This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2005)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2005 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

NADPH, a Resource of Electrons

by

Liang Zhang

Department of Anatomy and Cell Biology
The University of Iowa
Iowa City, IA 52242-1109

For 77:222, Spring 2005
24. February 2005

Abbreviations

$CO_3^{\bullet-}$	Carbonate radical
GPx	Glutathione peroxidase
GR	Glutathione (disulfide) reductase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid-chromatographic
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidized form
$NADP^{\bullet}$	Nicotinamide adenine dinucleotide phosphate radical
NADPH	Nicotinamide adenine dinucleotide phosphate
NMN	Nicotinamide nucleotide
$NO^{\bullet}$	Nitric oxide
$NO_2^{\bullet}$	Nitrogen dioxide
$ONOO^-$	Peroxynitrite
$O_2^{\bullet-}$	Superoxide
Prx	Peroxydase
Trx	Thioredoxin
$Trx(SH)_2$	Thioredoxin, reduced form
TrxSS	Thioredoxin, oxidized form

Outline

Abstract

In all living cells, NADPH, the reduced form of nicotinamide-adenine dinucleotide phosphate, plays important roles as an electron donor in antioxidation and many other cellular processes. By helping with the regeneration of important antioxidants such as glutathione, NADPH has long been considered as an indirect antioxidant. However, recent research also suggested the ability of NADPH to directly participate in the elimination of free radicals generated by peroxynitrite. In this article, I will discuss the chemistry and biochemistry of NADPH as an antioxidant. The detection methods of NADPH will also be addressed.

Introduction

Undesired free radicals inside cells can cause detrimental effects by spontaneously attacking biological molecules, such as proteins, lipids and DNA. In order to prevent these harmful effects, the cell has developed a variety of mechanisms using antioxidants to suppress those radical-induced reactions [1-2]. Important antioxidants include vitamin E, vitamin C, and glutathione, which play their counterattacking roles by scavenging toxic radicals and/or repairing biomolecule-derived radicals. An effective antioxidative system requires these molecules to be regenerated after their antioxidation reactions. This regeneration process needs electron donors to reduce the oxidized antioxidants. The nicotinamide dinucleotide phosphates (NADPH and $NADP^+$) and their homologues nicotinamide dinucleotides (NADH and NAD^+) were initially identified as the hydrogen transferring coenzymes for many cellular dehydrogenases [3]. The majority of cellular nicotinamide dinucleotide phosphates exist in the reduced form (*i.e.* NADPH), while the majority of cellular nicotinamide dinucleotides are NAD^+ [3-4]. The property of NADPH as a powerful electron donor has made it the major electron resource for the regeneration of cellular antioxidants. In addition, NADPH has been suggested to directly scavenge cellular radicals [5]. This review addresses the basic chemistry of NADPH as well as its biochemistry as an indirect and direct antioxidant. The detection of NADPH is discussed in the end.

NADPH Chemistry: a Good Electron-donor

The structure of NADPH can be envisaged as a 2'-phosphorylated 5'AMP linked to a nicotinamide nucleotide (NMN) by a pyrophosphate bond. There are two high energy linkages in

this molecule. One is the pyrophosphate bond and the other is the β-N-glycosidic linkage between the nicotinamide and the ribosyl group (**Figure 1) [3]**.

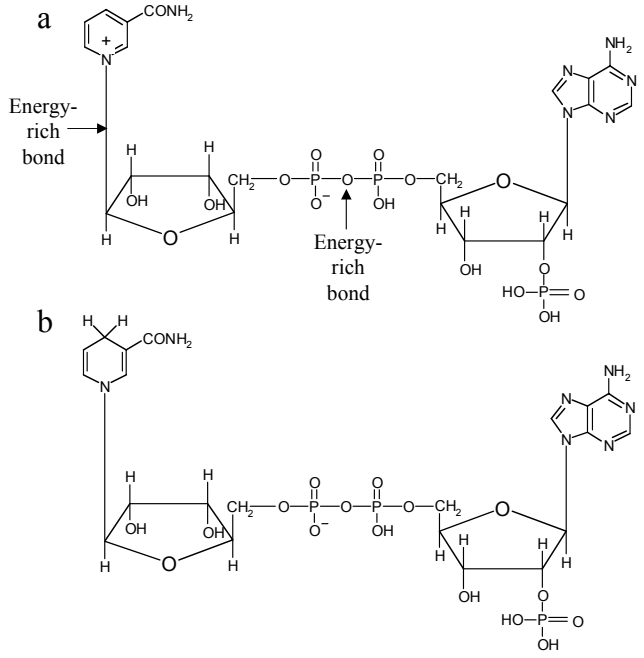

Figure 1. Chemical structure of $NADP^+$ (panel a) and NADPH (panel b). The two energy-rich bonds are indicated on the $NADP^+$ structure. Modified from [3].

