Review TRENDS in Biochemical Sciences Vol.29 No.3 March 2004

The new life of a centenarian: signalling functions of NAD(P)

Felicitas Berger[1], María H. Ramírez-Hernández[1] and Mathias Ziegler[1,2]

[1]Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany
[2]Department of Molecular Biology, University of Bergen, Thormøhlens gt. 55, N-5020 Bergen, Norway

Since the beginning of the last century, seminal discoveries have identified pyridine nucleotides as the major redox carriers in all organisms. Recent research has unravelled an unexpectedly wide array of signalling pathways that involve nicotinamide adenine dinucleotide (NAD) and its phosphorylated form, NADP. NAD serves as substrate for protein modification including protein deacetylation, and mono- and poly-ADP-ribosylation. Both NAD and NADP represent precursors of intracellular calcium-mobilizing molecules. It is now beyond doubt that NAD(P)-mediated signal transduction does not merely regulate metabolic pathways, but might hold a key position in the control of fundamental cellular processes. The comprehensive molecular characterization of NAD biosynthetic pathways over the past few years has further extended the understanding of the multiple roles of pyridine nucleotides in cell biology.

Most probably, Sir Arthur Harden did not envision the versatility of the low molecular weight compound that he deduced to exist when studying sugar fermentation in yeast at the beginning of the last century (Box 1). This compound, nicotinamide adenine dinucleotide (NAD), is now regarded as a universal energy- and signal-carrying molecule. The electron-transferring properties of NAD (and its phosphorylated relative, NADP) and its function as co-factor in multiple redox reactions are widely recognized. Numerous enzymes – particularly dehydrogenases, which depend on NAD(P) – were discovered within the last century. Because almost all metabolic pathways rely on these pyridine nucleotides, it is beyond doubt that NAD(P) holds a key position in energy metabolism.

More recent research has revealed an entirely different role of NAD(P): apparent degradation of pyridine nucleotides turned out to represent major signalling events. Poly- and mono-ADP-ribosylation were the first recognized modes in which NAD participates as a substrate for protein modification (Box 2). Only a few years ago, NAD-dependent protein deacetylation was discovered. Besides covalent protein modification, NAD(P) also serves as precursor of the intracellular calcium-mobilizing agents cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). Even the redox state of NAD might directly influence important processes such as transcriptional activity [1]. The unexpected array of functions of NAD(P) consumption has prompted a tremendous surge of interest in the enzymes involved in NAD(P) biosynthesis during the past few years. It now appears that even enzymes of this pathway might be involved in important regulatory processes.

NAD biosynthesis – a key to longer life?

NAD synthesis is essential for all organisms. Given that the hydrogen transfer reactions do not involve a net consumption of pyridine nucleotides, their biosynthesis was long regarded to be of minor importance. However, in all known NAD-dependent signalling pathways, the *N*-glycosidic bond between the ADP-ribose moiety and nicotinamide is cleaved [see Box 2, Figure Ib(i–iv)]. In particular, poly-ADP-ribosylation might cause a marked decrease in the NAD level [2]. Consequently, there is a permanent demand for a supply of NAD to these processes. This realization has prompted enormous efforts to examine the enzymes of NAD(P) biosynthesis. Even though the enzymatic activities catalysing NAD synthesis (reviewed in [3]) have been known for decades, the primary structures of various eukaryotic enzymes involved in this pathway and some important regulatory relationships have been largely established within the past five years [4–9]. The reactions of NAD synthesis are depicted in [see Box 2, Figure Ia(ii–vi)]. The three precursors quinolinate, nicotinate and nicotinamide can be transferred onto phosphoribosyl pyrophosphate (PRPP) by the respective phosphoribosyl transferase [see Box 2, Figure Ia(ii–iv)]. The resulting mononucleotides [nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NAMN)] are converted into the corresponding dinucleotides, NAD or NAAD, by nicotinamide mononucleotide adenylyltransferase (NMNAT) [see Box 2, Figure Ia(v)]. Finally, NAAD is amidated to NAD by NAD synthase [see Box 2, Figure Ia(vi)].

The tremendous advances in the research of human NMNATs illustrate the rapid progress in the field. These enzymes are essential because they catalyse the final step of NAD biosynthesis [see Box 2, Figure Ia(v)]. The first isoform (hNMNAT1) was cloned in 2001 [10,11] and soon thereafter the 3D structure was reported by three independent groups (reviewed in [12]). At the same time, a second isoform (hNMNAT2) was discovered [13], followed by a third one (hNMNAT3) for which the X-ray structure has also been solved [14]. One exciting result of the discovery of multiple NMNAT isoforms is their differential

Corresponding author: Mathias Ziegler (Mathias.Ziegler@mbi.uib.no).

 0968-0004/$ - see front matter doi:10.1016/j.tibs.2004.01.007

Box 1. The discovery of NAD(P)

Since the beginning of the last century numerous scientists, among them the four Nobel laureates (Figure I), contributed to the discovery of nicotinamide adenine dinucleotide (NAD) and the solution of its structure and function. In 1904, Sir Arthur Harden separated Buchner's yeast juice into high molecular and low molecular fractions. Neither of these fractions could perform fermentation. However, recombining them restored this capability. Harden, therefore, inferred the existence of a high molecular ferment (enzyme) and a low molecular coferment or 'cozymase'.

In subsequent years, cozymase was established as a virtually universal factor participating in fermentation, respiration and glycolysis in a variety of organisms. Unfortunately, owing to its low concentration, isolation turned out to be difficult and frustrated scientists over a period of almost thirty years. Eventually, Hans von Euler-Chelpin succeeded in isolating cozymase from yeast extracts in the late 1920s, and 'von Euler's cozymase' became a dictum thereafter. He determined its chemical composition of a sugar, an adenine and a phosphate, and later also described the dinucleotide character of NAD and the combination of the two mononucleotides, adenosine monophosphate (AMP) and nicotinamide mononucleotide (NMN), the latter containing the pyridine-like nicotinamide. The actual function of NAD was established by Otto Warburg in the mid-1930 s. Based on his work on alcoholic fermentation, Warburg discovered the capability of NAD to transfer hydrogen from one molecule to another. He also ascribed this electron transfer to the pyridine moiety of NAD and discovered another coenzyme, 'cozymase II' – which we now refer to as NADP – with similar properties.

