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Research review paper

Improved strategies for electrochemical 1,4-NAD(P)H_2 regeneration: A new era of bioreactors for industrial biocatalysis

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ABSTRACT

Industrial enzymatic reactions requiring 1,4-NAD(P)H_2 to perform redox transformations often require convoluted coupled enzyme regeneration systems to regenerate 1,4-NAD(P)H_2 from NAD(P) and recycle the cofactor for as many turnovers as possible. Renewed interest in recycling the cofactor via electrochemical means is motivated by the low cost of performing electrochemical reactions, easy monitoring of the reaction progress, and straightforward product recovery. However, electrochemical cofactor regeneration methods invariably produce adventitious reduced cofactor side products which result in unproductive loss of input NAD(P). We review various literature strategies for mitigating adventitious product formation by electrochemical cofactor regeneration systems, and offer insight as to how a successful electrochemical bioreactor system could be constructed to engineer efficient 1,4-NAD(P)H_2-dependent enzyme reactions of interest to the industrial biocatalysis community.

1. Introduction

Nicotinamide adenine dinucleotide, commonly written as NAD^+/NADH, and its phosphorylated variants nicotinamide adenine dinucleotide phosphate, $NADP^+$/NADPH, are biochemical redox cofactors responsible for the transfer of electrons and protons in biological redox reactions. Formally, this allows the transfer of a molecule of hydrogen between metabolic intermediates. The oxidized form of the cofactor accepts electrons, balanced by protons, and in doing so can assume a number of isomeric structures as well as a dimer. These are shown in Fig. 1.

The naming convention for the species in Fig. 1, however, is not consistent in the literature, and the common NAD(P)+ terminology causes confusion when considering electrochemical reactions. As explained further in Section 2.1, the oxidized form will be termed NAD(P), and the biologically active reduced form will be termed 1,4-NAD(P)H_2. The C-2 and C-6 isomers of the reduced form will be termed 1,2-NAD(P)H_2 and 1,6-NAD(P)H_2, respectively, and the more general term NAD(P)H_2 will be used when specifying a particular isomer of the reduced cofactor is not necessary.

Nearly 20% of known oxidoreductases require cofactors to supply stoichiometric quantities of reducing equivalents. For nearly 700 known classes of redox enzymes (Ullah et al., 2015), 1,4-NAD(P)H_2 is the required cofactor. Since the total intracellular pool of both the oxidized and reduced forms of the cofactor is fairly low, the cofactor undergoes reduction and oxidation turnovers on the order of 10^3 to 10^5 in typical reactions (Angelastro et al., 2017; Ströhle et al., 2016). The current technology for regeneration of 1,4-NAD(P)H_2, exemplified in Fig. 2, includes 1) sacrificial substrates, e.g., a non-valuable alcohol is present in the reaction mixture and is simultaneously oxidized to an aldehyde (or acid). This was used by Whitesides and Wong (1982), Wong and Whitesides (1983) to utilize all of the reducing power

Abbreviations: NAD, oxidized nicotinamide adenine dinucleotide; NADP, oxidized nicotinamide adenine dinucleotide phosphate; $NADH_2$, reduced nicotinamide adenine dinucleotide (any isomer); $NADPH_2$, reduced nicotinamide adenine dinucleotide phosphate (any isomer); $[NAD]_2$, nicotinamide adenine dinucleotide dimer; $[NADP]_2$, nicotinamide adenine dinucleotide phosphate dimer; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; 1,*n*-$NADH_2$, reduced 1,*n*-nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; HPLC, high performance liquid chromatography; MSE, saturated mercury sulfate electrode; mt, metric tonne; SCE, saturated calomel electrode; Poly-His, polyhistidine; HRP, horseradish peroxidase