NADPH is a major electron-donor for many reduction reactions. The nicotinamide group of NADPH can provide two electrons, resulting in the formation of H^+ and $NADP^+$, the oxidized form. The reaction can be summarized as follow:

$$NADPH \rightarrow NADP^+ + 2e^- + H^+ \quad (1)$$

According to the Nernst equation, at 25°C, pH 7.0, the half-cell reduction potential for $NADP^+$/NADPH would be:

$$E_{hc} = E°' - (59.1/2) \log ([NADPH]/[NADP^+]) \text{ mV} \quad (2)$$

E°′, which stands for the two-electron reduction potential of the NADPH/$NADP^+$ couple, has been determined to be –315 mV at this condition (25°C, pH 7.0) [4].

From the Nernst equation, it can be inferred that the half-cell reduction potential for the $NADP^+$/NADPH couple in a system will not depend on the absolute concentration of either the reduced or the oxidized form, but on the relative ratio of these forms. As the percentage of $NADP^+$ in the coupled system increases, the E_{hc} value of $NADP^+$/NADPH becomes more positive (**Figure 2**) [4].

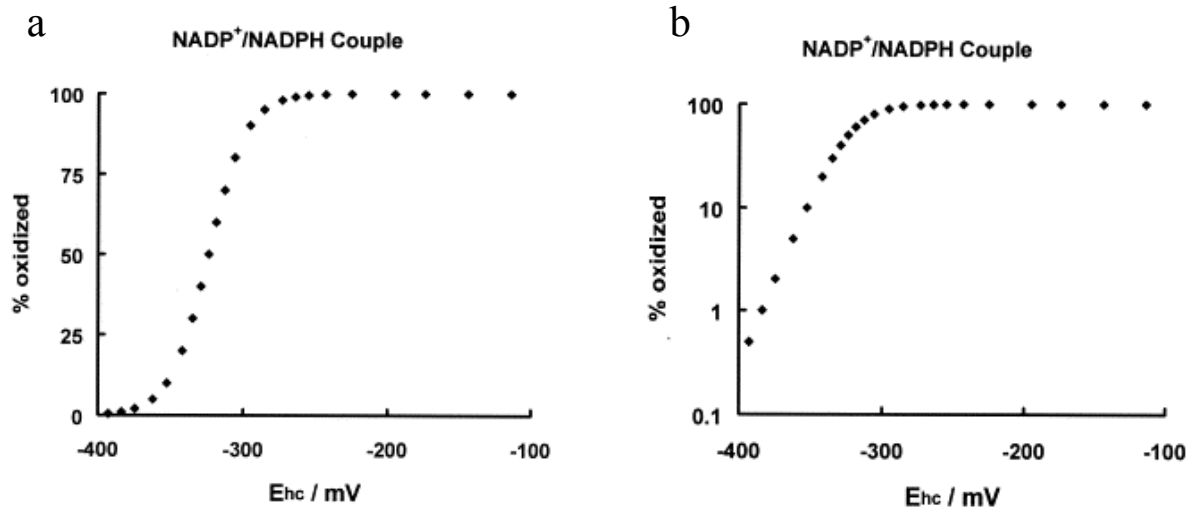

Figure 2. The half-cell reduction potential of the $NADP^+$/NADPH couple tends to be more positive as the oxidized form increases. At 25°C and pH 7.0, the midpoint potential is –315 mV. The behavior of this redox couple is reflected differently on the linear (panel a) or log (panel b) scale plots. From [4].

The involvement and function of $NADP^+$/NADPH inside cells can be implied by the ratio of its oxidized and reduced forms. In cells and tissues the [$NADP^+$]/[NADPH] ratio is about 1:100, while on the other hand, the ratio of [NAD^+]/[NADH] is from 10:1 to 1000:1, depending on different cell types [4]. Based on the above discussion of E_{hc}, it is not difficult to understand that NADPH generally functions as a coenzyme for reductive biosynthesis, while NAD^+ usually functions as a trap for electrons in oxidative catabolic processes [4].

NADPH as an Antioxidant

Besides functioning as a coenzyme, the reducing feature of cellular NADPH makes it a major resource of electrons for cellular antioxidative processes. It is well understood that NADPH acts as an indirect co-antioxidant by helping with the re-reduction of oxidized antioxidants, which is crucial for the cell to defend against oxidative stress and maintain the normal redox environment [4]. However, recent research also suggests that NADPH has the potential to be a direct antioxidant by directly scavenging free radicals and repair damaged biomolecules [5]. The rest of this section will discuss both the indirect and direct antioxidant activity of NADPH.