The synthesis of NAD was first described by Arthur Kornberg who was on a quest to investigate the synthesis of nucleic acids starting with a simple dinucleotide. He discovered the crucial step of NAD-synthesis in 1954 by detecting an enzymatic activity in yeast extracts that catalysed the condensation of ATP with nicotinamide mononucleotide to form NAD. It took another 55 years until the first primary structure of this enzyme, nicotinamide mononucleotide adenylyltransferase (NMNAT), was determined. Further information regarding the contributions of the Nobel Prize Laureates is available at www.nobel.se.

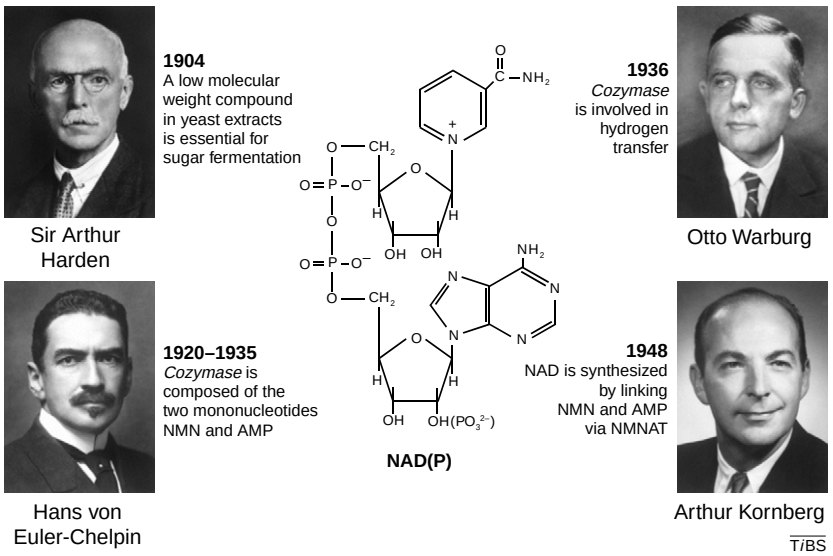

Figure I. Nobel Prize Laureates who contributed to the discovery of structure and functions of NAD(P). Key observations of the individual scientists are indicated. The photographs were kindly provided by the Nobel Foundation (© The Nobel Foundation). The structural formula of NAD(P) is also shown. Abbreviations: AMP, adenosine monophosphate; NAD(P), nicotinamide adenine dinucleotide (phosphate); NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase.

subcellular localization. Although hNMNAT1 was found to be localized to the nucleus [11], the other two isoforms appear to be cytosolic, and hNMNAT3 might also reside within mitochondria [14]. The reasons for such a compartmentation of NAD synthesis are unknown. However, the origin of NAD in mammalian mitochondria, where up to 70% of the entire cellular pool might be sequestered [15], is still unknown. Therefore, an autonomous mitochondrial NAD metabolism appears plausible (Box 3). Indeed, at least in yeast, one of three NAD kinase isoforms is mitochondrial [16]. As the known human NAD kinase [17] [see Box 2, Figure Ic(i)] appears to be cytosolic, a second, mitochondrial isoform could also exist in humans.

Surprisingly, increasing the activity of NAD biosynthetic enzymes has been recently demonstrated to extend lifespan [18,19]. This effect is mediated by NAD-dependent histone deacetylases [see Box 2, Figure Ib(iv)] the subsequent activation of which increases lifespan. Overexpression of enzymes of the NAD biosynthetic pathway such as nicotinate phosphoribosyltransferase, NMNAT or nicotinamidase in yeast results in higher activity of the NAD-dependent histone deacetylase Sir2p and concomitant lifespan extension [20–22]. Unexpectedly, the NAD level and the NAD^+:NADH ratio were unaffected by the overexpression of any of these proteins. It turned out that nicotinamide, a side-product of NAD-dependent deacetylation, is an endogenous strong inhibitor of Sir2p [20,23]. Therefore, the positive effect of enhanced NAD biosynthesis on lifespan was probably due to accelerated reutilization of nicotinamide, thereby, releasing the inhibition of Sir2p. It appears possible that overexpression of NMNAT in mammalian cells has a comparable effect. Intriguingly, the slow Wallerian degeneration mouse overexpresses an N-terminally extended, catalytically active NMNAT [24]. Similar to the results obtained in yeast, the NAD concentration and

Box 2. Eukaryotic NAD(P)-metabolizing enzyme activities

Metabolic and signalling reactions of nicotinamide adenine dinucleotide (phosphate) [NAD(P)] are shown in Figure I. NAD synthesis is commonly divided into the *de novo* and the salvage pathways. The NAD precursors of the salvage pathway are the degradation products of NAD, nicotinamide and nicotinate (niacin, vitamin B3) [70]. Nicotinamide can be deamidated to nicotinate by nicotinamidase (Figure Ia), except in mammals. Quinolinate, a degradation product of tryptophan, is the precursor of the *de novo* pathway. These three precursors are transferred onto phosphoribosyl pyrophosphate (PRPP) by specific phosphoribosyltransferases yielding nicotinate mononucleotide or nicotinamide mononucleotide [Figure Ia(ii–iv)]. Both mononucleotides are converted into the corresponding dinucleotides via an adenylation reaction that is catalysed by nicotinamide mononucleotide adenylyltransferase (NMNAT) [Figure Ia(v)]. The products of this reaction are NAD or nicotinic acid adenine dinucleotide (NAAD), the latter can be amidated to NAD by NAD synthase [Figure Ia(vi)]. NAD-consuming processes include formation of (cyclic) ADP-ribose by NAD glycohydrolases, mono- and poly-ADP-ribosylation, by mono-ADP-ribosyltransferases (ARTs) and poly-ADP-ribose polymerases (PARPs), respectively, and NAD-dependent deacetylation by protein deacetylases [Figure Ib(i–iv)]. These reactions involve the cleavage of the glycosidic bond between nicotinamide and the adjacent ribose. Nicotinamide is released and the original positive charge of the nicotinamide moiety remains temporarily on the terminal ribose of ADP-ribose, thereby, representing a target for nucleophilic agents. For example, for NAD glycohydrolase [Figure Ib(i), Ic(ii)], one preferred nucleophile would be the N^1 of the adenine ring (of the same molecule of ADP-ribose) leading to the synthesis of cyclic ADP-ribose [36]. NADP synthesis is accomplished by NAD kinase, which phosphorylates NAD at the 2′ position [Figure Ic(i)].

