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Citation for final published version:

Angelastro, Antonio ORCID: <https://orcid.org/0000-0002-4023-7411>, Dawson, William M., Luk, Louis Y. P. ORCID: <https://orcid.org/0000-0002-7864-6261> and Allemann, Rudolf K. ORCID: <https://orcid.org/0000-0002-1323-8830> 2017.  
A versatile disulfide-driven recycling system for NADP+ with high cofactor turnover number. ACS Catalysis 7 (2) , pp. 1025-1029.  
10.1021/acscatal.6b03061 file

Publishers page: <http://dx.doi.org/10.1021/acscatal.6b03061>  
<<http://dx.doi.org/10.1021/acscatal.6b03061>>

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# A Versatile Disulfide-Driven Recycling System for NADP<sup>+</sup> with High Cofactor Turnover Number

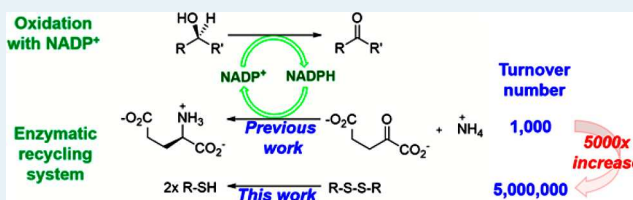
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## Supporting Information

**ABSTRACT:** NADP<sup>+</sup>-dependent enzymes are important in many biocatalytic processes to generate high-value chemicals for the pharmaceutical and food industry; hence, a cost-effective, efficient, and environmentally friendly recycling system for the relatively expensive and only marginally stable enzyme cofactor NADP<sup>+</sup> offers significant benefits. NADP<sup>+</sup> regeneration schemes have previously been described, but their application is severely limited by the low total turnover numbers (TTN) for the cofactor. Here, we report a glutathione-based recycling system that combines glutaredoxin from *E. coli* (EcGRX) and the glutathione reductase from *S. cerevisiae* (ScGR) for NADP<sup>+</sup> regeneration. This system employs inexpensive latent organic disulfides such as oxidized cysteine or 2-hydroxyethyl disulfide (HED) as oxidizing agents and allows NADP<sup>+</sup> recycling under both aerobic and anaerobic conditions with a TTN in excess of  $5 \times 10^5$ , indicating that each regeneration cycle is 99.9998% selective toward forming the cofactor. Accordingly, for each 1 mol of product generated, less than \$0.05 of cofactor is needed. Finally, the EcGRX/ScGR pair is compatible with eight enzymes in the guanosine monophosphate (GMP) biosynthetic pathway, giving the corresponding isotopically labeled nucleotide in high yield. The glutathione-based NADP<sup>+</sup> recycling system has potential for biocatalytic applications in academic and industrial settings.

**KEYWORDS:** biocatalysis, biosynthesis, cofactor/coenzyme recycling, enzyme oxidation, biotechnology



## INTRODUCTION

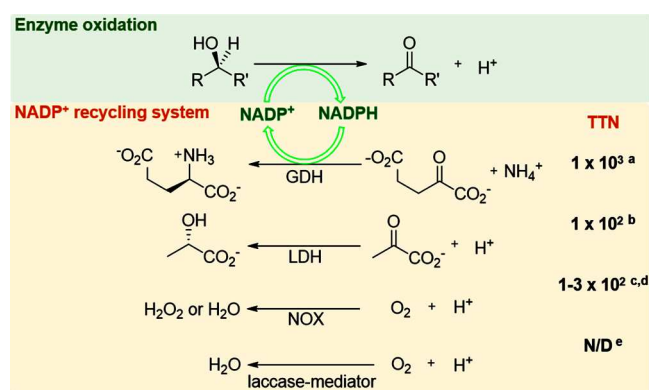
Refining the performance of enzyme-catalyzed redox processes remains at the frontier of biocatalysis research<sup>1,2</sup> and is of central importance to the development of sustainable chemical production processes.<sup>3</sup> Oxidoreductases are a large group of enzymes,<sup>4</sup> which due to their good catalytic efficiency, general applicability, and nontoxic nature have been used widely in industrial processes, ranging from kilogram-scale chiral resolutions<sup>5</sup> to intricate syntheses of pharmaceuticals.<sup>6,7</sup> Since oxidoreductases almost exclusively require the use of structurally complex and expensive cofactors such as nicotinamide adenine dinucleotide phosphate NADP(H), cost-efficient recycling schemes have been developed. Currently, there are a number of in situ enzymatic systems that efficiently recycle NADPH, including engineered glucose/glucose 6-phosphate dehydrogenase,<sup>8,9</sup> formate dehydrogenase,<sup>10,11</sup> and phosphite dehydrogenase.<sup>12</sup> These recycling schemes have been extensively used in NADPH-dependent enzymatic reductions, such as the production of the HMG-CoA reductase inhibitor atorvastatin.<sup>6</sup> On the other hand, the current options for the regeneration of the corresponding oxidized cofactor NADP<sup>+</sup> are limited,<sup>13</sup> even though enzymatic oxidation processes play an important role in contemporary synthetic chemistry and should be fully integrated into chemical manufacturing.<sup>14–23</sup> Hence, there is a pressing need for the design of a flexible, noninterfering NADP<sup>+</sup> recycling system.<sup>24–27</sup>

