

NAD(P)H, a directly operating antioxidant?

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ABSTRACT Endogenous oxygen- and nitrogen-centered free radicals are considered to play a decisive role in a variety of diseases such as neurodegenerative disorders, atherosclerosis, or cancer. Directly operating antioxidants limit the action of freely diffusing radicals by scavenging the attacking, oxidizing radical and re-reducing the oxidized biomolecule, i.e., the biomolecule-derived radical. From textbooks of biochemistry it is understood that NAD(P)H acts as a hydride (hydrogen anion) donor in a variety of enzymatic processes. One example is the re-reduction of GSSG to GSH, catalyzed by glutathione reductase. Because of this reaction, NADPH has been suggested to also act as an indirectly operating antioxidant, thus maintaining the antioxidative power of glutathione. To the best of our knowledge, however, neither NADPH nor NADH has been considered to be directly operating antioxidants. Based on recently published data, new experiments, and theoretical considerations, we propose that NAD(P)H represents a decisive, directly operating antioxidant that should be considered of major importance in the mitochondrial compartment. NAD(P)H fulfills this task both by scavenging toxic free radicals and repairing biomolecule-derived radicals.—Kirsch, M., de Groot, H. NAD(P)H, a directly operating antioxidant? *FASEB J.* 15, 1569–1574 (2001)

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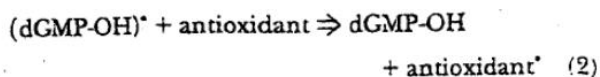
DIRECTLY AND INDIRECTLY OPERATING ANTIOXIDANTS

ENDOGENOUSLY PRODUCED FREE radicals such as peroxyl (ROO[•]) and hydroxyl radicals (•OH), as well as trioxocarbonate (1-) (CO₃^{•-}) and nitrogen dioxide (NO₂[•]), spontaneously attack proteins, nucleic acids, lipids, and other biologically important molecules, thereby altering their structure and properties. As a consequence, cell- and tissue-injuring pathways are induced. Accordingly, these (undesired) radicals are believed to play an important role in many diseases like the neurodegenerative disorders, atherosclerosis, and cancer (1–3). For the suppression of radical-induced reactions, human beings are equipped with an arsenal of counterattacking compounds, commonly referred to as antioxidants. So-called 'directly operating' antioxidants, such as vitamin E (in hydrophobic regions) as well as vitamin C (ascorbate) and glutathione (GSH)

(in hydrophilic environments), counteract oxidizing radicals with two independent mechanisms (Fig. 1):

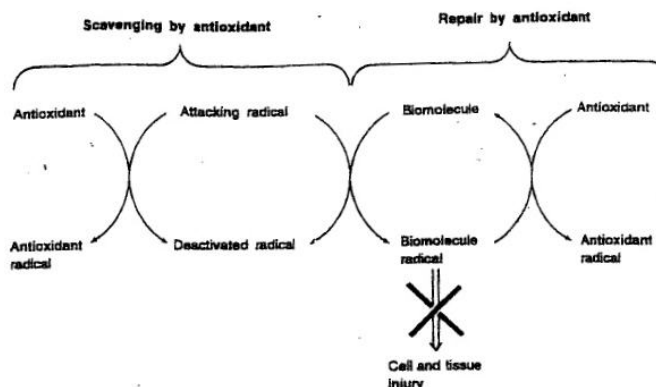
1) Following the 'classical' antioxidative mechanism, the antioxidant rapidly scavenges the attacking radical and thereby terminates its destructive pathways. This mechanism presumes that the resulting antioxidant-derived radical is a 'harmless' one (4), i.e., the reactivity of the antioxidant radical toward typical biomolecules must be low. In fact, vitamin C, but not GSH (see below), does accomplish this demand. 2) The antioxidant does not scavenge the attacking, oxidizing radical but regenerates the oxidized biomolecule, in most cases by re-reducing the primarily formed biomolecule radical. This antioxidative function is termed 'repair function'. Vitamin C as well as glutathione operate by this pathway in order to counteract the destructive action of radicals on biomolecules (5) in addition to the classical mechanism. The major advantage of the repair function becomes obvious when various types of attacking radicals are involved in the destructive process (Fig. 2).