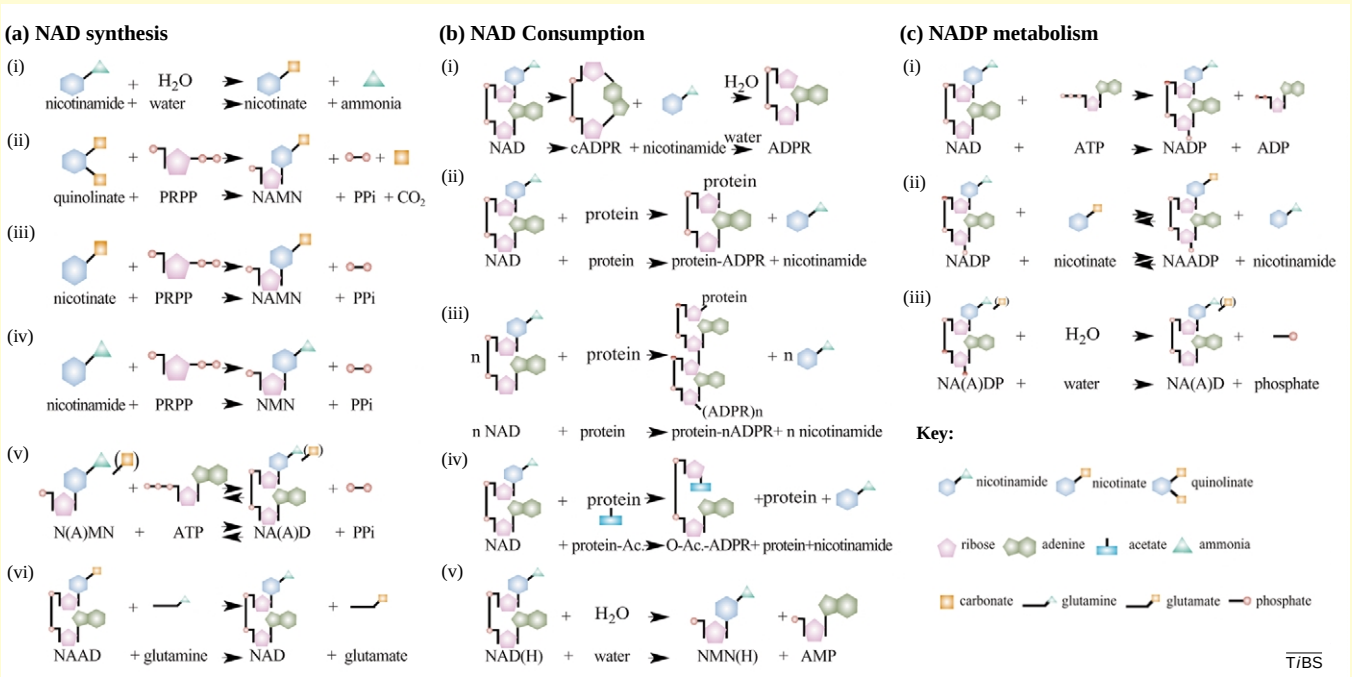

Figure I. Individual reactions of nicotinamide adenine dinucleotide (phosphate) [NAD(P)] synthesis and consumption. **(a)** NAD-synthesizing reactions are illustrated, the enzymes involved are as follows: (i) nicotinamidase (EC 3.5.1.19) is an enzyme of the salvage pathway. It has been localized to the cytosol, nucleus and peroxisomes, and is absent in mammals; (ii) quinolinate-phosphoribosyltransferase (EC 2.4.2.11; predicted to be cytosolic) is an enzyme of the *de novo* pathway, it might prevent neuronal disorders by degrading quinolinate, and is absent in *Caenorhabditis elegans* and *Drosophila melanogaster*; (iii) nicotinate-phosphoribosyltransferase (EC 2.4.11), found in the cytosol and nucleus in all known organisms, is involved in the salvage pathway; (iv) nicotinamide-phosphoribosyltransferase (EC 2.4.2.12) is a cytosolic enzyme of the salvage pathway that is involved in lymphocytic activation, present in mammals but absent in yeast, *C. elegans, D. melanogaster* and plants; (v) nicotinamide mononucleotide (NMN)-adenylyltransferase (EC 2.7.7.1) is localized in the nucleus (and possibly in the cytosol and mitochondria), it is an essential NAD-synthesizing enzyme, and is present in all known organisms; (vi) NAD synthase (EC 6.3.5.1; predicted to be cytosolic) is a NAD-synthesizing enzyme that is present in all known organisms. **(b)** NAD-consuming reactions; the catalysing enzymes are as follows: (i) NAD glycohydrolase (EC 3.2.2.5) is a plasma membrane, mitochondrial and cytosolic enzyme that is involved in the regulation of intracellular calcium release by synthesis of the calcium-releasing agent cyclic ADP-ribose (cADPR); (ii) mono-ADP-ribosyltransferase (EC 2.4.2.31), a glycosyl-phosphatidyl inositol (GPI)-anchored secretory enzyme that catalyses covalent protein modification and, thereby, inhibition of protein or peptide activity, it is present in all known organisms but absent in yeast; (iii) poly-ADP-ribose polymerase (EC 2.4.2.30) is localized in the nucleus, cytosol, golgi and centrosomes, it catalyses poly-ADP-ribosylation of proteins and, thereby, regulates fundamental functions (e.g. DNA synthesis and repair, and transcription); (iv) NAD-dependent protein deacetylase (EC 2.3.1.-) is found in the cytosol, nucleus and mitochondria, and is involved in regulation of protein function by deacetylation, it is present in all known organisms; (v) NAD(H) pyrophosphatase (EC 3.6.1.22), a transmembrane and peroxisomal enzyme that acts during NAD degradation, is present in all known organisms. **(c)** The following enzymes catalyse the NADP-metabolizing reactions shown: (i) NAD kinase (EC 2.7.1.23), a cytosolic (and mitochondrial in yeast), NADP-synthesizing enzyme involved in defense against oxidative stress, present in all organisms; (ii) NAD glycohydrolase or NADP nicotinate transglycosidase (EC 2.4.-.-) is found in the plasma membrane, mitochondria and cytosol, it is presumably involved in regulation of intracellular calcium release by synthesis of the calcium-releasing agent NAADP; (iii) NA(A)DP phosphatase (EC 3.1.3.-) – localization is not determined, but it is known to be involved in the degradation of NAADP and/or NADP. Abbreviations: Ac., acetyl; NAMN, nicotinic acid mononucleotide; PPi, inorganic pyrophosphate; PRPP, phosphoribosyl pyrophosphate.

the NAD^+:NADH ratio remain unchanged. Still, the elevated level of NMNAT causes a considerable delay of axon degeneration after injury, which could perhaps be equivalent to extended lifespan. Future studies will have to establish whether this phenomenon is attributable to an activation of the mouse Sir2p homologue.