The industrial relevance of any cofactor-regenerating scheme is primarily assessed by its total turnover number (TTN), which is defined as the total number of moles of product formed per mole of cofactor.<sup>2,28,29</sup> While the minimal TTN required for each specific cofactor depends on its cost and the value of the product yielded from the biocatalytic process, it is generally anticipated that the TTN should fall in the range of  $10^4$ – $10^6$  to be economically viable on an industrial scale.<sup>2,28,29</sup> This generalization is applicable to the use of NADP<sup>+</sup>; its current cost is ~\$22k per mole (Table S1 in the Supporting Information) and it cannot be used as a stoichiometric reagent for large-scale synthetic reactions. Additionally, in order to be practical, the cofactor-recycling process needs to be specific yet compatible with the designed chemical reactions. Enzymes are often characterized by high chemical selectivity and specificity, and they are biodegradable, so that enzymatic methods to regenerate a cofactor have significant advantages.<sup>29</sup> Therefore, it is perhaps surprising that there are only a few published enzymatic NADP<sup>+</sup> recycling schemes, but all of these have drawbacks that limit their applications. The glutamate dehydrogenase (GDH) system is the most widely used method to regenerate NADP<sup>+</sup> (Figure 1).<sup>25,30–32</sup> In this system, a stoichiometric amount of ammonium  $\alpha$ -ketoglutarate is

Received: October 26, 2016

Revised: December 3, 2016

Published: December 19, 2016



**Figure 1.** Currently available  $\text{NADP}^+$ -recycling systems and their corresponding total turnover number (TTNs): glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), NADPH oxidase (NOX) and laccase/mediator system. N/D = not determined. For a–e see refs 25 and 34–37, respectively.

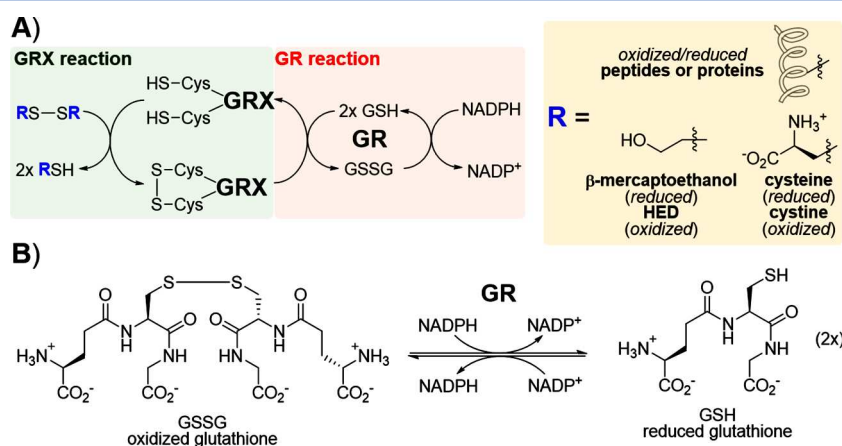
converted to glutamate to regenerate the oxidized cofactor. A major drawback of this system is the modest activity of GDH whose maximum steady-state turnover number of approximately  $40 \text{ s}^{-1}$  restricts the maximal TTN to less than  $1 \times 10^3$ ,<sup>25,33</sup> significantly lower than the value required for an economically viable process. Furthermore,  $\alpha$ -ketoglutarate contains a highly electrophilic carbonyl group that is prone to cross reaction with other components of the system, resulting in the production of unwanted byproduct(s); in addition, the byproduct glutamate has been shown to complicate product isolation.<sup>29</sup> Consequently, other regeneration schemes, including the D-lactate dehydrogenase (LDH),<sup>34</sup> NADPH oxidase (NOX),<sup>35,36</sup> and the laccase/mediator system,<sup>37</sup> have been developed. The calculated TTNs of the LDH and NOX systems are rather low, however, and range from  $1 \times 10^2$  to  $3 \times 10^2$ ,<sup>35,36</sup> whereas the laccase/mediator system has not been optimized for  $\text{NADP}^+$  and its TTN has not been determined.<sup>37</sup> Moreover, both NOX and the laccase/mediator system are oxygen-dependent and hence not suitable for most anaerobic biocatalysts such as cytochrome P450 dependent enzymes. Hence, a recycling system that uses simple, inert, latent oxidizing reagents and is characterized by high TTNs is urgently required.

