6]. A study with the NADH-NADPH utilizing *Scheffersomyces stipitis* xylose reductase suggested formation of glucose 6-phosphate via gluconeogenesis was limiting [4]. The expression of NADH oxidase to diminish cofactor imbalance lowered glycerol and xylitol yields while improving the ethanol yield [7]. Models suggested cofactor balancing in the engineered D-xylose utilization pathways would generate an increase in ethanol production while reducing time and suggested harmonizing cofactor usage would be worthwhile [16].

The driving force for reactions in vivo was examined in 1-butanol synthesis. A modified clostridial 1-butanol pathway was introduced into *E. coli* and steps were taken to create NADH and acetyl-CoA to help direct the flux. Competing pathways in the host were removed and *E. coli atoB* was used for formation of acetoacetyl-CoA. The intracellular NADH driving force was further stimulated by employing formate dehydrogenase from *C. boidinii* [8]. The group also used an ATP driven step to form acetoacetyl-CoA for butanol formation in a cyanobacterium [59]. A model of butanol and isobutanol biosynthesis noted the importance of cofactor utilization [17].

Other examples include the production of thymidine, where a mutation in *pgi* to divert more flux through the pentose pathway and employment of a NAD kinase and transhydrogenase UdhA, each increased yields of thymidine [60]. A lactic acid bacteria based system that could convert fructose to mannitol, in high yield also employed a cofactor regeneration system [61].

Cofactor recycling: specialized enzyme systems.

Cytochrome P450 reactions require extensive cofactor recycling. These oxygenases are important in drug metabolism and can perform specific chiral introduction of functional groups. Cytochrome P450-199A2 from *Rhodopseudomonas palustris* oxidizes para-substituted benzoic acids and has an associated [2Fe-2S] ferredoxin, palustrisredoxin, rather than ferredoxin [9]. Expression of a similar P450 in *E. coli* from a tricistronic construct generated a whole cell biocatalyst that efficiently oxidized (1R)-(+)-camphor to 5-exo-hydroxycamphor and limonene to (-)-perillyl alcohol [10]. *Gordonia* SoCg *alkB* (alkane 1-monooxygenase) was expressed in *E. coli* BL21 and in *Streptomyces coelicolor* M145, and both hosts acquired the ability to transform n-hexadecane into 1-hexadecanol [62]. An *E. coli* system composed of P450pyr triple mutant, ferredoxin reductase, glucose dehydrogenase and ferredoxin genes, was engineered to show a high activity and high chiral efficiency in forming valuable pharmaceutical intermediates [11].

An in vitro system for producing and using NADPH was made from 12 enzymes and demonstrated high-yield formation of NAD(P)H from cellobiose when coupled with NADPH consumption by xylose reductase [63]. Such in vitro processing of sugars may allow a new route for biohydrogenation reactions. Another in vitro system used non-covalent immobilization of a cofactor regeneration system on functionalized single-walled carbon nanotubes to make the pharmaceutical intermediate 4-hydroxy-2-butanone [12].

Conclusions

Fundamental studies of individual enzymes, pathway fluxes, and global physiological responses have provided essential information for cofactor engineering as an aid for industrial production of fuels and chemicals. Protein engineering of enzyme cofactor specificity and formation of artificially created active complexes have impact for future work. Advances in engineering of the

host on a larger and more precise scale have contributed to improved availability of the desired cofactor. Such combinations of an engineered host with enhancement of a novel or native pathway have generated effective biocatalysts for bulk and specialty chemicals. The refinement of ideal redox chemistry through cofactor manipulation seems to have considerable potential in metabolic engineering and fits with trends in synthetic biology circuits and genome engineering.

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Bibliography

1. Bera AK, Ho NW, Khan A, Sedlak M: **A genetic overhaul of Saccharomyces cerevisiae 424A(LNH-ST) to improve xylose fermentation**. *Journal of industrial microbiology & biotechnology* 2011, **38**:617-626.

•2. Guo ZP, Zhang L, Ding ZY, Shi GY: **Minimization of glycerol synthesis in industrial ethanol yeast without influencing its fermentation performance**. *Metabolic engineering* 2011, **13**:49-59.

The article demonstrates how altering the cofactor produced through glycolysis more toward NADPH can reduce byproduct glycerol formation in the widely used yeast xylose fermentation.

3. Guo ZP, Zhang L, Ding ZY, Wang ZX, Shi GY: **Improving ethanol productivity by modification of glycolytic redox factor generation in glycerol-3-phosphate dehydrogenase mutants of an industrial ethanol yeast**. *Journal of industrial microbiology & biotechnology* 2011, **38**:935-943.

