Cofactor NAD(P)H Regeneration: How Selective Are the Reactions?

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Abstract

The regeneration of NAD(P)H can be achieved *via* catalysis, but the unequivocal determination of the species involved has yet been fully realized. The current analytical methods of reactant/product analysis based on UV-vis spectroscopy, enzymatic assays, NMR spectroscopy, and HPLC are critically examined here with suggestions for future development.

Keywords: Cofactor regeneration; analytical methods; conversion; selectivity; mass balance.

Reduced Nicotinamide Adenine Dinucleotide (NAD(P)H) is a critical reductant in biochemistry, participating in numerous biological transformations (Box 1). Regeneration of NAD(P)H (i.e., the reduction of NAD(P)$^+$ to NAD(P)H) has thus far taken on six different forms: enzymatic, chemical, electrocatalytic, photocatalytic, homogeneous, and heterogeneous regeneration, and is a topic of regular reviews.

Albeit a topic of significant recent research, thorough and unequivocal quantification of 1,4-NAD(P)H and other products has not been fully achieved. Upon the conversion of NAD(P)$^+$ to NAD(P)H, four possible isomers may be formed: 1,2-; 1,4-; and 1,6-NAD(P)H; and the NAD$_2$ dimers, with the former three suffering from possible further decay and 1,4-NAD(P)H being the only enzymatically active isomer (i.e., the target product, Figure I). All these isomers contain chromophores and exhibit λ_{max} at 395, 340, 345, and ~340 nm respectively, whereas their common adenine group exhibits λ_{max} at 260 nm (Figure I). Considering this, it is surprising that a significant proportion of literature uses ultraviolet-visible (UV-vis) spectroscopy to track the absorbance at 340 nm solely (Figure 1A, or sometimes the ratio of the absorbance at 260 nm and 340 nm [5]) to quantify the concentration of 1,4-NAD(P)H [7]. Using the λ_{max} at 340 nm and applying the molar absorption coefficient for 1,4-NAD(P)H will undoubtedly lead to erroneous results when the reaction is unselective. Specifically, the molar absorption coefficients for 1,4-, 1,6- and the dimer are 6.22, 6.5, and 6.5-7.8 L mol^{-1} cm^{-1} [8], respectively, so the activity (and selectivity) of the regeneration system will be overestimated. Given the booming interest and effort in this field [3-12], it is crucial that the concentrations of the products and unreacted reactant are unambiguously quantified.

In addition to using UV-vis spectroscopy, it is also fairly common practice to use a biological assay. A direct assay typically consists of a substrate (e.g., acetaldehyde or CO_2), a sample (the NAD mixture from a reaction) and an enzyme (e.g., alcohol dehydrogenase (ADH) or formate dehydrogenase (FDH)) that only utilizes the enzymatically active 1,4-NAD(P)H (Figure 1B). Tracking the initial absorbance at 340 nm and monitoring the consumption as the reaction progresses (typically in comparison with a commercial 1,4-NAD(P)H sample) will determine the concentration of 1,4-NAD(P)H in the system (Figure 1C) [3,9]. Although using a combination of UV-vis spectroscopy and biological assays is an improvement on relying on UV-vis spectroscopy alone, it only correctly measures the amount of 1,4-NAD(P)H formed and

still does not provide a complete picture of what is occurring when other products are being generated. Often, authors tend to speculatively suggest some isomers have been formed, but this has not clarified the reaction mechanism or helped to develop better catalytic materials.

A further improvement to using a direct biological assay with UV-vis spectroscopy is using combined biological assays. Unlike a direct biological assay, a cyclic assay makes use of two enzymes and two substrates, catalyzing the reduction of one substrate (e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) using 1,4-NADH and oxidizing the other substrate (e.g., lactate) using NAD^+ (Figure 1D). Tracking the formation of one of the products will give the total concentration of 1,4-NADH and NAD^+ in the system (Figure 1E). Using this idea, we have recently developed an analytical method that can track NAD^+ (for conversion determination) and the monomers of NADH using a combination of UV-vis spectroscopy (340, 395 and 565 nm) and two biological assays (for 1,4-NADH and the total of 1,4-NADH and NAD^+) [7]. Although this is a facile and relatively easy method to perform in the laboratory, it cannot examine the potential formation of NAD_2 dimers.