In nature,  $\text{NADP}^+$  can be generated by coupling to enzymatic disulfide bond reduction.<sup>38</sup> One major example of this

chemistry is the glutathione (GSH) reductase system, which plays an essential role in maintaining a reducing environment within the cell.<sup>39</sup> In plants, mammals, some bacteria, and archaea, this system is composed of a pair of enzymes, glutaredoxin (GRX) and glutathione reductase (GR), and a pair of redox reagents, glutathione (GSH) and its oxidized counterpart GSSG.<sup>40</sup> GRX contains catalytic cysteine residue(s), which are used to reduce organic disulfide bonds.<sup>41,42</sup> The resulting oxidized disulfides within the active site of GRX are then recycled by reduced glutathione to regenerate the enzyme in its reducing form (Figure 2A). In turn, to maintain a sufficient pool of glutathione, GSSG is reduced by GR, an enzymatic process that oxidize a stoichiometric amount of  $\text{NADPH}$  to  $\text{NADP}^+$  (Figure 2B).

The glutathione coupling system presents itself as an ideal surrogate for a  $\text{NADP}^+$  recycling system. Glutathione and its oxidized counterpart are relatively inert in comparison to oxygen and  $\alpha$ -ketoglutarate and are thus more compatible with most biocatalytic processes. Importantly, while GR is directly responsible for generating  $\text{NADP}^+$ , GRX is capable of generating GSSG by reducing a wide range of disulfide species from oxidized proteins to small, inexpensive organic molecules such as 2-hydroxyethyl disulfide (HED or oxidized  $\beta$ -mercaptoethanol) and cystine (Figure 2A).<sup>43,44</sup> Such substrate promiscuity can be exploited in that the oxidizing agent can be carefully chosen to suit a particular transformation and complications in product isolation can be minimized. The reduced thiol byproduct (e.g.,  $\beta$ -mercaptoethanol and cysteine) will also protect the substrates and biocatalysts from oxidative damages. This system requires the use of two cooperating enzymes, and it provides clear competitive advantages over what is offered by all of the currently used systems.

We have developed a glutathione-based recycling system that employs the enzymes GR and GRX and is capable of using small, latent organic disulfides as oxidizing reagents to regenerate  $\text{NADP}^+$  with a maximal TTN in excess of  $5 \times 10^5$ , a value noticeably higher than those of the existing  $\text{NADP}^+$  recycling system and acceptable at an industrial standard. This system is compatible with various enzymes and can be used to generate important sugar intermediates such as 6-phosphoglucuronate (6-PG), ribulose 5-phosphate (Ru5P), and GMP.

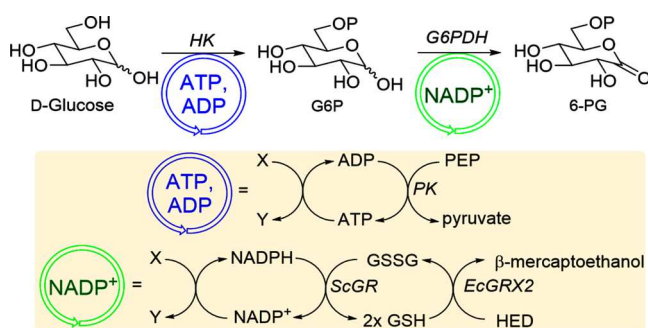


**Figure 2.** (A) Coupling of the glutaredoxin (GRX) and glutathione reductase (GR) reactions and (B) the disulfide bond reduced in the GR reaction.

## RESULTS AND DISCUSSION

Glutathione reductase from *S. cerevisiae* (ScGR) and glutaredoxin 2 from *E. coli* (EcGRX2) were chosen to construct a glutathione recycling system.<sup>45–48</sup> ScGR is commercially available at a reasonable price with a relatively high turnover number  $k_{\text{cat}}$  of 240 s<sup>-1</sup> at pH 7.0,<sup>46</sup> while recombinant EcGRX2 can be produced in large quantities by expression in *E. coli* (Table S2 in the Supporting Information),<sup>47</sup> which yields an enzyme with a high  $k_{\text{cat}}$  value ( $\sim 550$  s<sup>-1</sup>).<sup>47</sup> On the basis of their kinetic parameters, the functional pair of EcGRX2 and ScGR should recycle NADP<sup>+</sup>  $8.64 \times 10^5$  times within 1 h, resulting in a high TTN.