Considering that nicotinamide appears to have an important regulatory function related to lifespan, it is

Box 3. NAD(P)-mediated signalling events and pyridine nucleotide biosynthesis in eukaryotic cells

Cytosolic nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) can be converted to the calcium-mobilizing agents cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), respectively, by the multifunctional enzyme NAD glycohydrolase (NADase) (Figure I). NAADP causes the release of calcium from lysosome-related organelles independent of cytosolic [Ca^{2+}], whereas cADPR mobilizes Ca^{2+} from the endoplasmic (or sarcoplasmic) reticulum by a calcium-induced calcium release mechanism that is similar to inositol (1,4,5)-trisphosphate-mediated calcium release (not shown). Thereby, ordered calcium waves can be generated. The prevalent NAD-consuming reaction in the nucleus is poly-ADP-ribosylation. This protein modification can be catalysed by different poly-ADP-ribose polymerases (PARPs) causing, for example, modulation of transcriptional activity or telomere elongation. NAD-dependent protein deacetylases (sirtuins) transfer the acetyl group attached to proteins (e.g. histones) onto NAD, thus, forming *O*-acetyl-ADP-ribose. Deacetylation of histones causes gene silencing in the respective region of the chromatin. Note that NAD-dependent protein deacetylases are also present in the cytosol and mitochondria (not shown). Although present inside cells (not shown), mono-ADP-ribosylation reactions catalysed by mono-ADP-ribosyltransferases (ARTs) have been best characterized on the cell surface. There they appear to regulate immune functions. The well-studied mammalian ectoenzyme CD38 has also been associated with immunological functions. Owing to its NADase activity it can synthesize cADPR or NAADP. Internalization of CD38 has been reported and could, therefore, also contribute to intracellular NADase activity in mammals.

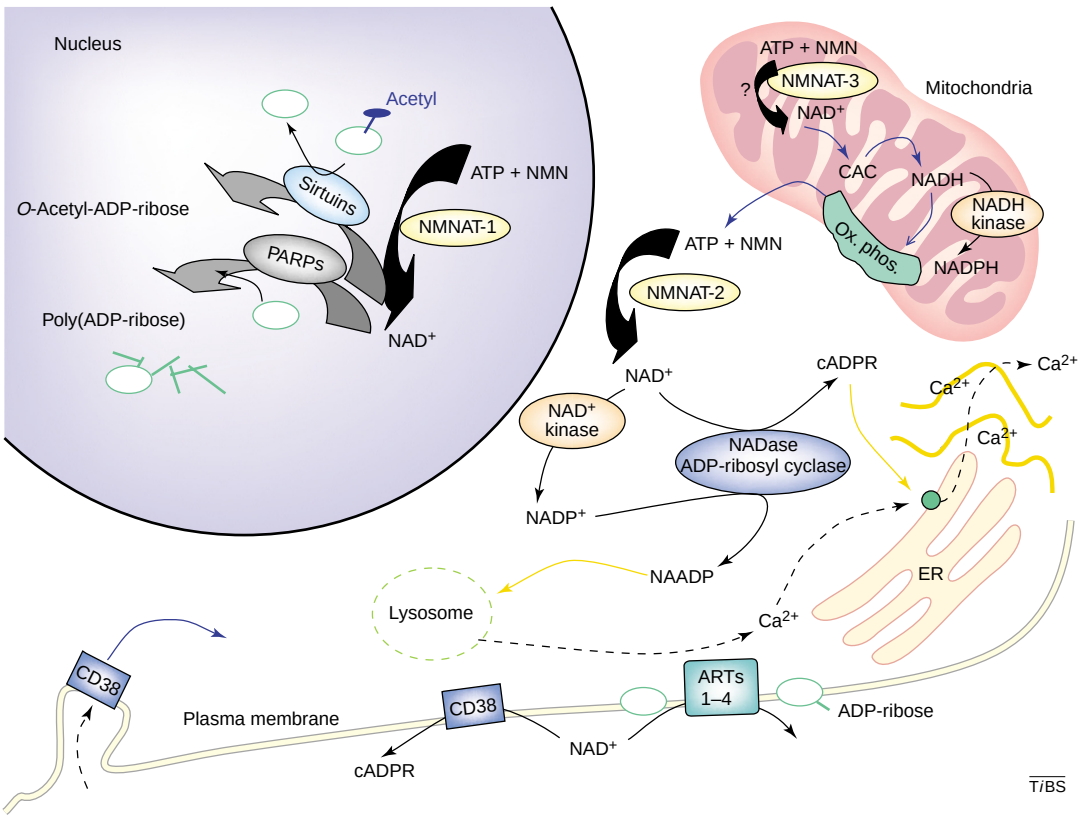

Figure I. Compartmentation of nicotinamide adenine dinucleotide (phosphate) [NAD(P)] biosynthesis and major NAD(P)-mediated signalling pathways in eukaryotic cells. The major reactions of NAD(P)-mediated signalling and potential compartmentation of the final steps of NAD(P) biosynthesis are summarized. Not all pathways occur necessarily in the same cell. The question mark indicates that the subcellular localization of the enzyme has not been clearly established. Abbreviations: ART, mono-ADP-ribosyltransferase; CAC, citric acid cycle; cADPR, cyclic ADP-ribose; ER, endoplasmic reticulum; NAADP, nicotinic acid adenine dinucleotide phosphate; NADase, bifunctional NAD glycohydrolase/ADP-ribosyl cyclase; NMNAT, nicotinamide mononucleotide adenylyltransferase; ox. phos., oxidative phosphorylation; PARP, poly-ADP-ribose polymerase.

intriguing to note that mammalians differ from other species in their pathway of nicotinamide metabolism. Detailed analyses of available sequence databases suggest that only mammals can directly re-use nicotinamide, the degradation product of NAD, because they possess nicotinamide phosphoribosyltransferase, which is absent in other eukaryotes [9]. Therefore, non-mammalian organisms require nicotinamidase to metabolize nicotinamide [see Box 2, Figure Ia(i)]. By contrast, mammals lack nicotinamidase [9] and are therefore unable to generate endogenous nicotinate, which is a potential precursor of NAADP synthesis.

