

Nicotinamide adenine dinucleotide, a metabolic regulator of transcription, longevity and disease

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Nicotinamide adenine dinucleotide (NAD) is a ubiquitous biological molecule that participates in many metabolic reactions. Recent studies show that NAD also plays important roles in transcriptional regulation, longevity, calorie-restriction-mediated life-span extension and age-associated diseases. It has been shown that NAD affects longevity and transcriptional silencing through the regulation of the Sir2p family, which are NAD-dependent deacetylases. Many human diseases are associated with changes in NAD level and/or the NAD : NADH ratio, raising the possibility that the Sir2p family might play a role in these diseases.

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Abbreviations

CR	calorie restriction
CtBP	carboxyl-terminal binding protein
DHEA(S)	dehydroepiandrosterone sulphate
NaAD	deamido-NAD
NAD	nicotinamide adenine dinucleotide, oxidised form
NADH	nicotinamide adenine dinucleotide, reduced form
Nam	nicotinamide
PARP	poly(ADP-ribose) polymerase
SIR2	silent information regulator 2
TCA	tricarboxylic acid (Krebs) cycle

Introduction

A change in metabolism has been implicated in the mechanisms of several age-associated diseases such as diabetes, cancers and neurodegenerative diseases (e.g. Parkinson's disease) [1,2*,3]. Calorie restriction (CR) has been shown to decrease the incidence or delay the onset of some of these diseases [4,5]. It is currently unclear how CR ameliorates these diseases. CR extends the life span of a spectrum of organisms and so far it is the only regimen known to extend the life span of mammals [4,5]. Recent studies show that the benefit of CR requires nicotinamide adenine dinucleotide (NAD) and Sir2p [6*], a key regulator of life span in both yeast and animals [7,8]. Sir2p exhibits an NAD-dependent histone deacetylase activity

that is conserved among Sir2p-family members and is required for chromatin silencing and life-span extension [9–11]. The requirement of NAD for Sir2p activity suggests that Sir2p is likely to be regulated by the metabolic state of cells. It is therefore likely that CR delays and/or prevents age-associated diseases by regulating NAD metabolism or Sir2p activity, or both.

In this review, we discuss the emerging roles of NAD as a signalling factor in transcriptional regulation, ageing and human diseases. We also discuss the roles the Sir2p family, one molecular target of NAD-mediated metabolic regulation, in age-associated diseases.

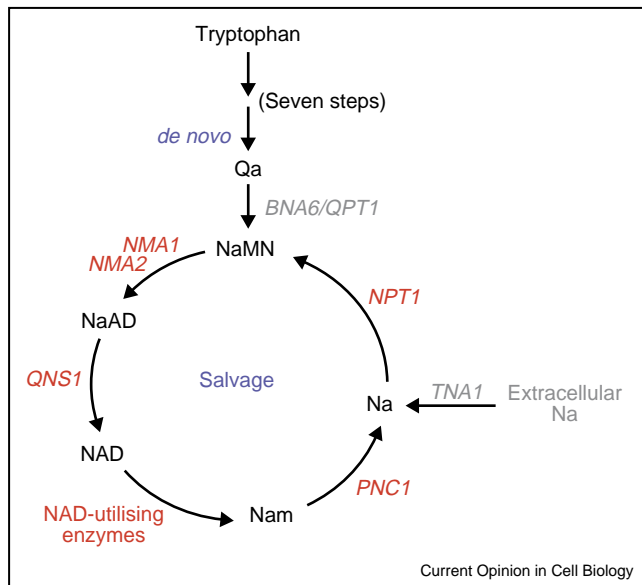
NAD metabolism and biological functions

NAD is synthesised via two major pathways in both prokaryotic and eukaryotic systems (Figure 1) [12–14,15*,16]. In one pathway, NAD is synthesised from tryptophan (the *de novo* pathway). In the other pathway, NAD is generated by recycling degraded NAD products such as nicotinamide (the salvage pathway). In yeast, the *de novo* pathway consists of six enzymatic steps (catalysed by the *BNA* genes) and one non-enzymatic reaction. The last enzymatic reaction is catalysed by a quinolinate phosphoribosyl transferase, encoded by the *BNA6/QPT1* gene, which converts quinolinate to nicotinic acid mononucleotide. At this point the *de novo* pathway converges with the salvage pathway. In the salvage pathway, the nicotinic acid may come from the hydrolysis of nicotinamide (Nam). This reaction is catalysed by a nicotinamidase, encoded by the *PNC1* gene. A nicotinate phosphoribosyl transferase, encoded by the *NPT1* gene, converts the nicotinic acid to nicotinic acid mononucleotide, which is then converted to deamido-NAD (NaAD) by a nicotinate mononucleotide adenyl transferase, encoded by the *NMA1* and/or *NMA2* genes. An NAD synthase (possibly encoded by *QNS1*) converts NaAD to NAD, which completes the final step in NAD synthesis.