Being the most abundant redox couple in a cell, GSSG/2GSH is considered to be the principle determinant factor of cellular redox environment [4]. Unlike $NADP^+$/NADPH, the half-cell reduction potential of GSSG/2GSH is dependent on both the ratio of [GSSG]/[GSH] and the absolute concentration of GSH [4]. Hence in order to prevent an oxidizing environment, maintaining the level of the reduced form GSH is critical. GSH can be consumed through its participation in antioxidative reactions. For instance, the peroxidized biomolecules (ROOH) can be removed by glutathione peroxidase (GPx) as in the following reaction:

$$\mathrm{ROOH + 2GSH \xrightarrow{GPx} ROH + H_2O + GSSG} \quad (3)$$

In this reaction, two GSH molecules provide two protons and two electrons for the reduction of ROOH and result in the production of GSSG [6]. It is important for the cell to recycle GSSG back to two GSH in order to maintain stabilized [GSSG]/[GSH] ratio and GSH level. NADPH serves as the electron-donor for the re-reduction of GSSG by the glutathione (disulfide) reductase (GR):

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad (4)$$

Thus, NADPH can function as an indirectly operating antioxidant through regenerating reduced glutathione.

Besides the glutathione system, NADPH is also known to provide electrons to the thioredoxin (Trx) system, which is important in gene expression by specific transcription factors. By donating two electrons, the reduce form of Trx ($Trx(SH)_2$) changes the cysteine moieties on transcription factors to the reduced status, which are critical for their DNA-binding activities:

$$Trx(SH)_2 \rightarrow TrxSS + 2e^- + 2H^+ \quad (5)$$

$Trx(SH)_2$ also provides electrons to the peroxidase (Prx) system to eliminate hydrogen peroxide (H_2O_2), resulting in the formation of water.

$$Trx(SH)_2 + H_2O_2 \xrightarrow{Prx} TrxSS + 2H_2O \quad (6)$$

The oxidized Trx (TrxSS) needs be reduced back to $Trx(SH)_2$, which is catalyzed by the thioredoxin reductase, and NADPH provides electrons for this regeneration [4]. Therefore, due to its property as the electron source for the regeneration of antioxidants, NADPH can be considered as an indirectly operating antioxidant, or a co-antioxidant.

Generally, NADPH is not considered to be a directly operating antioxidant. However, recent studies have proposed that NADPH may work directly against radicals [5, 7]. Peroxynitrite ($ONOO^-$/ONOOH) is formed through a reaction between nitric oxide ($NO^\bullet$) with superoxide ($O_2^{\bullet-}$):

$$NO^\bullet + O_2^{\bullet-} \rightarrow ONOO^- \qquad k_7 = 1 \times 10^{10}\ M^{-1}\ s^{-1} \quad (7)$$

In physiological conditions peroxynitrite can react with CO_2 to produce new radicals carbonate radical ($CO_3^{\bullet-}$) and nitrogen dioxide ($NO_2^{\bullet}$) [8]:

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow CO_3^{\bullet-} + NO_2^{\bullet} \quad (8)$$

The rate constant for the first step of rection (8) is 3×10^4 M^{-1} s^{-1}, and the second step is very fast [9]. Both of the newly formed radicals are able to damage biomolecules and need to be eliminated. Recently, Kirsch *et al.* reported that NADPH, as well as NADH, can terminate $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ through the following reactions [7]:

$$CO_3^{\bullet-} + NADPH \rightarrow HCO_3^- + NADP^{\bullet} \qquad k_9 = 7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \text{ [9]} \quad (9)$$

$$NADP^{\bullet} + O_2 \rightarrow NADP^+ + O_2^{\bullet-} \qquad k_{10} = 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \text{ [9]} \quad (10)$$

The superoxide generated in reaction (8) then react with $NO_2^{\bullet}$ and terminate both radicals:

$$NO_2^{\bullet} + O_2^{\bullet-} \rightarrow O_2NOO^- \qquad k_{11} = 4.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \text{ [9]} \quad (11)$$

$$O_2NOO^- \rightarrow O_2 + NO_2^- \qquad k_{12} = 0.8 \text{ s}^{-1} \text{ [9]} \quad (12)$$

Based on these findings and other observations, Kirsch *et al.* proposed that NADPH and NADH could act as directly operating antioxidants [5]. However, since most of their experiments were performed on *in vitro* systems, the *in vivo* occurrence of these reactions needs to be confirmed and their physiological significance needs to be established.

Detection of NADPH

On UV-spectra, NADPH has an absorption peak where λ_{max} = 340 nm and ε = 6200 M^{-1} cm^{-1} [10]. Additionally, after absorbing light, NADPH can emit a bluish fluorescence with a peak at 460 nm, which provides the basis for fluorimetric methods **(Figure 3)** [10]. The spectrophotometric method allows accurate measurement of NADPH at concentration of more than 0.2 μM. The fluorimetric methods can measure even smaller concentration to as low as 0.05

μM [10]. In the actual experiment analyzing the formation and/or the presence of NADPH, it is important to prevent the oxidation of NADPH during the measurement. Usually the stabilization can be achieved by alkaline solution [10].