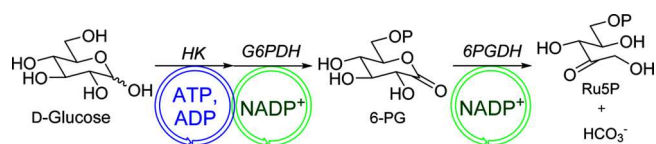
Oxidized sugar intermediates are often used in both traditional chemoenzymatic synthesis and contemporary synthetic biology developments.<sup>31,32,49–52</sup> Hence, the efficiency of the ScGR/EcGRX2 recycling system was tested for the production of 6-phosphogluconate (6-PG), a common intermediate in glucose-utilizing metabolic pathways such as the pentose-phosphate<sup>53</sup> and Entner–Doudoroff pathways.<sup>54</sup> To this end D-glucose was incubated with the commercially available enzymes hexokinase (HK), which catalyzes the addition of phosphate to C-6 of D-glucose, and glucose 6-phosphate dehydrogenase (G6PDH) (Figure 3). The gluta-



**Figure 3.** Conversion of D-glucose to 6-phosphogluconate by hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PDH). The ATP/ADP and NADP<sup>+</sup> recycling systems are composed of pyruvate kinase (PK), glutathione reductase (ScGR), and glutaredoxin (EcGRX2).

thione coupling enzymes ScGR and EcGRX2, the recycling reagent GSH, and the latent oxidizing reagent HED were added to regenerate NADP<sup>+</sup>. A downfield shift from  $\sim 96$  and 92 ppm (64%  $\beta$  and 36%  $\alpha$  anomer, respectively) of the resonance of C-1 to  $\sim 178$  ppm in the <sup>13</sup>C NMR spectrum showed that the substrate was efficiently converted to 6-PG (Figure S1 in the Supporting Information). The maximum TTN achieved with the enzyme pair ScGR/EcGRX2 recycling system was  $5 \times 10^5$  (Table S3 in the Supporting Information), which together with the low cost of HED (\$3.27 per mole) shows that ScGR/EcGR can form a NADP<sup>+</sup> recycling system that is commercially viable on an industrial scale. This system is also compatible with pyruvate kinase, which is used to recycle ATP in the first step of the pathway.<sup>50</sup> Cysteine, though sparingly soluble, can also act as a latent oxidizing agent; it efficiently recycled NADP<sup>+</sup> and converted glucose to 6PG (see Supporting Information).

To further examine the potential of the ScGR/EcGRX2 recycling system, an additional NADP<sup>+</sup>-dependent enzyme, 6-phosphogluconate dehydrogenase (6-PGDH), on the pathway to guanosine monophosphate (GMP) was added (Figure 4). 6-PGDH catalyzes the conversion of 6-PG to ribulose 5-



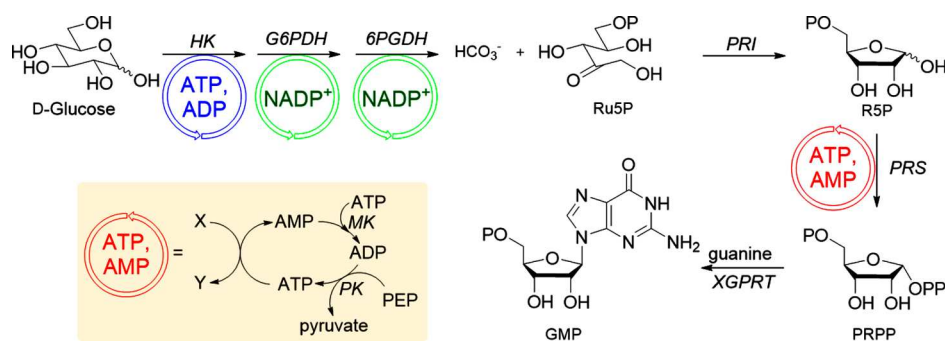
**Figure 4.** Conversion of D-glucose to ribulose 5-phosphate (Ru5P) by hexokinase (HK), glucose 6-phosphate dehydrogenase (G6PDH), and 6-phosphogluconate dehydrogenase (6-PGDH). Details of the ATP and NADP<sup>+</sup> recycling systems are described in Figure 3 and in the text.

phosphate (Ru5P).<sup>53</sup> Two characteristic downfield chemical shifts corresponding to C-2 of Ru5P ( $\delta$  213 ppm) and the byproduct bicarbonate ( $\delta$  160 ppm) indicated complete oxidative decarboxylation (Figure S2 in the Supporting Information).