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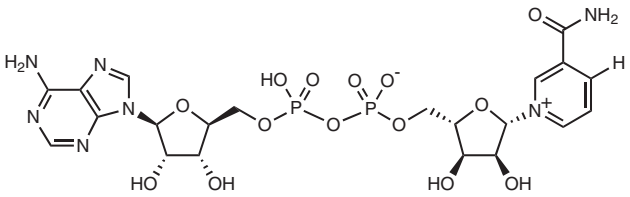
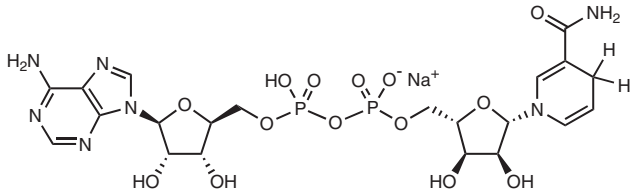
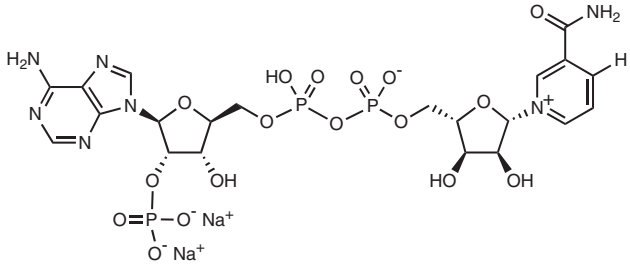


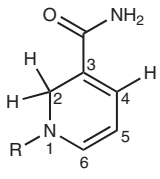
NAD (zwitterion)



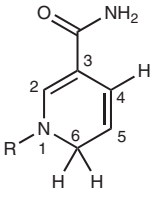
1,4-NADH$_2$ (sodium salt)



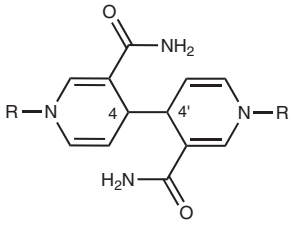
NADP (disodium salt)
pKa$_1$ = 3.9; pKa$_2$ = 6.1



1,2-NADH$_2$



1,6-NADH$_2$



4,4'-dimer; [NAD]$_2$

R =

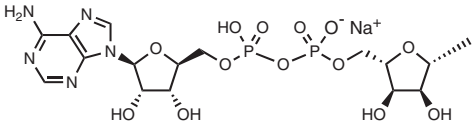


Fig. 1. The numbered chemical structures for NAD(P) cofactor material. The isomers of NAD(P)H and a representative example of an NAD dimer are also shown here.

available by employing three enzymes and oxidizing methanol all the way to CO_2; 2) whole cell methods, using resting cells or active fermentations, in which a nutrient such as glucose is metabolized to provide reducing equivalents (Zaks and Dodds, 1997); and 3) combined chemoenzymatic methods (Eikeren, 1989). While all of these methods can be used, they require handling whole cells, multiple enzymes, and/or other molecules, such as the sacrificial substrate. Direct regeneration of the 1,4-NAD(P)H$_2$ would be simpler and less expensive if electrons and protons could be provided directly to the oxidized form of NAD(P). Electrochemical methods, under specific reaction conditions, are able to achieve exactly that.

Formally, the reduced form of the cofactor, NAD(P)H$_2$, carries one molecule of H_2, which is provided to the reaction being catalyzed by the enzyme requiring reducing power. Reduced cofactor is thus the chemical equivalent of hydrogen, and competition with other sources of hydrogen must be considered when planning a chemical process that could use NAD(P)H$_2$.

Historically, the regeneration of 1,4-NAD(P)H$_2$ by electrochemical means has been well reported, but has never attained wide-spread practical application due to the barrier caused by the formation of biologically inactive isomers of NAD(P)H$_2$ and dimers of NAD(P). The recognition of these well-known chemistries has held back the application of electrochemical 1,4-NAD(P)H$_2$ regeneration to industrial processes. As discussed in Section 4.2, strategies for dimer mitigation have been recognized for decades, but no similar approaches for isomer mitigation were known. However, naturally occurring enzymes (renalases) were recently discovered, as discussed in Section 4.2.1, in both eukaryotic and prokaryotic cells whose primary function is the recovery of the biologically inactive forms of 1,2- and 1,6-NAD(P)H$_2$ by re-oxidizing them back to NAD(P). This recent discovery of nature's system for conserving the biologically active NAD(P) molecule within the cell can be incorporated into electrochemical systems for removing this barrier.

An electrochemical bioreactor must achieve a number of cofactor