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Synopsis
Nicotinamide adenine dinucleotide (NAD^+) is well known as a crucial cofactor in the redox balance of metabolism. Moreover, NAD^+ is degraded in ADP-ribosyl transfer reactions which are important components of multitudinous signalling reactions. These include reactions linked to DNA repair and ageing. Here, using the concept of elementary flux modes (EFMs), we established all potential routes in a network describing NAD^+ biosynthesis and degradation. All known biosynthetic pathways, which include *de novo* synthesis starting from tryptophan as well as the classical Preiss-Handler pathway and NAD^+ synthesis from other vitamin precursors, were detected as EFMs. Moreover, several EFMs were found that degrade NAD^+, represent futile cycles or have other functionalities. The systematic analysis and comparison of the networks specific for yeast and humans documents significant differences between species with regard to both the use of precursors, biosynthetic routes and NAD^+-dependent signalling.

Key words
NAD^+ synthesis, NAD^+ salvage pathways, niacin, elementary flux modes, Preiss-Handler pathway, Sirtuins

Abbreviations used: Bna6, quinolinate phosphoribosyl transferase; Isn1, IMP-specific 5'-nucleotidase 1; NA, nicotinic acid; NaAD, deamino-NAD^+; Nam, nicotinamide; NaMN, nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyl transferase; NAR, nicotinate D-ribonucleoside; Nma1,2, nicotinamide/nicotinate-nucleotide adenylyltransferase 1,2; NMN, nicotinamide mononucleotide; Npt1, nicotinate phosphoribosyl transferase; Npy1, NAD^+ pyrophosphatase; NR, N-ribosylnicotinamide; Nrk1, nicotinamide riboside kinase; Pnc1, nicotinamidase 1; Pnp1, purine nucleoside phosphorylase 1; PRPP, 5-phospho-α-D-ribose-1-diphosphate; Prs1-5, ribose-phosphate pyrophosphokinases 1-5; QA, quinolinic acid; Qns1, glutamine-dependent NAD^+ synthase; ribose-P, ribose-phosphate; Sdt1, pyrimidine 5'-nucleotidase 1; Urh1, uridine hydrolase 1.

1. Introduction

Nicotinamide adenine dinucleotide (NAD^+) is well known as a co-enzyme of pivotal importance in the redox balance of metabolism, as it is continuously interconverted between an oxidized (NAD^+) and reduced (NADH) state. In the terminology of network theory [1], it is a "hub" because of its high connectivity, that is, the large number of reactions in which it participates. The reaction database in KEGG (release 51.0, [2]) identifies around 1600 reactions that use pyridine nucleotides for hydride transfer (all organisms stored in KEGG taken together).

Consumption of NAD^+ involves release of nicotinamide (Nam) and transfer of the remaining ADP-ribose moiety onto acceptor molecules. ADP-ribosyl transfer reactions are key constituents of intracellular signalling networks that control vital processes [3]. Protein acceptors are modified by mono-ADP-ribosylation, poly-ADP-ribosylation or NAD^+-dependent deacetylation and thereby undergo changes of their functional properties [3]. Poly-ADP-ribosylation appears to be confined to the nucleus of multicellular organisms and is involved in a variety of processes involving DNA rearrangements, transcription and repair [4]. NAD^+-dependent deacetylases (EC 3.5.1.-) represent a conserved class of enzymes [5] that transfer the acetyl group from modified proteins onto the ADP-ribose moiety liberated from NAD^+. They are also referred to as Sirtuins (based on the founding member, yeast Sir2), which also include structurally related mono-ADP-ribosyltransferases [5]. Sirtuins have a critical impact on the regulation of nuclear events (e.g., by histone deacetylation), but also on central metabolic enzymes such as acetyl-CoA synthase, carbamoyl phosphate synthase and glutamate dehydrogenase [5, 6]. Sirtuins have been found in prokaryotes and eukaryotes including yeast and mammals. However, neither mono- nor poly-ADP-ribosylation have been detected in yeast. Moreover, NAD^+ glycohydrolase (NADase) activity is also absent from yeast. NADases (EC 3.2.2.5) cleave NAD^+ into ADP-ribose (that is, ADP-ribosyl transfer onto water) or generate cyclic ADP-ribose by forming an intramolecular bond between the terminal ribose and the adenine ring [7]. Both ADP-ribose and cyclic ADP-ribose are potent intracellular calcium messengers acting on TRPM2 channels [8] or ryanodine receptors [9], respectively. Given the wide spectrum and permanent activity of NAD^+ consuming reactions, it is obvious that cellular NAD^+ pools need to be continuously replenished [10].