Cyclic ADP-ribose and NAADP – a new calcium wave

Early studies on sea urchin eggs demonstrated a significant change in pyridine nucleotide concentrations after fertilization, and suggested an influence on the concomitant changes of intracellular calcium. Indeed, both NAD^+ and $NADP^+$ trigger calcium release from intracellular stores following conversion into cADPR and NAADP, respectively [25] (Box 3).

Calcium regulates many cellular processes [26]. The existence of two additional intracellular calcium-releasing agents, besides the well-known inositol (1,4,5)-trisphosphate ($Ins(1,4,5)P_3$), has important implications. For

example, it indicates that the complexity of calcium signalling might be a consequence of independent release mechanisms. Although there are similarities between Ins(1,4,5)P_3 and cADPR, NAADP seems to be unique in several aspects. Both Ins(1,4,5)P_3 and cADPR release calcium by a calcium-induced calcium release (CICR) mechanism, whereas the effect of NAADP is independent of cytosolic calcium [27,28]. Moreover, Ins(1,4,5)P_3 and cADPR activate calcium stores of the endoplasmic (or sarcoplasmic) reticulum [25,28–31]. By contrast, NAADP acts on a distinct store of calcium that has recently been identified as reserve granules in sea urchin eggs, which are the equivalent of lysosomes [32] (Box 3). Release of calcium by cADPR is mediated by the ryanodine receptor, whereas the NAADP-receptor is a presently unidentified membrane-associated protein [28,29].

Intracellular calcium release triggered by NAADP or cADPR has been observed in a variety of organisms including plants, invertebrates and mammals [25,28–31]. In sea urchin eggs, NAADP initiates long-term calcium oscillations during fertilization that are amplified by the CICR mechanism via Ins(1,4,5)P_3- and cADPR-sensitive stores [33]. Such a hierarchy between the three mechanisms was also observed in pancreatic acinar cells and starfish oocytes [30,34].

Important cellular events related to cADPR- and NAADP-triggered calcium mobilization include muscle contraction, ion fluxes, catecholamine secretion, insulin secretion and T-cell activation [25,28–31]. A recent study has suggested that stimulation of pancreatic β cells with glucose induces an NAADP-mediated intracellular calcium increase [35].

Synthesis of cyclic ADP-ribose is accomplished by NAD glycohydrolases (NADases), enzymes that had previously been known to just hydrolyse NAD^+ to ADP-ribose (ADPR) and nicotinamide [36] [see Box 2, Figure Ib(i)]. That is, formation of a cyclic product (cADPR) containing a new intramolecular bond between the adenine ring and the terminal ribose, or hydrolytic cleavage of the glycosidic bond between nicotinamide and the terminal ribose (yielding the non-cyclic product ADPR) are catalysed by the same enzyme. Therefore, these enzymes are now also referred to as bifunctional NADases or ADP-ribosyl cyclases. Initially, cADPR synthesis was observed in the mollusc *Aplysia californica* [25]. Two mammalian NADase homologues, the ectoenzymes CD38 and bone marrow stromal antigen-1 (BST-1 or CD157), have also been identified. Although CD38 is a plasma membrane glycoprotein, it was proposed that it might catalyse intracellular cADPR synthesis following internalization [37,38] (Box 3). In sea urchin eggs, two intracellular NADases – a membrane-bound and a soluble form – exist [29]. Earlier reports of a mitochondrial NADase in mammalian tissues are supported by a recent study of pancreatic acinar cells. In addition, a cytosolic NADase has been detected that is stimulated by cGMP [39], a phenomenon that was also observed in sea urchin eggs and rat parotid acinar cells [25,28–31].

Given that cADPR is a potent intracellular messenger, it is most surprising that NADases themselves are also the only enzymes known to inactivate cADPR by hydrolysing it to ADPR [25,36] [see Box 2, Figure Ib(i); note the sequential reaction from NAD to cADPR and then to ADPR]. Moreover, NADases also catalyse a transglycosylation reaction leading to the synthesis of NAADP. Using NADP as substrate they exchange the nicotinamide to nicotinate and form NAADP [36] [see Box 2, Figure Ic(ii)]. To date, this is the only known mechanism to generate NAADP. In sea urchin eggs, this exchange activity is activated by cAMP, which suggests a reciprocal regulation of cADPR and NAADP synthesis by cGMP and cAMP [25,28]. However, this transglycosylation requires high amounts of nicotinate and acidic pH, which argues against its physiological relevance. Even though NAADP is effective at nanomolar concentrations and NADases are able to produce such amounts at pH 7 (in the presence of 5 mM nicotinate), the origin of nicotinate in mammals, if not nutritional, is obscure because they are unable to convert nicotinamide to nicotinate [cf. see Box 2, Figure Ia(i)]. Alternatively, NAADP could be generated, for example, by deamidation of NADP or phosphorylation of NAAD, but there are no observations supporting such possibilities.

Poly-ADP ribosylation – nuclear chain reactions

Forty years ago, NMN-stimulated formation of adenine-containing polymers was observed in nuclear extracts [40]. These polymers were eventually identified as poly-ADP-ribose, a molecule that is formed from NAD^+ by poly-ADP-ribose polymerases [PARPs; see Box 2, Figure Ib(iii)]. Poly-ADP-ribosylation has been established as post-translational protein modification occurring in nearly all cells of higher eukaryotes. PARPs are currently a major focus of biological and medical investigations because of their crucial involvement in fundamental cellular events, including apoptosis, cell-cycle regulation, DNA repair and transcription [2,41,42]. The mechanism of these multiple effects is often ascribed to the negative charge of the ADP-ribose chains that are attached to the acceptor protein.

For many years, PARP1 was thought to represent the sole enzymatic activity catalysing poly-ADP-ribosylation. Investigations of PARP1 knockout mice have greatly contributed to the understanding of the role of this protein [43]. Moreover, they have enabled the unexpected detection of other proteins with PARP activity. PARPs now constitute a new family of proteins [44,45] of which six human members have been characterized.