4. Hector RE, Mertens JA, Bowman MJ, Nichols NN, Cotta MA, Hughes SR: **Saccharomyces cerevisiae engineered for xylose metabolism requires gluconeogenesis and the oxidative branch of the pentose phosphate pathway for aerobic xylose assimilation**. *Yeast* 2011, **28**:645-660.

5. Kim JW, Chin YW, Park YC, Seo JH: **Effects of deletion of glycerol-3-phosphate dehydrogenase and glutamate dehydrogenase genes on glycerol and ethanol metabolism in recombinant Saccharomyces cerevisiae**. *Bioprocess and biosystems engineering* 2012, **35**:49-54.

6. Tamakawa H, Ikushima S, Yoshida S: **Ethanol production from xylose by a recombinant Candida utilis strain expressing protein-engineered xylose reductase and xylitol dehydrogenase**. *Bioscience, biotechnology, and biochemistry* 2011, **75**:1994-2000.

7. Zhang GC, Liu JJ, Ding WT: **Decreased xylitol formation during xylose fermentation in Saccharomyces cerevisiae due to overexpression of water-forming NADH oxidase**. *Applied and environmental microbiology* 2012, **78**:1081-1086.

••8. Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC: **Driving forces enable high-titer anaerobic 1-butanol synthesis in Escherichia coli**. *Applied and environmental microbiology* 2011, **77**:2905-2915.

The article shows the importance of driving forces in producing reduced cofactor in generating optimal flow through an engineered pathway for production of a major chemical in *E. coli*.

9. Bell SG, Xu F, Johnson EO, Forward IM, Bartlam M, Rao Z, Wong LL: **Protein recognition in ferredoxin-P450 electron transfer in the class I CYP199A2 system from Rhodopseudomonas palustris**. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2010, **15**:315-328.
10. Kim D, Ortiz de Montellano PR: **Tricistronic overexpression of cytochrome P450cam, putidaredoxin, and putidaredoxin reductase provides a useful cell-based catalytic system**. *Biotechnology letters* 2009, **31**:1427-1431.
11. Pham SQ, Gao P, Li Z: **Engineering of recombinant E. coli cells co-expressing P450pyrTM monooxygenase and glucose dehydrogenase for highly regio- and stereoselective hydroxylation of alicycles with cofactor recycling**. *Biotechnology and bioengineering* 2012.
12. Wang L, Zhang H, Ching CB, Chen Y, Jiang R: **Nanotube-supported bioproduction of 4-hydroxy-2-butanone via in situ cofactor regeneration**. *Applied microbiology and biotechnology* 2012, **94**:1233-1241.
13. Xiao Z, Lv C, Gao C, Qin J, Ma C, Liu Z, Liu P, Li L, Xu P: **A novel whole-cell biocatalyst with NAD+ regeneration for production of chiral chemicals**. *PloS one* 2010, **5**:e8860.
14. Celton M, Goelzer A, Camarasa C, Fromion V, Dequin S: **A constraint-based model analysis of the metabolic consequences of increased NADPH oxidation in Saccharomyces cerevisiae**. *Metabolic engineering* 2012, **14**:366-379.
15. Celton M, Sanchez I, Goelzer A, Fromion V, Camarasa C, Dequin S: **A comparative transcriptomic, fluxomic and metabolomic analysis of the response of Saccharomyces cerevisiae to increases in NADPH oxidation**. *BMC genomics* 2012, **13**:317.
16. Ghosh A, Zhao H, Price ND: **Genome-scale consequences of cofactor balancing in engineered pentose utilization pathways in Saccharomyces cerevisiae**. *PloS one* 2011, **6**:e27316.
17. Trinh CT: **Elucidating and reprogramming Escherichia coli metabolisms for obligate anaerobic n-butanol and isobutanol production**. *Applied microbiology and biotechnology* 2012, **95**:1083-1094.
18. Agapakis CM, Silver PA: **Modular electron transfer circuits for synthetic biology: insulation of an engineered biohydrogen pathway**. *Bioengineered bugs* 2010, **1**:413-418.
•19. Bastian S, Liu X, Meyerowitz JT, Snow CD, Chen MM, Arnold FH: **Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in Escherichia coli**. *Metabolic engineering* 2011, **13**:345-352.
The article shows the effect protein engineering of cofactor specificity can make to optimize compatibilty and increace production through a major introduced pathway for a commodity chemical.
20. Hoelsch K, Suhrer I, Heusel M, Weuster-Botz D: **Engineering of formate dehydrogenase: synergistic effect of mutations affecting cofactor specificity and chemical stability**. *Applied microbiology and biotechnology* 2012.
21. Liu X, Bastian S, Snow CD, Brustad EM, Saleski TE, Xu JH, Meinhold P, Arnold FH: **Structure-guided engineering of Lactococcus lactis alcohol**