The use of ^{1}H NMR to assess the catalytic performance of a regeneration method has been used to varying degrees of success. A combination of UV-vis spectroscopy, direct biological assays, and ^{1}H NMR has been used to deduce the activity and selectivity towards 1,4-NAD(P)H. Typically, the absorbance at 340 nm is used to calculate an 1,4-NAD(P)H concentration, a direct assay is used to determine the enzymatically active component, and ^{1}H NMR is used to show the appearance of a peak at 6.96 ppm which is characteristic of 1,4-NADH (Figure 1F) [10]. It would be reasonable then to claim that this regeneration system is fully selective; however, this evidence is not fully conclusive. Because 100% conversion is frequently not achieved (i.e., the mass balance is not closed), it cannot be said with complete certainty that other products (e.g., the enzymatically inactive isomer 1,2-NAD(P)H) have not been formed. This is because the concentrations of reactant used in the literature are typically very low (< 1 mM) and ^{1}H NMR signals are rather weak for potential side product formation (with even lower concentrations) and the majority of publications show only selectively part of the spectrum, where a full picture could not be assessed. Moreover, 1,2-NAD(P)H is known for its instability, especially in pH ~7 phosphate buffers in which most reactions are performed in. So it is possible that this isomer could be formed and would go undetected by the

quantification method. Similarly, misleading results may be obtained using only the integrals (I) of the signals at 9.33 ppm (NAD$^+$) and 6.96 ppm (1,4-NADH) according to Yield (%) = $I_{6.96}/(I_{6.96}+I_{9.33})\times100\%$ [11].

There have been some attempts on using high-performance liquid chromatography (HPLC) to monitor the progress of the regeneration reaction. However, to the best of our knowledge, it has thus far been limited to the quantification of NAD(P)$^+$ and NAD(P)H generally, and not the specific isomers. For example, some authors have used HPLC in combination with UV-vis spectroscopy to track the formation of NAD(P)H *via* the increase in the absorbance at 340 nm (UV-vis spectroscopy) and the conversion of NAD$^+$ by the change in the ratio of NAD$^+$/NADH (HPLC) [12]. Although this method provides a good indication of the conversion of the reaction by means of tracking the reactant consumption, it still does not reveal the true selectivity of the regeneration method. Beaupre and colleagues reported an HPLC method that could separate and quantify both NAD$^+$ and 1,2-, 1,4- and 1,6-NADH (Figure 1G) [8]. Although the paper is not investigating the regeneration of 1,4-NAD(P)H, it shows the feasibility of using HPLC to separate and quantify the different NADH isomers, albeit except for the dimers, which was not attempted. From a reaction engineering perspective, mass/materials balance is of great importance in studying any reactive systems. Without such fundamental information, it would be impossible to understand reaction mechanisms and design better catalysts. Although combined UV-vis spectroscopy and biological assays can realize the same level of product qualifications as the use of HPLC (i.e., dimers not detected), we suggest HPLC be used as a routine tool for this particular application as it allows more sensitive detection of potential by-product formation and room for method development.

In summary, the dependence on UV-vis spectroscopy alone affords inaccurate and often misleading results. As such, it is not a good descriptor of catalytic performance of the NAD(P)H regeneration systems. The use of ^{1}H NMR together with UV-vis spectroscopy provides a clearer picture still, allowing the identification of the unselective isomers, but as of now, quantification is still unattainable. Combining UV-vis spectroscopy and two biological assays or the use of HPLC is the most thorough and unequivocal form of analysis, providing quantitative analysis of both reactant, desired product, and by-products. Although both procedures have yet involved the quantification of any dimeric species, it is hoped that in the

near future with the help of analytical chemists and engineers, all species can be fully accounted for to reveal comprehensive reaction paths for the design of more efficient and cleaner strategies of cofactor regeneration for sustainable biosynthesis.

Acknowledgements

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Box 1. Biocatalytic transformations and the necessity of NAD(P)H regeneration.