A well known route of NAD^+ synthesis is the Preiss-Handler pathway [11, 12]. In that pathway, nicotinic acid (NA) is converted to NAD^+ by consecutive addition of ribose-phosphate (from phosphoribosyl pyrophosphate, PRPP), AMP (from ATP) and an amino group (form ammonium or glutamine). Another widely found biosynthetic pathway of NAD^+, often referred to as *de novo* synthesis, starts from tryptophan (cf. [10, 13]). A number of lower organisms can alternatively synthesize NAD^+ *de novo* from aspartate [13]. These routes, too, require amidation of the NA moiety to NAD^+. Nam is also used as NAD^+ precursor. Interestingly, some, primarily lower, organisms deamidate Nam to NA, which is then used in the Preiss-Handler pathway, whereas other organisms convert Nam more directly to NAD^+ via nicotinamide mononucleotide (NMN) formation (cf. Fig. 1). NA and Nam are collectively known as niacin or vitamin B_3 and represent physiological precursors of NAD^+ in mammals (cf. [14]). Lack of niacin in the diet leads to the disease of pellagra. Tryptophan appears to be insufficient to fully maintain mammalian NAD^+ levels [14], but it may have an important role as NAD^+ precursor in some tissues (cf. [15]). Recent work identified the ribosides of NA and Nam (NAR and NR, respectively) as additional precursors which enter NAD^+ synthesis by alternative routes [16]. Whether riboside precursors can fully complement mammalian niacin deficiency is not known.

To understand and explore the emerging complexity of NAD^+ metabolism, a systematic analysis of the network comprising its biosynthesis and catabolic conversions is required. This can be performed by elementary-modes analysis [17]. Many metabolites can be synthesized or degraded on multiple routes, possibly with different molar yields. Elementary

modes provide a powerful tool for determining the degree of multiplicity and, thus, of redundancy [18] and to identify routes with maximum yields or minimal energy expenditure [17, 19]. An elementary flux mode (EFM) is a minimal set of enzymes that is stoichiometrically and thermodynamically feasible at steady state [17]. Any flux distribution in the living cell is a superposition of EFMs [20]. EFM analysis has been successfully applied to various systems such as central metabolism in *Escherichia coli* [18] and fatty acid synthesis in plants [21]; for review see [19]. Moreover, EFMs can readily be used to assess the impact of nutrients. This is done by checking which modes start from given nutrients and, thus, would be lost in their absence [22], and which conversions are infeasible even in their presence because the routes are stoichiometrically not balanced [23].

In this study, we first focus on a theoretical analysis of NAD^+ biosynthetic pathways in a generic network. We then compare the network characteristics of NAD^+ synthesis in mammals and yeast. As pointed out above, in yeasts, the variety of NAD^+-degrading signalling reactions appears to be limited to a single type (NAD^+-dependent deacetylation), whereas in mammals, it is far more extensive with regard to both the number of reaction types and processes they control. Therefore, comparative analysis of these two clades may provide information about the evolution of signal transduction. The analyses presented here provide a basis for the experimental verification of the role of identified EFMs and the development of a kinetic model of NAD^+ biosynthesis.

2. Methods

2.1. Metabolic model and data sources

The reaction scheme analysed here is depicted in Fig. 1 and is described in more detail in the Supplementary material. It represents a generic model covering the situation in many organisms, notably mammals and many yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* (cf. [13]). Organism-specific routes will be discussed below. The directionality of reactions was defined according to the binary distinction made in the original literature and the database BRENDA [24]. Information about the directionality is in many cases scarce. When necessary, we complemented that information based on established knowledge about similar reactions in other biochemical pathways.

In EFM analysis (and also in other modelling analyses of metabolism), a distinction is made between internal and external metabolites [17, 20]. Internal substances are assumed to be at steady state by a balance between production and consumption. External metabolites are assumed to have constant concentrations, because they are available in large excess or are exchanged across the boundaries of the system. The network (Fig. 1) contains the input of quinolinic acid (QA) generated from tryptophan and five exchange reactions associated with the metabolic pools of nicotinamide (Nam), nicotinic acid (NA), nicotinate D-ribonucleoside (NAR), N-ribosylnicotinamide (NR) and NAD^+. Very little is known about transport reactions associated with these NAD^+ precursors and intermediates and about the exact localization of the enzymes in the network. Therefore, we do not consider compartmentation. Moreover, the exchange reactions are to be interpreted in a more general sense. The corresponding substances might be degraded, converted into other substances or built from other compounds. Nevertheless, it is known that at least some of these substances can enter the cell, for example Nam and NA (vitamin B_3) or NR coming from the diet [16]. We denoted the exchange reactions by the suffix "_pool" and the metabolites present in these pools by the suffix "_ex".