dehydrogenase LlAdhA for improved conversion of isobutyraldehyde to isobutanol. *Journal of biotechnology* 2012, **164**:188-195.
22. Ma C, Zhang L, Dai J, Xiu Z: **Relaxing the coenzyme specificity of 1,3-propanediol oxidoreductase from Klebsiella pneumoniae by rational design**. *Journal of biotechnology* 2010, **146**:173-178.
23. Paladini DH, Musumeci MA, Carrillo N, Ceccarelli EA: **Induced fit and equilibrium dynamics for high catalytic efficiency in ferredoxin-NADP(H) reductases**. *Biochemistry* 2009, **48**:5760-5768.
24. Lovley DR: **Electromicrobiology**. *Annual review of microbiology* 2012, **66**:391-409.
25. Lan EI, Liao JC: **Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources**. *Bioresource technology* 2012.
26. Sieber JR, McInerney MJ, Gunsalus RP: **Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation**. *Annual review of microbiology* 2012, **66**:429-452.
27. Cai X, Servinsky M, Kiel J, Sund C, Bennett GN: **Analysis of redox responses during TNT transformation by Clostridium acetobutylicum ATCC 824 and mutants exhibiting altered metabolism**. *Applied microbiology and biotechnology* 2012.
28. Choi O, Um Y, Sang BI: **Butyrate production enhancement by Clostridium tyrobutyricum using electron mediators and a cathodic electron donor**. *Biotechnology and bioengineering* 2012, **109**:2494-2502.
29. Li N, Zhang Y, Ye Q, Chen Y, Chen X, Wu J, Bai J, Xie J, Ying H: **Effect of ribose, xylose, aspartic acid, glutamine and nicotinic acid on ethyl (S)-4-chloro-3-hydroxybutanoate synthesis by recombinant Escherichia coli**. *Bioresource technology* 2012, **118**:572-575.
30. Baroni S, Pandini V, Vanoni MA, Aliverti A: **A single tyrosine hydroxyl group almost entirely controls the NADPH specificity of Plasmodium falciparum ferredoxin-NADP+ reductase**. *Biochemistry* 2012, **51**:3819-3826.
31. Komori H, Seo D, Sakurai T, Higuchi Y: **Crystal structure analysis of Bacillus subtilis ferredoxin-NADP(+) oxidoreductase and the structural basis for its substrate selectivity**. *Protein Sci* 2010, **19**:2279-2290.
32. Muraki N, Seo D, Shiba T, Sakurai T, Kurisu G: **Asymmetric dimeric structure of ferredoxin-NAD(P)+ oxidoreductase from the green sulfur bacterium Chlorobaculum tepidum: implications for binding ferredoxin and NADP+**. *Journal of molecular biology* 2010, **401**:403-414.
33. Poehlein A, Schmidt S, Kaster AK, Goenrich M, Vollmers J, Thurmer A, Bertsch J, Schuchmann K, Voigt B, Hecker M, et al.: **An ancient pathway combining carbon dioxide fixation with the generation and utilization of a sodium ion gradient for ATP synthesis**. *PloS one* 2012, **7**:e33439.
34. Schut GJ, Adams MW: **The iron-hydrogenase of Thermotoga maritima utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production**. *Journal of bacteriology* 2009, **191**:4451-4457.
••35. Buckel W, Thauer RK: **Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation**. *Biochimica et biophysica acta* 2012.
The review covers multienzyme complexes that catalyze bifuricating reactions between ferredoxin and NAD(P)H or H_2 that are important in energy conservation in bacteria.

36. Wang S, Huang H, Moll J, Thauer RK: **NADP+ reduction with reduced ferredoxin and NADP+ reduction with NADH are coupled via an electron-bifurcating enzyme complex in Clostridium kluyveri**. *Journal of bacteriology* 2010, **192**:5115-5123.

••37. Delebecque CJ, Lindner AB, Silver PA, Aldaye FA: **Organization of intracellular reactions with rationally designed RNA assemblies**. *Science* 2011, **333**:470-474.

The article describes a new way of placing enzymes of a biosynthetic pathway on designed RNA scaffold structures in vivo and the consequences of the complexes on channeling of reaction intermediates.

38. Gao H, Tiwari MK, Kang YC, Lee JK: **Characterization of H2O-forming NADH oxidase from Streptococcus pyogenes and its application in l-rare sugar production**. *Bioorganic & medicinal chemistry letters* 2012, **22**:1931-1935.