Biocatalysis is playing an increasingly important role industrially, capable of performing complex chemical transformations under relatively benign reaction conditions. Due to their inherently high activity, selectivity, and specificity, enzymes are ideal candidates for a wide variety of reactions [1]. Among the enzymes used in biosynthesis, oxidoreductases are the largest class of commercially applied enzymes and are capable of performing a vast array of oxidative and reductive chemical transformations [1], involving the production of chiral intermediates, amino acids, steroids, and other pharmaceutically important chemicals, as well as the fixation and subsequent conversion of molecules such as CO_2 [2-6]. These transformations are dependent on NAD(P)H, a cofactor that acts as a source of hydride and is subsequently oxidised to NAD(P)$^+$ during the reaction (Figure I). Unlike the enzymes themselves, the cofactor NAD(P)H is stoichiometrically consumed and due to its high cost ($2600/mol), regeneration of the cofactor is necessary for its practical use [7].
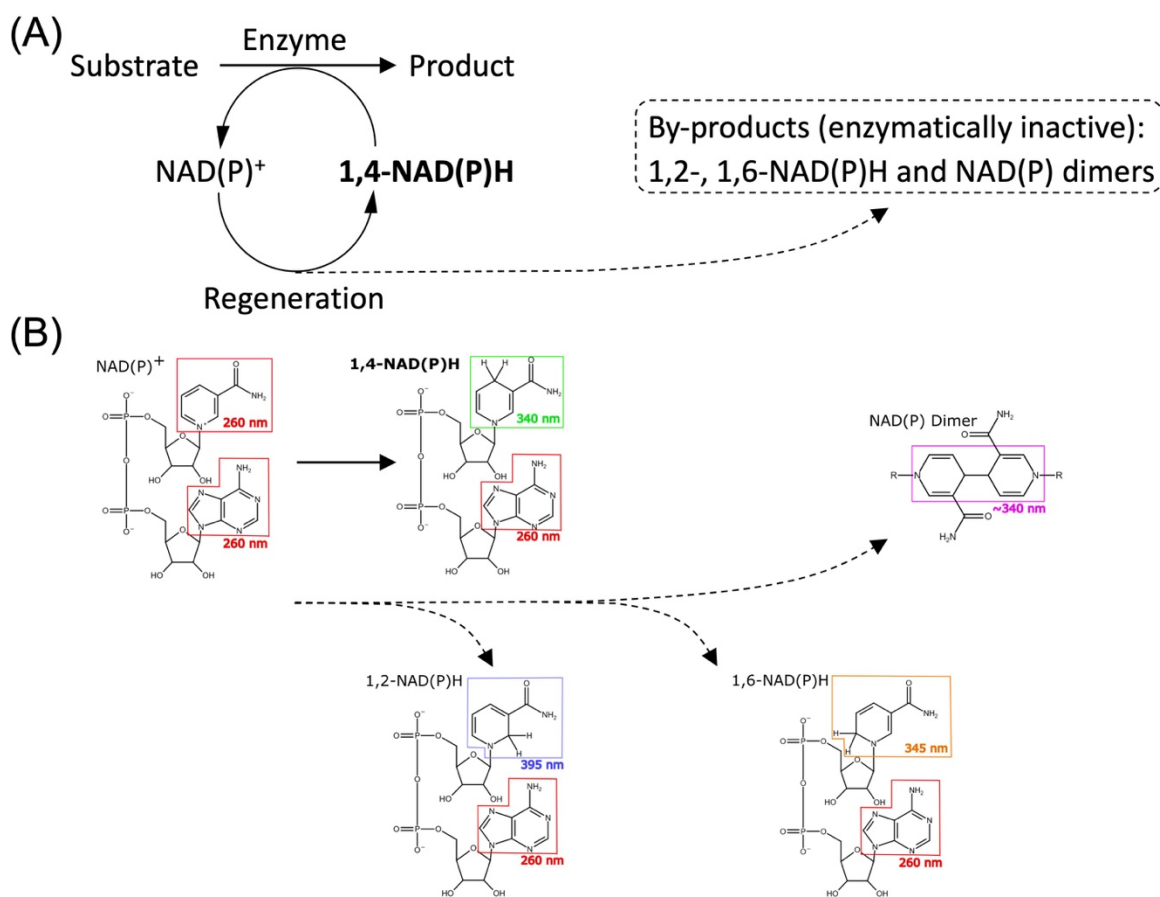