An additional set of metabolites, such as ATP, ribose, ribose phosphate (ribose-P; corresponding both to ribose 5-phosphate and ribose 1-phosphate), glutamine, and glutamate were also considered external. This simplification was made in order to reduce the complexity of the problem and thereby focus only on the balance of the pyridine ring and 5-phospho-α-D-

ribose-1-diphosphate (PRPP). We included the reaction catalysed by ribose-phosphate pyrophosphokinases 1-5 (Prs1-5) so that the overall reaction associated to each EFM can be represented correctly in terms of ATP, ribose or ribose-P requirements. Since PRPP is an energy-rich compound, fixing it as external would lead to underestimation of the ATP demand. Note that the inclusion of Prs1-5 does not change the number nor the structure of the elementary flux modes computed from the network.

The presence of the corresponding genes in yeast and various human cells such as hepatocytes was verified based on the databases KEGG [2], BioCyc [25] and the literature (see Supplementary material for more details).

[Figure 1 here]

The presence of nicotinamidase (Pnc1, EC 3.5.1.19), in many bacteria and fungi (including yeasts) has been known for a long time. Recently, homologues of nicotinamidase were identified in *Caenorhabditis elegans* [26] and in *Drosophila melanogaster* [27]. NAD^+ synthase (Qns1, EC 6.3.5.1), in humans operates by using glutamine [28]. The consumption (labelled ADP-ribosyl transfer reaction in Fig. 1) reaction corresponds to a set of transformations all starting with the release of the ADP-ribose moiety. This set includes the mere hydrolysis, hydrolysis and subsequent conversion into cyclic ADP ribose by NAD^+ glycohydrolases (EC 3.2.2.5), hydrolysis and subsequent polymerisation to poly-ADP ribose by poly(ADP-ribose) polymerase (EC 2.4.2.30) and the transfer of ADP ribose to other moieties, such as arginine or acetyl (e.g. by SIRTUINs, see Introduction). We use the term ADP-ribosyl transfer as a general term even if ADP-ribose is just released or transformed into cyclic or poly-ADP ribose.

The export reaction Nam_pool involves, among others, the accumulation of Nam and its methylation by nicotinamide N-methyltransferase (EC 2.1.1.1). The enzyme NAMPT is absent from yeast and constitutes the main route of Nam salvage in humans [29]. Recently, yeast uridine hydrolase, Urh1, was found to catalyse the conversion of NAR and NR to NA and Nam, respectively [30, 31].

The reactions catalyzed by Npt1 and NAMPT were set to irreversible in the direction of ribonucleotide synthesis. Indeed, in the presence of ATP, the reaction equilibrium is shifted towards the ribonucleotide formation, because it is coupled with the hydrolysis of ATP [32, 33]. Although the exact ATP stoichiometry is unknown in the case of NAMPT, because the coupling is weak [33], we here use a 1:1 stoichiometry for both reactions of ribonucleotide formation.

2.2. EFM analysis

EFMs were computed by the program Metatool 5.1 [34] which is freely available from our website http://pinguin.biologie.uni-jena.de/bioinformatik/networks/. In the network depicted in Fig. 1, several enzymes can catalyze two reactions due to broad substrate specificity. In the model, these reactions were distinguished by adding a suffix '_2' to one of the reactions. In this analysis we considered the presence of a given enzyme when at least one of the associated reactions is present. The EFMs are classified in several subsets according to their presence in the organisms and to their functionality.

A tool for visualizing EFMs was developed using the software GraphMLViewer (*y*Works GmbH, Tübingen, Germany). The tool can be used online on our website http://pinguin.biologie.uni-jena.de/bioinformatik/networks/ (Access credential during the review process- username:BiochemicalJ password:kynurenine26).

3. Results

3.1. Overview of EFMs

The generic model gave rise to 113 EFMs listed in the Supplementary Material. They can be visualized by the tool mentioned in Section 2.2 (http://pinguin.biologie.uni-jena.de/bioinformatik/networks/; see above the details to access the tool). Three out of these EFMs contain enzymes absent from humans (Urh1 and Pnc1) and NAMPT not present in yeast. We did not immediately discard them, because they might be relevant in other organisms. In Fig. 2, eight selected EFMs are depicted.