PARP1 is a nuclear protein (Box 3) and exhibits by far the highest catalytic activity of all described PARP enzymes. Several transcription factors including p53, YY1, nuclear factor κB (NFκB) and TATA-binding protein (TBP) serve as polymer acceptors. As a consequence of their modification they lose affinity to their cognate DNA sequences, which results in altered gene expression [46–48]. A characteristic property of PARP1 is its selective binding to DNA single-strand breaks, which triggers the catalytic activity of the enzyme. The prompt execution of poly-ADP-ribosylation and subsequent recruitment of DNA-repair proteins has established PARP1 as a major surveillance factor of the integrity of the genome [2,41–47]. PARP1 has also been recognized as an essential component of cell-death pathways. It has been shown that poly-ADP-ribosylation can trigger the release of apoptosis-inducing

factor (AIF) from mitochondria [49] and, thereby, effectively mediate apoptosis. When DNA damage is extensive, PARP1 is overactivated resulting in massive NAD^+ consumption and eventually leading to necrotic cell death [50,51]. In fact, overactivation of PARP1 could occur in many pathophysiological situations, such as ischaemia-reperfusion or inflammatory injury, which are accompanied by oxidative stress that causes DNA damage. Because prevention of PARP1 overactivation has a considerable beneficial effect, this protein has become a promising target for pharmacological intervention. Indeed, PARP1 inhibition is highly effective in reducing the extent of necrotic damage in different models of ischaemia-reperfusion injury [50,51].

Owing to the fact that other PARP enzymes have only been discovered very recently, much less is known about their physiological functions. Nevertheless, the newly identified PARP enzymes have already been established as important regulators of fundamental cellular events. PARP2, like PARP1, is involved in DNA base-excision repair and a double knockout in mice is lethal [52]. PARP3 resides preferentially in the daughter centriole and modulates G1/S cell-cycle progression [53]. As PARP1 also occurs in the centrosome, a joint function of PARP1 and PARP3 might be DNA-damage surveillance at the mitotic fidelity checkpoint [54]. A cytoplasmic PARP [PARP4 or vault poly(ADP-ribose) polymerase (VPARP)] is a component of vaults, which are barrel-shaped cytosolic ribonucleoprotein particles. So far, the function of poly-ADP-ribosylation in these large complexes has remained unclear [44]. The discovery of tankyrases 1 and 2 (PARP5 and PARP6) established a link between poly-ADP-ribosylation and telomere elongation [44,55]. Both tankyrases 1 and 2 release the inhibitory effect of telomere-repeat binding factor-1 (TRF1) on telomere elongation. The mechanism is reminiscent of the transcriptional regulation by PARP1 in that the tankyrases modify TRF1, which then loses affinity to telomeric DNA [55,56]. Taken together, poly-ADP-ribosylation is involved in an impressive array of regulatory pathways. Nevertheless, the number of PARPs and their activities are almost certain to grow further because analyses of the human genome suggest the existence of at least 18 proteins carrying a typical PARP signature (J-C. Amé and G. de Murcia, pers. commun.).

Mono-ADP-ribosylation – a bacterial invention

Mono-ADP-ribosylation was initially identified as a catalytic activity of bacterial toxins. Classical examples are the toxins of *Vibrio cholerae*, *Bordetella pertussis* and *Corynebacterium diphtheriae* [57]. Today, a variety of bacterial toxins that deregulate important physiological functions by modifying host cell proteins with ADP-ribose are known.

Endogenous mono-ADP-ribosylation in higher eukaryotes appears to modulate the immune response, cell adhesion, signal and energy metabolism [58]. The modification is catalysed by mono-ADP-ribosyltransferases [ARTs; see Box 2, Figure Ib(ii)]. Several of these enzymes have been cloned and shown to modify arginine residues of the acceptor proteins. Recently, α-defensin-1 – an antimicrobial, arginine-rich protein secreted by immune cells – was demonstrated to be modified by ART-1, which is expressed on the surface of airway epithelia. The modification caused a loss of the antimicrobial effect of defensin-1 [59].

Somewhat surprisingly, all identified ARTs represent ectoenzymes that are anchored in the plasma membrane or secreted [60]. However, a variety of intracellular targets of endogenous mono-ADP-ribosylation have been identified *in vitro* [58]. Moreover, the β subunit of a heterotrimeric G-protein [61], glutamate dehydrogenase [62], and the glucose regulatory protein 78 [63] have been shown to be modified and thereby inhibited *in vivo*. Recently, an entirely unexpected target of ADP-ribosylation was discovered. Deoxyguanosine bases in double-stranded DNA can be mono-ADP-ribosylated by pierisin-1 (a protein of the cabbage butterfly). Although the function of this modification has not been elucidated, it is of significant interest that pierisin-1 causes apoptosis following uptake into mammalian cells [64].

NAD-dependent protein deacetylation – a silencer makes noise

Another exciting contribution highlighting the versatility of NAD is the discovery that yeast silent information regulator protein 2 (Sir2p) is an NAD-dependent histone deacetylase [18,19] [see Box 2, Figure Ib(iv)]. Sir2p has been recognized as an essential factor in gene silencing in the yeast *Saccharomyces cerevisiae*. It mediates hypoacetylation of histones to form transcriptionally inactive chromatin. The requirement of Sir2p for NAD distinguishes its reaction from all other known deacetylations. The acetyl group is transferred from the target protein onto NAD, giving rise to the deacetylated protein, nicotinamide, and a novel NAD derivative, *O*-acetyl-ADP-ribose [18,19] [see Box 2, Figure Ib(iv); Box 3]. Microinjection of this new NAD metabolite into oocytes blocks maturation and embryo cell division in blastomeres [65]. Conceivably, *O*-acetyl-ADP-ribose might have messenger functions that will have to be established in the future.

Sir2p homologues, so called sirtuins, are highly conserved from prokaryotes to mammals [66]. Recent studies have also revealed functional similarities in different organisms. Yeast Sir2p silencing activity is targeted at the mating-type loci HML and HMR, it contributes to the stability of telomeric repeats by silencing the telomeres and inhibits the formation of extrachromosomal rRNA-encoding DNA (rDNA) [18,19]. The occurrence of extrachromosomal rDNA is one limiting factor for the replicative lifespan of yeast. Indeed, overexpression of SIR2 prevents formation of extrachromosomal rDNA and causes lifespan extension of up to 60%, whereas catalytically inactive Sir2p mutants exhibit shortened lifespan [18,19].

Similarly, the Sir2p-homologue of *Caenorhabditis elegans*, Sir-2.1, prolongs lifespan of adult animals by negatively regulating an insulin-like signalling pathway [18,19]. The mouse homologue, Sir2α, is involved in repression of rDNA transcription by RNA polymerase I [67], whereas a human counterpart, SIRT1, deacetylates and thereby inhibits p53 by decreasing its DNA affinity.