The ScGR/EcGRX2 recycling system was then combined with eight enzymes to produce in a one-pot reaction <sup>13</sup>C-labeled GMP,<sup>31,32,49</sup> which is used in many biochemical studies, including structural and functional analysis of RNA<sup>51,55,56</sup> and metabolomic investigations.<sup>57–59</sup> The above Ru-5P biosynthetic pathway was extended by incorporating phosphoriboisomerase (PRI) and ribose-phosphate pyrophosphokinase (PRS), which catalyze the isomerization of Ru-5P and the addition of pyrophosphate to the C-1 position, respectively (Figure 5). The resulting intermediate phosphoribose pyrophosphate (PRPP) is chemically labile at room temperature and was transformed into GMP in situ with xanthine-guanine phosphoribosyl transferase (XGPRT). Guanine is only sparingly soluble at neutral pH, and while it has been suggested previously that the reaction can proceed as a slurry,<sup>49</sup> the heterogenous nature of the reaction led to poor reproducibility and increased reaction times of up to 1 day. Hence, guanine was dissolved at increased pH (50 mM KOH), where it shows good solubility, and added in a dropwise fashion to the reaction mixture. Under these conditions 70–80% GMP (Figure S3 in the Supporting Information) was reproducibly obtained within 2 h from D-glucose. Because PRPP formation requires ATP, which is converted to AMP as the byproduct, myokinase (MK) was included to generate ADP, which is subsequently converted to ATP in a PK-catalyzed reaction that uses phosphoenol pyruvate (PEP) as the phosphate donor. Together, this work illustrated that ScGR/EcGRX2 pair is compatible with eight enzymes, including six biosynthetic enzymes and two ATP-recycling enzymes.

## CONCLUSIONS

Developing an efficient and highly compatible NADP<sup>+</sup> recycling process is an essential step toward integrating enzymatic oxidation into the production of high-value chemicals.<sup>14,15</sup> With small, inert organic disulfides as oxidizing agents, the ScGR/EcGRX2 pair can regenerate NADP<sup>+</sup> up to  $5 \times 10^5$  times, well in excess of the best TTNs of  $1 \times 10^3$  reported so far.<sup>25</sup> Accordingly, each cycle of regeneration is 99.9998% selective for the formation of the active cofactor. With such selectivity, the cost of NADP<sup>+</sup> can be reduced to < \$0.05 per mole of product formed. In practice, the ScGR/EcGRX2 pair was shown to be compatible with several biosynthetic enzymes, including ATP-recycling kinases, in the production of crucial synthons such as 6-PG, 5RuP, and GMP. This system also offers an attractive synthetic pathway for the production of isotopically labeled compounds. In addition, GRX is able to produce useful “byproducts” such as  $\beta$ -mercaptoethanol, which can protect enzymes, reagents, and



**Figure 5.** Conversion of D-glucose to GMP by hexokinase (HK), glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), phosphoribose isomerase (PRI), ribose-phosphate pyrophosphokinase (PRS) and xanthine-guanine phosphoribosyl transferase (XGPRT). The ATP/AMP recycling system composed of myokinase (MK), pyruvate kinase (PK), and phosphoenol pyruvate (PEP) is illustrated. The ATP/ADP and NADP<sup>+</sup> recycling systems are described in Figure 3 and in the text.

products from unwanted oxidative damage. GRX has previously been shown to enhance protein stability by preventing proteins from oxidative misfolding and aggregation.<sup>60–62</sup> The system developed here is atom efficient in that there are reagents serving more than one role. Finally, unlike other NADP<sup>+</sup> recycling schemes, including the NOX and laccase/mediator systems, the ScGR/EcGRX2 pair is oxygen-independent and is functional in aerobic as well as anaerobic environments and is therefore compatible with oxygen-sensitive biocatalysts such as P450s. The ScGR/EcGRX2 based NADP<sup>+</sup> recycling systems is superior to all existing methods for cofactor regeneration and offers many advantages for commercial and academic users.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.6b03061.

Full experimental procedures and tabulated experimental data for TTN (PDF)

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The authors thank Dr. James R. Williamson at the Scripps Research Institute for his kind gift of the *prsA* gene in a pET22-HT plasmid. This work was supported by Cardiff University through a President's Research Scholarship to A.A. and the UK's Biotechnology and Biological Sciences Research Council (BBSRC) through grants BB/J005266/1 and BB/L020394/1 to R.K.A.

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