[Figure 2 here]

In the metabolic network under study, there are 12 EFMs that produce NAD^+. The *de novo* synthesis from QA (kynurenine pathway) is represented in EFM 26 (Fig. 2a) and the Preiss-Handler pathway converting NA to NAD^+ [11, 12] (including the balancing of PRPP by synthesis from ribose-P) is represented in EFM 25 (Fig. 2b). There are three pathways associated with NAD^+ synthesis from NAR, all experimentally verified. One of them (mode 22) is Nrk-dependent [35] and two are Nrk-independent [31]. Among the latter, one is yeast specific, using Urh1 (mode 24) and the other is present in both organisms, using Pnp (mode 23). Both involve the Preiss-Handler pathway. There are 7 EFMs that start from amidated precursors, two from Nam and five from NR. One of the pathways for NAD^+ synthesis from Nam is yeast-specific, using Pnc1 and then the Preiss-Handler pathway (mode 20) [36], while the other is specific for humans. Mode 19 uses NAMPT to convert Nam to NMN, which is then converted to NAD^+ by NMNAT1-3 (the human homologues of the yeast enzymes Nam1,2) [37, 38]. Starting from NR, there is one EFM specific for humans (mode 29), which is Nrk1-independent and uses NAMPT and PNP (Pnp1 in yeast), and two that are exclusive for yeast (represented by modes 27 and 28, the latter is shown in Fig. 2e). Both take a detour via NA involving the yeast-specific enzyme Pnc1 and then use the Preiss-Handler pathway [39] and are Nrk1-independent as well. The Nrk-dependent pathway represented by mode 21 is present in both organisms [16].

Net consumption of NAD^+ leads to the generation of NA, NAR, Nam or NR in yeast and Nam or NR in humans. QA cannot be produced from NAD^+ due to the irreversibility of the reaction catalyzed by Bna6. There are in total 18 routes with NAD^+ net consumption, four of which proceed via the ADP-ribosyl transfer reaction and therefore, play a role in signalling.

An interesting observation is the number of EFMs that constitute interconversions between NAD^+ precursors. These EFMs account for ~2/3 of the EFMs computed from this network. For these interconversions enzymes Qns1 and Nma1,2 play an important role, being present in ~80% of them (Fig. 3). However, the relevant number is likely to be lower, because of compartmentation – NMNATs 2 and 3 (human homologues of yeast Nma1,2) are located in the Golgi complex and mitochondria, respectively [40]. It is largely unknown which NAD^+ derivatives can cross intracellular membranes and how the biosynthetic enzymes are distributed. Permeability constraints may thus reduce the number of these pathways.

[Table 1 here]

It is of interest to note that several EFMs in the network constitute futile cycles. Generally, futile cycles are defined as routes with no net transformation except hydrolysis of energy-rich compounds. There are five EFMs satisfying this definition. Two of them (modes 41 and 42) represent the overall transformation ATP → P_i + ADP and consist of the enzymes Nrk1 and Sdt1/Isn1. Mode 40 has the overall stoichiometry ATP + H_2O → PP_i + AMP and is composed of the enzymes Nam1,2 and Npy1. Interestingly, only four of these futile cycles are present in yeast, whereas the human model involves one additional futile cycle (mode 47, Fig. 2c)

composed of the reactions catalyzed by NAMPT, NT5 (the human homologue of the yeast enzymes Sdt1 and Isn1) and PNP. In a less strict sense, cyclic modes with no net consumption of NAD^+ or its precursors can be considered as futile cycles as well. Applying this definition, we find another six futile cycles in the network (Supplementary Material). Two of them result in the release of the amino group from glutamine. These modes require the enzymes Pnc1 and Qns1. Two of the futile cycles in the broader sense convert ribose-P into ribose and another two perform both this conversion and the deamidation of glutamine. Interestingly, these more broadly defined futile cycles require enzymes specific to yeast and are therefore absent in humans.

The presence of many futile cycles in the network can be explained by the existence of several enzymes catalysing similar reactions with opposite directions. Whether these futile cycles are of physiological relevance depends on compartmentation (not considered here) and on regulation in the cell. In fact, it was recently shown that Sdt1 and Isn1 are highly regulated [41]. For example, it was shown that Isn1 levels decrease when yeast is growing in low glucose medium [41]. Taking into account the fact that Isn1 takes part in many of the futile cycles, the regulatory effect can be seen as a way to reduce energy dissipation and thereby avoiding wasting resources. Therefore, detection of potential futile cycles by EFM analysis may indeed be helpful to identify critical nodes that require regulation (cf. [17]). Further data and studies on enzyme regulation and localization are required to determine the physiological significance of these cycles.