In yeast, both pathways play redundant yet essential roles in cell growth [6*,14]. However, deleting the *NPT1* gene decreases the NAD level 2.5-fold, whereas deleting the *QPT1* gene has no effect [13,14]. These studies suggest that, under normal conditions, the salvage pathway plays a more important role in NAD synthesis.

NAD participates in many biological processes, including the regulation of energy metabolism [17], DNA repair [18,19] and transcription [2*,13,15*,20*]. In addition to serving as a coenzyme, NAD is utilised as a substrate. Enzymes that utilise NAD as a substrate include some

Figure 1

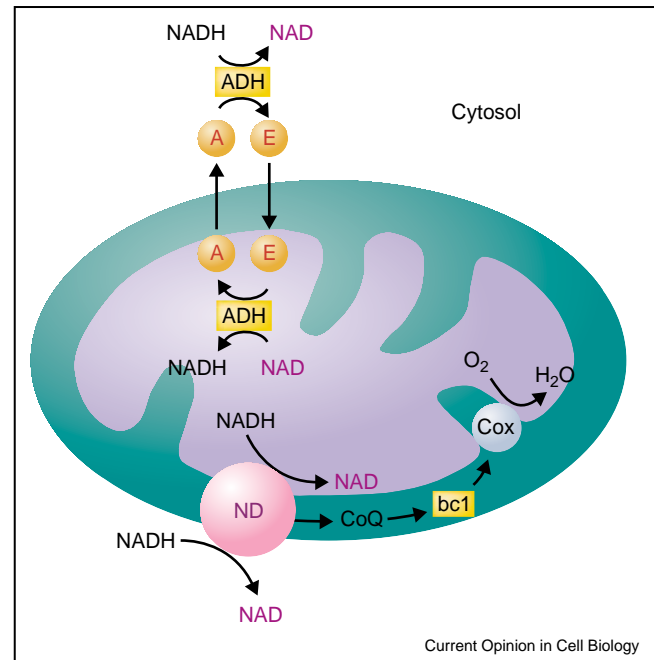


Summary of the NAD synthetic pathways in yeast. In the *de novo* pathway, NAD is synthesised from tryptophan, which is converted to quinolinic acid (Qa), then to nicotinic acid mononucleotide (NaMN). The salvage pathway recycles the NAD breakdown products, such as nicotinamide (Nam), generated by the NAD-utilising enzymes. Genes encoding enzymes in the salvage pathway are highlighted in red. Extracellular nicotinic acid (Na) can be imported into the cell through nicotinic acid permease, encoded by the *TNA1* gene [14,45]. Na is then converted to NaMN, deamido-NAD (NaAD) and NAD. NAD is degraded to Nam, which is converted back to Na. In mammals, Nam in the salvage pathway is directly converted to nicotinamide mononucleotide, bypassing Na as an intermediate. NaMN is converted to deamido-NAD (NaAD) by the NaMN adenylate transferase [46].

NAD-dependent DNA ligases [18], NAD-dependent oxidoreductases [17], poly(ADP-ribose) polymerase (PARP) [19] and the recently characterised Sir2p family, NAD-dependent deacetylases [9–11,21,22]. The reduced form of NAD, NADH, is a substrate for the NADH dehydrogenase of the mitochondrial respiratory chain, which transfers electrons to coenzyme Q, generating NAD [17]. A derivative of NAD, the NADP(H) coenzyme, is involved in many assimilatory pathways as well as maintaining the intracellular redox state together with glutathione [17].

NAD is converted to NADH mostly in catabolic reactions including glycolysis and the tricarboxylic acid (TCA/Krebs) cycle [17]. To maintain a proper redox state, NADH needs to be re-oxidised constantly via several mechanisms. During respiratory growth, both cytosolic and mitochondrial NADH are re-oxidised primarily by the respiratory chain [23,24]. Owing to the impermeability of the inner mitochondrial membrane for NAD and NADH, several shuttle systems exist to transfer permeable redox equivalents across this barrier. One example is the ethanol–acetaldehyde shuttle (Figure 2) [23].