An effective protection of the biomolecule via the repair mechanism can often be achieved at very low concentrations of the antioxidant. This is because the reactivity to just one radical must be high, namely, the one produced from the biomolecule. By contrast, when the antioxidant protects the biomolecule via the scavenging function, the antioxidant must rapidly react with the variety of possible attacking radicals. Although the repair function guarantees an enormous antioxidative capacity, its success strictly depends on the way in which the undesirable radical attacks the target molecule, i.e., oxidation of the biomolecule by either electron transfer or hydrogen abstraction (directly or indirectly via an addition/elimination mechanism). In situations where the attacking radical acts via an intermolecular addition (as, for example, in the reaction of the •OH radical with DNA bases; see ref 6), the biomolecule adduct radical can also be 'repaired' by the antioxidant, i.e., reconverted into a nonradical compound, though its structure is generally not restituted (reactions 1 and 2).



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Figure 1. Action of directly operating antioxidants.



Thus, the repair function represents a powerful, but rather specific line of cellular defense.

In contrast to directly operating antioxidants, indirectly working antioxidants interact with neither the attacking radicals nor the biomolecule radicals. They contribute to the antioxidative potential of the cell by regenerating the oxidized antioxidant. A classical example is NADPH. As the coenzyme (NADPH+H⁺) of glutathione reductase, it formally donates a hydrogen molecule, thereby re-reducing GSSG to GSH. To the best of our knowledge, neither NADPH nor NADH have been considered to be directly operating antioxidants so far. In the present paper we will summarize the currently available evidence that NAD(P)H is a powerful, directly operating antioxidant equipped with both scavenging and repairing capabilities.

NAD(P)H SCAVENGING OF RADICALS DERIVED FROM PEROXYNITRITE

Peroxynitrite (ONOO⁻/ONOOH) is formed from the diffusion-controlled reaction of nitric oxide ([•]NO) with superoxide (O₂^{•-}) (7; reaction 3).

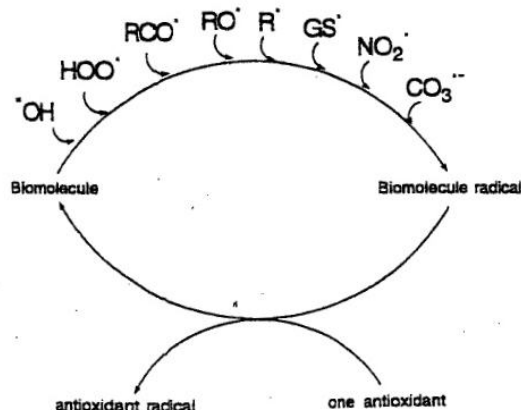
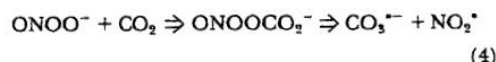


Figure 2. Advantage of the antioxidant-derived repair function.



This peroxide is considered to be involved in various inflammatory diseases (8). Under physiological conditions, peroxynitrite primarily reacts with CO₂ (9, 10; reaction 4).

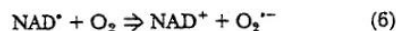


The putative intermediate, 1-carboxylato-2-nitrosodioxidane, fragments with a yield of ~30–35% (11–13) into the free radicals trioxocarbonate (1-) (CO₃^{•-}) (14, 15) and nitrogen dioxide (NO₂[•]) (16). Both radicals (CO₃^{•-} and NO₂[•]) are considered to mainly convey the damaging potential of peroxynitrite in vivo due to oxygenation, nitrosation, and nitration of biomolecules, respectively.

Recent in vitro experiments performed by us in which we reacted peroxynitrite with NAD(P)H demonstrated that NAD(P)H effectively terminates both CO₃^{•-} and NO₂[•]. Whereas the CO₃^{•-} radical is directly scavenged by NADH with a rate constant close to the diffusion-controlled limit (17, 18; reaction 5),



NO₂[•] is, unexpectedly, deactivated by action of oxygen, via formation of superoxide and subsequent formation of peroxynitrate (O₂NOO⁻) (18, 19), which rapidly decomposes to O₂ and nitrite (reactions 6 and 7):



NADPH acts in the same manner. However, in competition with the reaction with O₂^{•-}, the NO₂[•] radicals also directly attack NAD(P)H. This reduces the stationary concentration of NO₂[•] compared with that of O₂^{•-}. Consequently, H₂O₂ is formed from the excess O₂^{•-} radicals.