39. Guo T, Kong J, Zhang L, Zhang C, Hu S: **Fine tuning of the lactate and diacetyl production through promoter engineering in Lactococcus lactis**. *PloS one* 2012, **7**:e36296.

40. Pain J, Balamurali MM, Dancis A, Pain D: **Mitochondrial NADH kinase, Pos5p, is required for efficient iron-sulfur cluster biogenesis in Saccharomyces cerevisiae**. *The Journal of biological chemistry* 2010, **285**:39409-39424.

41. Shi F, Li Z, Sun M, Li Y: **Role of mitochondrial NADH kinase and NADPH supply in the respiratory chain activity of Saccharomyces cerevisiae**. *Acta biochimica et biophysica Sinica* 2011, **43**:989-995.

42. Hou J, Vemuri GN, Bao X, Olsson L: **Impact of overexpressing NADH kinase on glucose and xylose metabolism in recombinant xylose-utilizing Saccharomyces cerevisiae**. *Applied microbiology and biotechnology* 2009, **82**:909-919.

43. Hou J, Lages NF, Oldiges M, Vemuri GN: **Metabolic impact of redox cofactor perturbations in Saccharomyces cerevisiae**. *Metabolic engineering* 2009, **11**:253-261.

44. Lee WH, Kim JW, Park EH, Han NS, Kim MD, Seo JH: **Effects of NADH kinase on NADPH-dependent biotransformation processes in Escherichia coli**. *Applied microbiology and biotechnology* 2013, **97**:1561-1569.

45. Lindner SN, Niederholtmeyer H, Schmitz K, Schoberth SM, Wendisch VF: **Polyphosphate/ATP-dependent NAD kinase of Corynebacterium glutamicum: biochemical properties and impact of ppnK overexpression on lysine production**. *Appl Microbiol Biotechnol* 2010, **87**:583-593.

•46. Takeno S, Murata R, Kobayashi R, Mitsuhashi S, Ikeda M: **Engineering of Corynebacterium glutamicum with an NADPH-generating glycolytic pathway for L-lysine production**. *Appl Environ Microbiol* 2010, **76**:7154-7160.

The article shows that the redox cofactor produced by glycolysis can be altered by genetic substitution with an enzyme of different specificity and the increased NADPH leads to improved production of a large-scale amino acid.

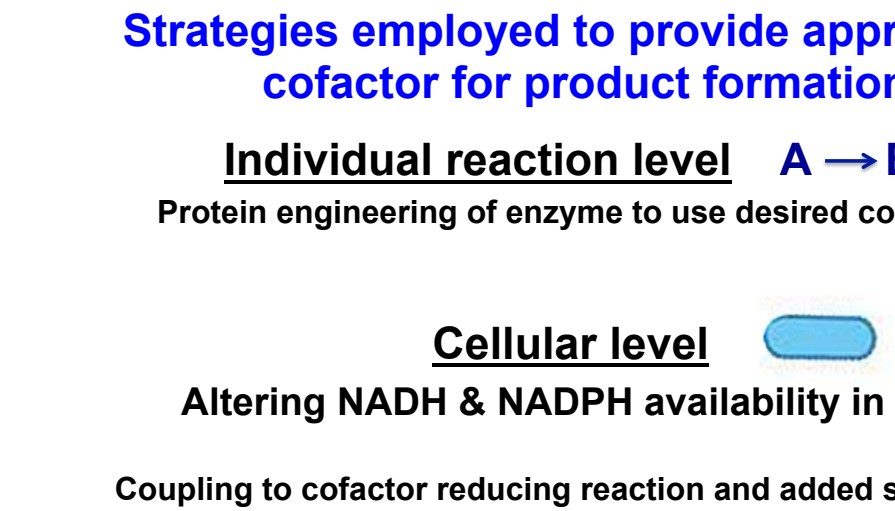
47. Chin JW, Cirino PC: **Improved NADPH supply for xylitol production by engineered Escherichia coli with glycolytic mutations**. *Biotechnol Prog* 2011, **27**:333-341.

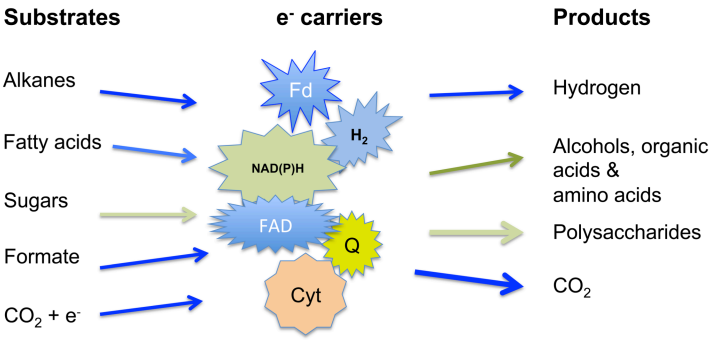
48. Kim YM, Cho HS, Jung GY, Park JM: **Engineering the pentose phosphate pathway to improve hydrogen yield in recombinant Escherichia coli**. *Biotechnol Bioeng* 2011, **108**:2941-2946.