Figure 2



Schematic presentation of the yeast mitochondrial respiratory chain and a NAD/NADH shuttle system. Mitochondrial NADH dehydrogenases (ND) re-oxidise NADH from both cytosolic and mitochondrial pools. Electrons then pass down the *trans* chain to an ultimate oxygen acceptor. The ethanol–acetaldehyde shuttle system balances the NAD : NADH ratio between the cytosolic and the mitochondrial pools. For example, an increase in mitochondrial NAD leads to the production of acetaldehyde (A) through mitochondrial alcohol dehydrogenases (ADH). Mitochondrial acetaldehyde diffuses freely to the cytosol, which is then reduced to ethanol (E) via cytosolic alcohol dehydrogenases, resulting in an increase in cytosolic NAD.

bc1, Cytochrome bc1 complex; Cox, cytochrome oxidase; CoQ, coenzyme Q.

The NAD : NADH ratio

The NAD : NADH ratio plays an important role in regulating the intracellular redox state and is often considered as a read out of the metabolic state. Many metabolic enzymes are regulated by the NAD : NADH ratio, such as the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase and the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA, a substrate for the TCA cycle [17]. It has been well documented that the NAD : NADH ratio fluctuates in response to a change in metabolism [25–29].

If NAD is indeed a metabolic regulator, one would assume that the ratio of the intracellular levels of NAD to NADH is close to 1. If this is the case, the NAD : NADH ratio should be regulated by a small/reasonable change in the NAD concentration. For example, if the ratio is very high (e.g. 500), regulation of the NAD : NADH ratio will be more sensitive to a change in the concentration of NADH not NAD. Intracellular NAD(H)

Table 1**Baseline ratios of the cytosolic NAD : NADH in various species.**

Species	Tissue type/ cell line	NAD : NADH ratio	References
Mice	Liver	4	[26]
Mice	Kidney	2.6	[26]
Mice	Brain	0.26	[25]
Mice	Blood	0.03	[25]
Mice	Pancreas	0.1	[29]
Swine	Liver	0.07	[28]
Rat	Heart	0.05	[27]
Monkey	Cos7	644	[2*]

exists in both the free and (mostly) bound form [30]. Reported ratios of total intracellular NAD : NADH are: 4–10 in *Escherichia coli* [31,32]; 3–10 in mammals [33,34]; 3 in yeast [15*]. These values suggest that NAD can function as a metabolic regulator of the NAD : NADH ratio.

Although the ratios of total (i.e. free + bound) NAD to total NADH in different organisms seem to be within a reasonable range for NAD regulation, the baseline ratios of the free pool of NAD : NADH have been debated. The free pool NAD : NADH ratio can be estimated from the concentrations of the intracellular metabolites, pyruvate and lactate (the pyruvate : lactate ratio). Using these criteria, the free pool (often referred to as the ‘cytosolic pool’) NAD : NADH ratios in various tissues from different species have been estimated and found to range from 0.1 to 10 (Table 1) [25–29]. A recent study, however, shows that the ratio of the free pool of NAD to NADH is around 600, measured by the pyruvate : lactate ratio [2*]. This number seems to correspond with the classical view in which the ratio is estimated from the cytosolic NAD redox potential [30]. Cytosolic NAD redox potential is about –250 mV in rat livers, which translates to a NAD : NADH ratio of 500 [30]. To understand the role of NAD as a metabolic regulator, it is therefore very important to investigate which reported number for the NAD : NADH ratio represents the real situation in cells.

A role for NAD in transcriptional regulation

Recent studies show that the NAD salvage pathway plays an important role in transcriptional silencing at the telomere and rDNA loci [13,14,15*]. The nucleolar silencing regulator Sir2p is required for silencing at these loci [13,14,15*]. The silencing status of these loci is thus considered as a read out of the Sir2p activity. Similar to Sir2p, components of the salvage pathway affect silencing in a dose-dependent manner [14,15*]. Therefore, the salvage pathway is thought to increase silencing by activating Sir2p, which requires NAD. Interestingly, increased cycling through the salvage pathway does not affect the overall steady-state NAD level or NAD : NADH ratio. It has been suggested that the salvage

pathway is compartmentalised in the nucleus, where NAD can be readily utilised by Sir2p [15*]. Consistent with this notion, components of the salvage pathway, such as Npt1p, are localised primarily to the nucleus, whereas components of the *de novo* pathway reside evenly throughout the cell and have no effect on silencing [14,15*].