Figure I. Schematic representation of enzymatic reaction using cofactor 1,4-NAD(P)H with *in situ* regeneration. (A) Enzymatic reduction consuming 1,4-NAD(P)H and its regeneration to various products. (B) Structures of NAD(P)H isomers and their associated absorption maxima, R = adenosine diphosphoribose.
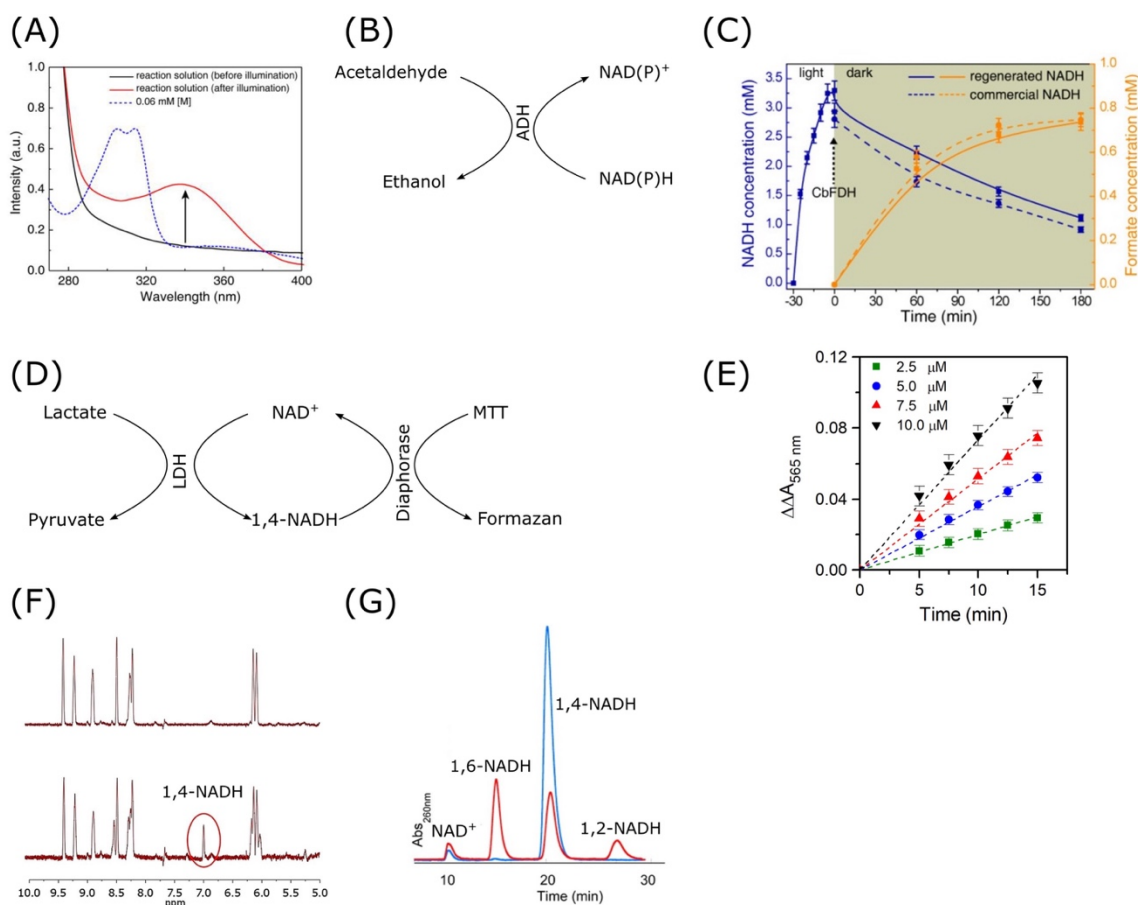
Figure 1. The analytical methods currently used for 1,4-NADH quantification. (A) Representative spectra showing the increase in the UV-vis spectroscopy absorbance at 340 nm as the reaction progresses, M, $[Cp*Rh\text{-}(bpy)H_2O]^{2+}$ ($Cp*$ = pentamethylcyclopentadienyl, bpy = 2,2'-bipyridyl), is a typical organic mediator used in photocatalytic/electrocatalytic regeneration. Adapted with permission from [3]. (B) An example of a direct biological assay. (C) Comparison of commercial 1,4-NADH and regenerated NADH when used in a direct biological assay (involving CO_2 to formate reaction, CbFDH: FDH from *Candida boidinii*). Adapted with permission from [3]. (D) An example of a cyclic biological assay (LDH: lactate dehydrogenase). (E) A time on stream profile showing the increase in the absorbance at 565 nm, corresponding to formation of formazan in a biological cyclic assay, with different total concentrations of 1,4-NADH and NAD^+. Adapted with permission from [7]. (F) ^{1}H NMR profile showing the formation of 1,4-NADH. Adapted with permission from [10]. (G) The separation of NAD^+ and the monomeric isomers of 1,4-NADH in HPLC. Adapted with permission from [8].