Hence, SIRT1 prevents cellular senescence and apoptosis under stress conditions [68,69]. It appears, therefore, that sirtuins exert a universal effect on aging in various organisms, although the mechanisms of the aging process might differ. It is also interesting to note that human homologues of Sir2p have been localized to several subcellular compartments, including the nucleus, the cytosol and mitochondria and their function is not limited to chromatin silencing [18].

Concluding remarks

Although the potential involvement of NAD in regulatory pathways has been realized for quite some time, our knowledge about the underlying mechanisms and new discoveries of NAD-mediated processes have grown at an astonishing pace over the past few years. The concurrent molecular characterization of NAD biosynthetic pathways has not only facilitated these advancements but, by itself, largely contributed to the understanding of the multiple roles of pyridine nucleotides in cell biology. The dualism of energy and signal transducing functions of NAD(P) is similar to ATP, the universal energy carrier in the cell. Nevertheless, in the versatility of regulatory reactions – from the formation of protein-bound polymers to small calcium releasing agents – NAD(P) has now probably taken the lead.

Acknowledgements

We thank the Nobel Foundation for providing photographs of Nobel Prize Laureates. Financial support by the Deutsche Forschungsgemeinschaft (ZI 541/3, ZI 541/4) is gratefully acknowledged.

References

1 Zhang, Q. *et al.* (2002) Regulation of corepressor function by nuclear NADH. *Science* 295, 1895–1897
2 D'Amours, D. *et al.* (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem. J.* 342, 249–268
3 Magni, G. *et al.* (1999) Enzymology of NAD^+ synthesis. *Adv. Enzymol. Relat. Areas Mol. Biol.* 73, 135–182
4 Fukuoka, S. and Shibata, K. (1999) Characterization and functional expression of the cDNA encoding human brain quinolinate phosphoribosyltransferase. *Adv. Exp. Med. Biol.* 467, 611–614
5 Rajavel, M. *et al.* (1998) Conversion of a cosubstrate to an inhibitor: phosphorylation mutants of nicotinic acid phosphoribosyltransferase. *Biochemistry* 37, 4181–4188
6 Emanuelli, M. *et al.* (1999) Identification and characterization of YLR328W, the *Saccharomyces cerevisiae* structural gene encoding NMN adenylyltransferase. Expression and characterization of the recombinant enzyme. *FEBS Lett.* 455, 13–17
7 Rongvaux, A. *et al.* (2002) Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur. J. Immunol.* 32, 3225–3234
8 Ghislain, M. *et al.* (2002) Identification and functional analysis of the *Saccharomyces cerevisiae* nicotinamidase gene, PNC1. *Yeast* 19, 215–224
9 Rongvaux, A. *et al.* (2003) Reconstructing eukaryotic NAD metabolism. *Bioessays* 25, 683–690
10 Emanuelli, M. *et al.* (2001) Molecular cloning, chromosomal localization, tissue mRNA levels, bacterial expression, and enzymatic properties of human NMN adenylyltransferase. *J. Biol. Chem.* 276, 406–412
11 Schweiger, M. *et al.* (2001) Characterization of recombinant human nicotinamide mononucleotide adenylyl transferase (NMNAT), a nuclear enzyme essential for NAD synthesis. *FEBS Lett.* 492, 95–100
12 Rizzi, M. and Schindelin, H. (2002) Structural biology of enzymes involved in NAD and molybdenum cofactor biosynthesis. *Curr. Opin. Struct. Biol.* 12, 709–720
13 Raffaelli, N. *et al.* (2002) Identification of a novel human nicotinamide mononucleotide adenylyltransferase. *Biochem. Biophys. Res. Commun.* 297, 835–840
14 Zhang, X. *et al.* (2003) Structural characterization of a human cytosolic NMN/NaMN adenylyltransferase and implication in human NAD biosynthesis. *J. Biol. Chem.* 278, 13503–13511
15 Di Lisa, F. and Ziegler, M. (2001) Pathophysiological relevance of mitochondria in NAD^+ metabolism. *FEBS Lett.* 492, 4–8
16 Outten, C.E. and Culotta, V.C. (2003) A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae*. *EMBO J.* 22, 2015–2024
17 Lerner, F. *et al.* (2001) Structural and functional characterization of human NAD kinase. *Biochem. Biophys. Res. Commun.* 288, 69–74
18 Denu, J.M. (2003) Linking chromatin function with metabolic networks: Sir2 family of NAD^+-dependent deacetylases. *Trends Biochem. Sci.* 28, 41–48
19 Hekimi, S. and Guarente, L. (2003) Genetics and the specificity of the aging process. *Science* 299, 1351–1354
20 Anderson, R.M. *et al.* (2003) Nicotinamide and PNC1 govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*. *Nature* 423, 181–185
21 Lin, S.J. *et al.* (2000) Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science* 289, 2126–2128
22 Anderson, R.M. *et al.* (2002) Manipulation of a nuclear NAD^+ salvage pathway delays aging without altering steady-state NAD^+ levels. *J. Biol. Chem.* 277, 18881–18890
23 Bitterman, K.J. *et al.* (2002) Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J. Biol. Chem.* 277, 45099–45107
24 Mack, T.G. *et al.* (2001) Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nat. Neurosci.* 4, 1199–1206
25 Lee, H.C. (2001) Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. *Annu. Rev. Pharmacol. Toxicol.* 41, 317–345
26 Berridge, M.J. *et al.* (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1, 11–21
27 Chini, E.N. and Dousa, T.P. (1996) Nicotinate-adenine dinucleotide phosphate-induced Ca^{2+}-release does not behave as a Ca^{2+}-induced Ca^{2+}-release system. *Biochem. J.* 316, 709–711
28 Patel, S. *et al.* (2001) Coordination of Ca^{2+} signalling by NAADP. *Trends Biochem. Sci.* 26, 482–489
29 Galione, A. *et al.* (2000) NAADP-induced calcium release in sea urchin eggs. *Biol. Cell.* 92, 197–204
30 Petersen, O.H. and Cancela, J.M. (1999) New Ca^{2+}-releasing messengers: are they important in the nervous system? *Trends Neurosci.* 22, 488–495
31 Guse, A.H. (2002) Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP): novel regulators of Ca^{2+}-signaling and cell function. *Curr. Mol. Med.* 2, 273–282
32 Churchill, G.C. *et al.* (2002) NAADP mobilizes Ca(2 +) from reserve granules, lysosome-related organelles, in sea urchin eggs. *Cell* 111, 703–708
33 Churchill, G.C. and Galione, A. (2001) NAADP induces Ca^{2+} oscillations via a two-pool mechanism by priming IP_3- and cADPR-sensitive Ca^{2+} stores. *EMBO J.* 20, 2666–2671
34 Santella, L. *et al.* (2000) Nicotinic acid adenine dinucleotide phosphate-induced Ca(2+) release. Interactions among distinct Ca(2+) mobilizing mechanisms in starfish oocytes. *J. Biol. Chem.* 275, 8301–8306
35 Masgrau, R. *et al.* (2003) NAADP. a new second messenger for glucose-induced Ca(2 +) responses in clonal pancreatic beta cells. *Curr. Biol.* 13, 247–251
36 Ziegler, M. (2000) New functions of a long-known molecule. Emerging roles of NAD in cellular signaling. *Eur. J. Biochem.* 267, 1550–1564
37 Ferrero, E. *et al.* (2000) The making of a leukocyte receptor: origin, genes and regulation of human CD38 and related molecules. *Chem. Immunol.* 75, 1–19