NAD also regulates gene expression at other genomic loci. Clock/BMAL1 and NPAS2/BMAL1 are heterodimeric transcription factors that regulate the expression of genes controlling the circadian clock. These factors are regulated by the redox state of NAD(H) and NADP(H) [20*]. This transcriptional feedback system, whose activity fluctuates as a function of the light–dark cycle, controls the circadian rhythms in many organisms. The reduced forms of the NAD cofactors, NADH and NADPH, enhance the heterodimerisation and DNA binding activity of these transcription factors, whereas the oxidised form, NAD and NADP, inhibit these activities. The effective concentrations of these NAD cofactors are in the low millimolar range. This corresponds with the total intracellular concentrations reported in the literature [23,34,35,36]. A recent study challenges the physiological relevance of this redox regulation since the effective concentrations (i.e. in the millimolar range) are much higher (about 1000 times) than the reported concentrations of the free pools of NADH and NAD [2*]. Assuming all these numbers are physiologically relevant, it is possible that the transition between light and dark cycles mobilises the bound pool of NAD(H), leading to a transient increase in the free NAD(H) pool to the required millimolar level, which then regulates these factors.

In another study, the binding of the co-repressor CtBP (carboxyl-terminal binding protein) to transcriptional repressors is also regulated by NAD and NADH [2*]. The co-repressor CtBP is involved in transcriptional pathways important for cell growth and differentiation, and transformation. NADH is two to three orders of magnitude more effective than NAD in enhancing binding. Levels of the free pool NAD(H) determined in this study using both two-photon excitation microscopy and conventional measurements (the pyruvate : lactate ratio) are within the low micromolar range, which corresponds with the levels required for half-maximal CtBP binding. The higher sensitivity of CtBP to NADH in this cell line suggests that changes in nuclear redox could manifest primarily through NADH.

A role for NAD in ageing

Yeast

Recent studies in yeast have provided insight into the molecular mechanisms underlying CR-mediated life-span extension [6*,37*]. Both Sir2p and NAD are essential for CR-mediated life-span extension: the benefit of CR is abolished by deleting either *SIR2* or *NPT1* [6*]. The requirement of NAD for Sir2p activity suggests CR

may work by increasing the available NAD pool for Sir2p activity. Interestingly, overexpression of Npt1p, which is assumed to increase the flux through the salvage pathway, functions in the same pathway as CR and Sir2p to extend life span [15^{*}]. However, overexpressing Npt1p does not increase the steady state level of the total NAD or the NAD : NADH ratio. This suggests that the salvage pathway activates Sir2p activity by increasing NAD regeneration in the nucleus, where it is more accessible for Sir2p [15^{*}]. It is also possible that overexpressing Npt1p increases the free NAD pool without significantly affecting the total level of NAD (free + bound). Increasing the NAD salvage activity might also work by decreasing the concentration of Nam, an inhibitor of Sir2p activity [15^{*},38]. Consistent with this hypothesis, overexpressing Pnc1p, a nicotinamidase that converts Nam to nicotinic acid in the salvage pathway, extends life span [38]. A recent study further discusses how CR affects metabolism to regulate Sir2p activity. This study indicates that the shunting of carbon metabolism toward the mitochondrial TCA cycle and the concomitant increase in respiration both play a central role in this process [37^{*}]. As discussed above, mitochondria play a major role in re-oxidising NADH [23,24]. It is thus possible that CR increases the free NAD pool and/or free NAD : NADH ratio to activate Sir2p by increasing respiration.

Higher eukaryotes

Ageing in higher eukaryotes is a much more complex process. Can we extrapolate the knowledge obtained from studies in yeast to multicellular organisms? Does NAD and the Sir2p family also play a role in ageing in higher eukaryotes? Recent studies show that *Caenorhabditis elegans* carrying extra copies of the *SIR2* orthologue *sir-2.1* exhibit a longer life span [8]. Although it is not yet clear whether nematode Sir-2.1 exhibits an NAD-dependent deacetylase activity, NAD is likely to play a role in regulating worm Sir2.1. Moreover, both human and mouse Sir2p have been shown to function as NAD-dependent p53 deacetylases [21,22], and deacetylation of p53 via Sir2 promotes cell survival under stress [21,22]. It is also suggested that p53 plays a role in ageing [39]. These studies strongly suggest that key regulators of the ageing process, such as Sir2p, are likely to be conserved. NAD might therefore play an important role in regulating longevity via the Sir2p family.