49. Siedler S, Bringer S, Blank LM, Bott M: **Engineering yield and rate of reductive biotransformation in Escherichia coli by partial cyclization of the pentose phosphate pathway and PTS-independent glucose transport**. *Applied microbiology and biotechnology* 2012, **93**:1459-1467.
50. Siedler S, Bringer S, Bott M: **Increased NADPH availability in Escherichia coli: improvement of the product per glucose ratio in reductive whole-cell biotransformation**. *Applied microbiology and biotechnology* 2011, **92**:929-937.
51. Chin JW, Cirino PC: **Improved NADPH supply for xylitol production by engineered Escherichia coli with glycolytic mutations**. *Biotechnology progress* 2011, **27**:333-341.
52. Wang Y, San KY, Bennett GN: **Improvement of NADPH Bioavailability in Escherichia coli through the use of Phosphofructokinase Deficient Strains**. *Applied microbiology and biotechnology* in press.
53. Akinterinwa O, Cirino PC: **Anaerobic obligatory xylitol production in Escherichia coli strains devoid of native fermentation pathways**. *Applied and environmental microbiology* 2011, **77**:706-709.
54. Rathnasingh C, Raj SM, Lee Y, Catherine C, Ashok S, Park S: **Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant Escherichia coli strains**. *Journal of biotechnology* 2012, **157**:633-640.
55. Blombach B, Riester T, Wieschalka S, Ziert C, Youn JW, Wendisch VF, Eikmanns BJ: **Corynebacterium glutamicum tailored for efficient isobutanol production**. *Applied and environmental microbiology* 2011, **77**:3300-3310.
56. Bartek T, Blombach B, Lang S, Eikmanns BJ, Wiechert W, Oldiges M, Noh K, Noack S: **Comparative 13C metabolic flux analysis of pyruvate dehydrogenase complex-deficient, L-valine-producing Corynebacterium glutamicum**. *Applied and environmental microbiology* 2011, **77**:6644-6652.
57. Ma C, Zhang L, Dai J, Xiu Z: **Relaxing the coenzyme specificity of 1,3-propanediol oxidoreductase from Klebsiella pneumoniae by rational design**. *J Biotechnol* 2010, **146**:173-178.
58. Hoelsch K, Suhrer I, Heusel M, Weuster-Botz D: **Engineering of formate dehydrogenase: synergistic effect of mutations affecting cofactor specificity and chemical stability**. *Appl Microbiol Biotechnol* 2012.
59. Lan EI, Liao JC: **ATP drives direct photosynthetic production of 1-butanol in cyanobacteria**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:6018-6023.
60. Lee HC, Kim JS, Jang W, Kim SY: **High NADPH/NADP+ ratio improves thymidine production by a metabolically engineered Escherichia coli strain**. *Journal of biotechnology* 2010, **149**:24-32.
61. Saha BC, Racine FM: **Biotechnological production of mannitol and its applications**. *Applied microbiology and biotechnology* 2011, **89**:879-891.
62. Lo Piccolo L, De Pasquale C, Fodale R, Puglia AM, Quatrini P: **Involvement of an alkane hydroxylase system of Gordonia sp. strain SoCg in degradation of solid n-alkanes**. *Applied and environmental microbiology* 2011, **77**:1204-1213.
•63. Wang Y, Huang W, Sathitsuksanoh N, Zhu Z, Zhang YH: **Biohydrogenation from biomass sugar mediated by in vitro synthetic enzymatic pathways**. *Chemistry & biology* 2011, **18**:372-380.

The article demonstrates an advance in in vitro technology using a multienzyme strategy to oxidize sugar and capture the reducing equivalents to form another product.

Figure 1. The illustration shows the different kinds of cofactor related manipulations, either at the individual enzyme level or at the cellular biocatalyst level, that are made to enhance formation of a desired product.




Substrates
e- carriers
Products
Alkanes
Fatty acids
Sugars
Formate
CO2 + e-
Fd
H2
NAD(P)H
FAD
Q
Cyt
Hydrogen
Alcohols, organic acids & amino acids
Polysaccharides
CO2