In line with the effective trapping of both CO₃^{•-} and NO₂[•], NADH efficiently prevents peroxynitrite-dependent nitration reactions (18). As shown here, the same is true for peroxynitrite-induced oxygenation and ni-

of as yet uncharacterized radical-NAD(P)H reactions. For instance, as aliphatic peroxy (ROO^\bullet) and alkoxy radicals (RO^\bullet) have reduction potentials of ~ 1 and 1.6 V (23), respectively, it can be inferred from Fig. 3 that NAD(P)H rapidly reacts with these radicals as well [$k(\text{ROO}^\bullet) \sim 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, $k(\text{RO}^\bullet) \sim 2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$]. In this case, NAD(P)H deactivates the attacking radical (an one-electron oxidant) by reducing it in an one-electron step to the corresponding anion, which is then rapidly protonated. Thus, NAD(P)H serves as hydride (hydrogen anion) donor when acting as either coenzyme or indirectly operating antioxidant, but it donates only one electron to the majority of undesirable free radicals. A prominent exception to this rule is the hydroxyl radical, which is reported to attack NADH via an intermolecular addition (17). Since a standard biochemical one-electron oxidant, $[\text{Fe}(\text{CN})_6]^{3-}$, reacts very slowly with NADH, the general view has emerged that NAD(P)H is relatively inert toward freely diffusing radicals. However, this conclusion neglects the fact that the oxidation potential of $[\text{Fe}(\text{CN})_6]^{3-}$ is low compared with oxygen-centered radicals, which react very fast with the reduced nicotinamides (Fig. 3).

REPAIR OF RADICALS BY NAD(P)H

The antioxidative capability of NAD(P)H toward freely diffusing radicals should be further increased when the repair function is operating as well. In fact, Forni and Willson (24) have demonstrated that the potentially harmful glutathyl radical (GS^\bullet) that is formed as the product of the free radical scavenging activity of GSH is rapidly repaired by NADH (24) (Fig. 4). Thus, NAD(P)H is expected to support GSH in its antioxidative action via two independent pathways: first, as an indirectly operating antioxidant (see above); and second, as a directly operating antioxidant by reduction of GS^\bullet . It must be emphasized that the NAD(P)H-mediated repair function is, in principle, not restricted to glutathyl radicals because tyrosyl radicals are also effectively restituted by NADH (24).

NAD(P)H AS ANTIOXIDANT: CYTOSOL VS. MITOCHONDRIA

In the cytosol of many cell types, concentrations of the three putative antioxidants GSH, vitamin C, and NAD(P)H are given by ~ 5 mM, 0.5 – 2 mM, and $\sim 10^{-3}$ mM (25–29), respectively. The high concentration of both GSH and vitamin C in conjunction with the low concentration of NAD(P)H apparently rules out any direct antioxidative function of NAD(P)H in the cytosol. However, in human erythrocytes and presumably in the cytosolic compartment of other cell types as well, NAD(P)H is mainly bound to soluble macromolecules (30). This bound NAD(P)H may be significant as an antioxidant for two reasons that have not been considered so far. First, bound NADPH may act as an 'in-

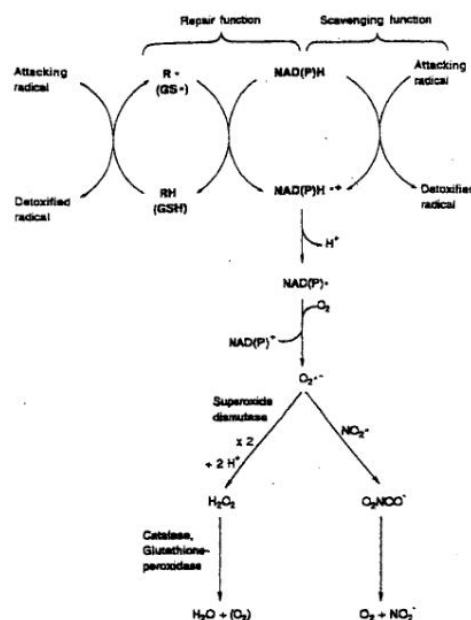


Figure 4. Proposed action of NAD(P)H as directly operating antioxidant. An anonymous referee suggested that vitamin C may act in a similar manner.

tramolecular antioxidant'. For example, four molecules of NADPH are tightly bound to human catalase (31). These NADPH molecules do not influence the activity of the enzyme but strongly increase the lifetime of the catalytically active catalase (31). In light of the findings presented here, this well-known behavior might also be explained by a NADPH-mediated scavenging of oxygen-centered radicals that may be artificially generated during the catalytic process. Second, protein binding may 'sensitize' NADH to react with radicals. For instance, the rate constant of reactions of $\text{HO}_2^\bullet/\text{O}_2^{\bullet-}$ with NADH increases by a factor of 10 – 10^4 when NADH is bound to either glyceraldehyde-3-phosphate dehydrogenase or lactate dehydrogenase (32, 33).