A very recent study shows that a decrease in the rate of decline of the adrenal steroid DHEAS (dehydroepiandrosterone sulphate) is correlated with longer life span in humans [40]. Decreased decline in DHEAS, reduced body temperature and plasma insulin are well-known biomarkers of CR in primates [5]. Since NAD plays an important role in CR in the model organism, it is likely to play a role in human CR and ageing. Interestingly, short-term feeding of DHEAS to rats causes an increase in liver NAD level and the NAD : NADH ratio [33]. This study

suggests that a change in the redox state mediated by NAD might be involved in the pleiotropic beneficial effects of DHEAS including, anti-ageing, anti-cancer and anti-obesity effects.

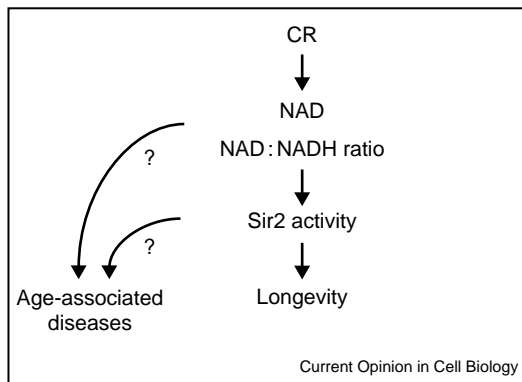
A role for NAD in human disease

Several age-related diseases have been directly or indirectly associated with a change in NAD level or NAD/NADH redox state. As discussed above, the NAD/NADH redox state regulates the co-repressor CtBP activity and therefore plays a role in carcinogenesis [2^{*}]. In addition, NAD/NADH may also regulate the tumour suppressor p53 via Sir2p [21,22]. PARP1 overactivation has been implicated in the mechanisms underlying type 1 diabetes. PARP1 utilises NAD as a substrate and participates in DNA-base excision repair, DNA-damage signalling, regulation of genomic function, transcription and proteasomal functions [19]. However, overactivation of PARP1 depletes intracellular NAD, and therefore, leads to cell death by necrosis [19,41]. NAD depletion is thus suggested to cause the cell destruction in insulin-producing β cells. NAD also plays a role in diabetic vascular dysfunction. Hyperglycaemia induces vascular complications in both type 1 and type 2 diabetes [42]. Hyperglycaemia causes a decrease in NAD : NADH ratio due to an increase in the carbon flux through the polyol pathway [42]. NAD depletion mediated by PARP1 overactivation also participates in diabetic endothelial dysfunction [1]. Furthermore, a point mutation in the mitochondrial gene *ND1* (NADH dehydrogenase) has been found in a patient with type 2 diabetes [43]. In another study, NADH dehydrogenase, a component of the respiratory chain complex I, isolated from type 2 diabetes patients exhibits lower activity [44]. These studies suggest that the ability to regulate NAD : NADH ratio is lower in diabetes patients, since mitochondrial NADH dehydrogenase is the major site for re-oxidising NAD. It is therefore likely that NAD may also play a role in other diseases associated with mitochondrial complex I dysfunction — such as Parkinson's disease [3].

Conclusions

NAD has emerged as a putative metabolic regulator of transcription, longevity and several age-associated diseases, including diabetes, cancer and neurodegenerative diseases. CR has been shown to decrease the incidence or delay the onset of some of these diseases. Studies in yeast suggest that CR might function by increasing the NAD level and/or the NAD : NADH ratio. It is thus possible that CR ameliorates these human diseases by a similar mechanism (Figure 3). Further studies are required to detail the mechanisms underlying these NAD-mediated metabolic regulations. One possible target of this metabolic regulation is the Sir2p family, which also plays an important role in regulating transcription and longevity in an NAD-dependent manner. In the future, therefore, it would be very interesting to

Figure 3



NAD as a putative metabolic regulator of longevity and disease. CR extends life span in an NAD- and Sir2-dependent manner and decreases the incidence and/or reduces the onset of several age-associated diseases, such as cancer, neurodegenerative diseases and diabetes. NAD and Sir2 might also play a role in CR-mediated amelioration of these diseases.

investigate whether the Sir2p family also plays a role in these diseases.

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