38 Zocchi, E. *et al.* (1999) Ligand-induced internalization of CD38 results in intracellular Ca^{2+} mobilization: role of NAD^{+} transport across cell membranes. *FASEB J.* 13, 273–283
39 Sternfeld, L. *et al.* (2003) Hormonal control of ADP-ribosyl cyclase activity in pancreatic acinar cells from rats. *J. Biol. Chem.* 278, 33629–33636
40 Chambon, P. *et al.* (1963) Nicotinamide mononucleotide activation of a new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. *Biochem. Biophys. Res. Commun.* 11, 39–43
41 Bouchard, V.J. *et al.* (2003) PARP-1, a determinant of cell survival in response to DNA damage. *Exp. Hematol.* 31, 446–454
42 Burkle, A. (2001) Physiology and pathophysiology of poly(ADP-ribosyl)ation. *Bioessays* 23, 795–806
43 Shall, S. and de Murcia, G. (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? *Mutat. Res.* 460, 1–15
44 Smith, S. (2001) The world according to PARP. *Trends Biochem. Sci.* 26, 174–179
45 Jacobson, M.K. and Jacobson, E.L. (1999) Discovering new ADP-ribose polymer cycles: protecting the genome and more. *Trends Biochem. Sci.* 24, 415–417
46 Kraus, W.L. and Lis, J.T. (2003) PARP goes transcription. *Cell* 113, 677–683
47 Smulson, M.E. *et al.* (2000) Roles of poly(ADP-ribosyl)ation and PARP in apoptosis, DNA repair, genomic stability and functions of p53 and E2F-1. *Adv. Enzyme Regul.* 40, 183–215
48 Hassa, P.O. and Hottiger, M.O. (2002) The functional role of poly(ADP-ribose) polymerase 1 as novel coactivator of NF-κB in inflammatory disorders. *Cell. Mol. Life Sci.* 59, 1534–1553
49 Yu, S.W. *et al.* (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* 297, 259–263
50 Chiarugi, A. (2002) Poly(ADP-ribose) polymerase: killer or conspirator? The 'suicide hypothesis' revisited. *Trends Pharmacol. Sci.* 23, 122–129
51 Virag, L. and Szabo, C. (2002) The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. *Pharmacol. Rev.* 54, 375–429
52 Menissier de Murcia, J. *et al.* (2003) Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. *EMBO J.* 22, 2255–2263
53 Augustin, A. *et al.* (2003) PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression. *J. Cell Sci.* 116, 1551–1562
54 Kanai, M. *et al.* (2003) Involvement of poly(ADP-Ribose) polymerase 1 and poly(ADP-Ribosyl)ation in regulation of centrosome function. *Mol. Cell. Biol.* 23, 2451–2462
55 Smith, S. *et al.* (1998) Tankyrase, a poly (ADP-ribose) polymerase at human telomeres. *Science* 282, 1484–1487
56 Kaminker, P.G. *et al.* (2001) TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon over-expression. *J. Biol. Chem.* 276, 35891–35899
57 Moss, J. and Vaughan, M. (1988) ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial toxins. *Adv. Enzymol. Relat. Areas Mol. Biol.* 61, 303–379
58 Corda, D. and Di Girolamo, M. (2003) Functional aspects of protein mono-ADP-ribosylation. *EMBO J.* 22, 1953–1958
59 Paone, G. *et al.* (2002) ADP ribosylation of human neutrophil peptide-1 regulates its biological properties. *Proc. Natl. Acad. Sci. U. S. A.* 99, 8231–8235
60 Okazaki, I.J. and Moss, J. (1998) Glycosylphosphatidylinositol-anchored and secretory isoforms of mono-ADP-ribosyltransferases. *J. Biol. Chem.* 273, 23617–23620
61 Lupi, R. *et al.* (2000) Endogenous ADP-ribosylation of the G protein β subunit prevents the inhibition of type 1 adenylyl cyclase. *J. Biol. Chem.* 275, 9418–9424
62 Herrero-Yraola, A. *et al.* (2001) Regulation of glutamate dehydrogenase by reversible ADP-ribosylation in mitochondria. *EMBO J.* 20, 2404–2412
63 Leno, G.H. and Ledford, B.E. (1989) ADP-ribosylation of the 78-kDa glucose-regulated protein during nutritional stress. *Eur. J. Biochem.* 186, 205–211
64 Takamura-Enya, T. *et al.* (2001) Mono(ADP-ribosyl)ation of 2′-deoxyguanosine residue in DNA by an apoptosis-inducing protein, pierisin-1, from cabbage butterfly. *Proc. Natl. Acad. Sci. U. S. A.* 98, 12414–12419
65 Borra, M.T. *et al.* (2002) Conserved enzymatic production and biological effect of *O*-acetyl-ADP-ribose by silent information regulator 2-like NAD^{+}-dependent deacetylases. *J. Biol. Chem.* 277, 12632–12641
66 Frye, R.A. (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem. Biophys. Res. Commun.* 273, 793–798
67 Muth, V. *et al.* (2001) Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. *EMBO J.* 20, 1353–1362
68 Langley, E. *et al.* (2002) Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *EMBO J.* 21, 2383–2396
69 Vaziri, H. *et al.* (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 107, 149–159
70 Kirkland, J.B. and Rawling, J.M. (2000) Niacin. In *Handbook of Vitamins*, 3rd edn, (Rucker, R. *et al.*, eds), pp. 211–252, Marcel Decker, New York