In rat liver mitochondria, the GSH concentration is ~ 10 mM and the total amount of NAD(P)H (3.8 ± 0.4 nmol/mg protein) is roughly equal to the total amount of GSH (3.7 ± 0.7 nmol/mg protein) (34). Because NAD(P)H is partly bound to proteins in the mitochondria as well (29), the free NAD(P)H concentration should be below 10 mM. Since the total amount of mitochondrial NADPH is obviously capable of providing reducing equivalents under conditions of oxidative stress (35), it can be estimated that the total amount of NADPH, i.e., 2.4 ± 0.2 nmol/mg protein (34), which corresponds to a concentration of ~ 6 mM, roughly reflects the lower limit of the concentration of the unbound form of NAD(P)H. The mitochondrial vitamin C concentration should be around 1 mM, because in rat brain mitochondria the amount of GSH is 10 -fold higher than the amount of vitamin C (36). Since all

three putative antioxidants react in a diffusion-controlled manner with highly oxidizing radicals (reduction potential $E^\circ > 1.8$ V), such as the $^{\bullet}\text{OH}$ radical (17, 37), these radicals should be scavenged mainly by the thiol, thereby yielding glutathionyl radicals. On the other hand, however, only the thiolate form of glutathione, i.e., GS^- , reacts fast with moderately oxidizing radicals. Since at physiological pH values only 1–2% of glutathione is present in the thiolate form, glutathione is not very effective at deactivating such types of radicals. NAD(P)H overcompensates for this weakness of GSH because it also reacts rapidly with moderately oxidizing radicals ($E^\circ \approx [1.8-0.9 \text{ V}]$) (Fig. 3). For instance, the $\text{CO}_3^{\bullet-}$ radical reacts ~260-fold faster with NADH than with GSH (17, 37). Furthermore, NAD(P)H quickly repairs the harmful glutathionyl radical (see above). The NO_2^{\bullet} radical as well as low oxidizing radicals ($E^\circ < 0.9$ V), i.e., the α -tocopheroxyl radical, are expected to react mainly with vitamin C. Other compounds, like α -lipoic/dihydrolipoic acid (38), may also be able to act as a directly operating antioxidant in the mitochondria, but their significance is largely limited by their very low concentrations. Taking into consideration the role of NADPH as an indirectly operating antioxidant (NADPH maintains glutathione in the reduced state and GSH obviously re-reduces dehydroascorbate to vitamin C; see ref 39) as well as its additional property of a directly operating antioxidant, i.e., to scavenge attacking radicals and to repair biomolecules (Fig. 4), NAD(P)H should be the decisive key antioxidant in mitochondria.

One may doubt that the reduced nicotinamides act as a directly operating antioxidant in mitochondria because $\text{O}_2^{\bullet-}$ and subsequently H_2O_2 , both of which can noxiously operate in the cell, are formed upon reaction of oxygen-centered radicals with NAD(P)H (40). Nevertheless, as superoxide dismutase and glutathione peroxidase are present in the mitochondrial matrix space (41), $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ are rapidly destroyed by these enzymes. Furthermore, formation of $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ generated from the reaction of NAD(P) $^{\bullet}$ radicals with O_2 may be of minor importance because the oxygen level in mitochondria is significantly lower than in the surrounding cytosol (42). Under such conditions, the NAD(P) $^{\bullet}$ radicals may directly scavenge the attacking radicals instead of reducing molecular oxygen. However, this interplay of reactions is not well characterized and its physiological significance remains to be established.

CONCLUSIONS AND FUTURE PROSPECTS

In the past 100 years, scientists have demonstrated that NAD(P)H is an essential coenzyme in several metabolic pathways, including those that led to the proposal that it is an indirectly working antioxidant. The evidence summarized here strongly suggests that NAD(P)H can also act as a directly operating antioxidant. It can perform this duty, which is proposed to be of major

importance in the mitochondrial matrix space, both by scavenging freely diffusing radicals and repairing biomolecule-derived radicals. [F]

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