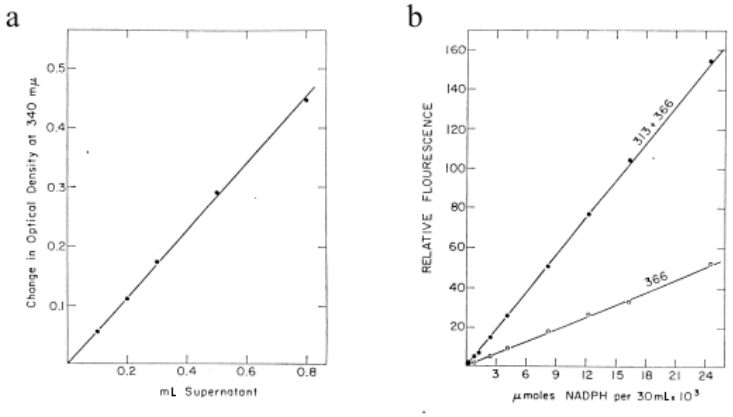

Figure 3. a. A standard curve for the determination of NADPH based on its absorption at 340 nm. NADPH of 1 μmol was added into a 3 mL system containing chloroplasts. Following incubation and termination of reaction, chloroplasts were centrifuged out. Increasing amounts of supernatant were taken for the measuring of absorbance at 340 nm. **b.** Standard curves for determination of NADPH based on its fluorescence after its absorption of UV. It showed that when a filter allowing the transmission of both 313 and 366 nm lines was used for the exciting light, one could detect lower concentrations of NADPH than only using 366 nm exciting light. From [11].

Chromatographic methods can also be used to determine NADPH and NADH. High-performance liquid-chromatographic (HPLC) methods are able to distinguish NADPH from its analogues and have been used to analyze commercially manufactured pyridine nucleotides [10]. Chromatographic technique combined with fluorimetric methods allows the isolation and analysis of NADPH [12].

Summary and Conclusions

Due to its reducing property, NADPH serves as an important electron-source for many cellular processes. By helping the regeneration of the reduced form of glutathione, NADPH indirectly

acts as an antioxidant. Whether it can directly participate in the scavenging of radicals under physiological conditions requires further evidence.

References

1. Sies H, ed. (1985) *Oxidative Stress.* London: Academic Press.

2. Halliwell B. (1999) Antioxidant defence mechanisms: from the beginning to the end (of the beginning). *Free Rad Res.* **31:**261-272.

3. Sies H. (1982) Nicotinamide nucleotide compartmentation. In: Sies H, ed. *Metabolic Compartmentation.* London: Academic Press; pp 205-231.

4. Schafer FQ, Buettner GR. (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med.* **30:**1191-1212.

5. Kirsch M, de Groot H. (2001) NAD(P)H, a directly operating antioxidant? *FASEB J.* **15:**1569-1574.

6. Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Riveri A, Schomburg D, Flohe L. (1995) Divesity of glutathione peroxidases. In: Packer L, ed. *Methods in Enzymology.* Vol. 252. San Diego: Academic Press. pp38-53.

7. Kirsch M, de Groot H. (1999) Reaction of peroxynitrite with reduced nicotinamide nucleotides, formation of hydrogen peroxide. *J Biol Chem.* **274:**24664-24670.

8. Lymar SV, Hurst JK. (1995) Rapid reaction between peroxonitrite ion and carbon dioxide: Implications for biological activity. *J Am Chem Soc.* **117:**8867-8868.

9. Goldstein S, Czapski G. (2000) Reactivity of peroxynitrite versus simultaneous generation of $NO^{\bullet}$ and $O_2^{\bullet-}$ toward NADH. *Chem Res Toxicol.* **13:**736-741.

10. Klingenberg M. (1985) Nicotinamide-adenine dinucleotides and dinucleotide phosphates (NAD, NADP, NADH, NADPH). In: Bergmeyev HU, ed. *Methods of Enzymtic Analysis.* Deerfield Beach, Florida: Verlag Chemie. pp251-271.

11. Ben-Hayyim G, Gromet-Elhanan Z, Avron M. (1969) A specific and sensitive method for the determination of NADPH. *Anal Biochem.* **28:**6-12.

12. Bernofsky C, Gallagher WJ. (1975) Liquid chromatography of pyridine nucleotides and associated compounds and isolation of several analogs of nicotinamide adenine dinucleotide phosphate. *Anal Biochem.* **67:**611-624.