There are 21 EFMs that include ADP-ribosyl transfer (indicated as producing ADP-ribosyl-X). These EFMs account for NAD^+ net consumption. Interestingly, the majority of EFMs including generation of ADP-ribosyl-X also involve interconversion of precursors. There are two cyclic EFMs that produce ADP-ribosyl-X without net consumption of precursors. One of them is present only in yeast (mode 51, Fig. 2g) with the overall stoichiometry 4 ATP + gln + 2 H_2O + ribose-P → 3 PP_i + ADP + glu + NH_3 + 2 AMP + ADP-ribosyl-X. The other is specific for humans (mode 52, Fig. 2h) with the overall stoichiometry 3 ATP + ribose-P → 2 PPi + AMP + ADP-ribosyl-X (Figs. 2g, h, respectively). The human pathway (Fig. 2h) is of great physiological significance, because NAMPT is considered to be rate limiting and is subject to circadian control [42, 43].

Considering the energetic requirements of NAD^+ biosynthetic pathways, it is evident that, on average, about five ATP are required to generate one molecule of NAD^+ (Table 1). There is one EFM (mode 30) that is not present in any of the two organisms because it involves both Urh and NAMPT, which do not occur together in either organism. The least expensive pathways correspond to the salvage of the amidated NAD^+ precursors, NR and Nam. For example, mode 21, present in both organisms, starts from NR following the Nrk1-dependent pathway and requires only two ATP. Three pathways require four ATPs. Two modes (modes 29 and 19) which use the amidated precursors NR and Nam, respectively are only present in humans. Mode 22 (Figure 2d) uses NAR as precursor. Four EFMs, exclusively present in yeast (EFMs 20, 24, 27 and 28), require more ATP for NAD^+ synthesis. One third of the pathways require one mole of ribose-P.

3.2. Importance of enzymes within pathways

In Fig. 3, the frequency by which each enzyme is found in an EFM belonging to a specific subset of EFMs is represented. For example, Pnc1, which generates ammonium, is absent in humans and, consequently, there is no futile cycle (in the broader sense) of glutamine deamidation involving Qns1, as opposed to what is observed for yeast. On the other hand, Nma1,2 is present in many EFMs, being essential for NAD^+ synthesis in both organisms.

It is interesting to see, in Fig. 3, that Sdt1 and Isn1 have a more preponderant role than ADP-ribosyl transfer in the EFMs consuming NAD^+. In humans, all the EFMs consuming NAD^+ convert it to Nam or NR. In general, our analysis indicates that the NAD^+ metabolism

of humans is more centered around the amidated ribonucleotides. In contrast, Npt1 plays an important role in the NAD^+ metabolism of yeast. In yeast, there are few EFMs producing NR from NAD^+ when comparing with the other three precursors.

In general, there are not many significant differences between the frequencies in which reactions occur in the subsets of both organisms, in spite of the network differences. It seems that the network differences have a bigger impact on the number of EFMs belonging to each subset. For example the subsets of EFMs corresponding to NAD^+ precursor inter-conversion and NAD^+ comsumption have more EFMs in yeast than in humans. Moreover, more than two thirds of the EFMs in yeast are specific for this organism.

[Figure 3 here]

4. Discussion

Here, we have performed a topological analysis of NAD^+ metabolism by using the concept of elementary flux modes (EFMs). This provides a systematic overview of the metabolic capabilities of that system including interesting effects on signalling. There is increasing interest in large-scale and even whole-cell metabolic models [44] and methods for topological analysis of such large networks are becoming available [45, 46]. However, medium-sized models describe in detail specific parts of metabolism and provide a valuable resource as well. They permit to derive non-trivial conclusions of great biochemical interest, which are easier to interpret in several aspects than the results from a large-scale model. Thus, we here consider a relatively small, yet complex system to study the interconnectivity between NAD^+ metabolism and signalling pathways. The network analysed here includes 23 reactions and is complicated enough that the entire set of pathways across the system cannot easily be seen by inspection. Consequently, we used computational methods to deal with the combinatorial complexity of the metabolic network and systematically, comprehensively and rapidly enumerated all the metabolic pathways enclosed in its structure. Moreover, we have included recently identified enzymatic reactions into the network, which are not yet included in whole-cell models.

The system under study only involves unit stoichiometric coefficients. Thus, path-finding methods based on graph theory [47] might be used. There has been a debate in the literature about the pros and cons of stoichiometric methods (as used here) and path-finding methods [23, 47, 48]. In our view, application of stoichiometric network methods was straightforward, rather than to check whether path-finding methods would have been sufficient to describe the metabolic system investigated.

We have considered the synthesis and degradation of NAD^+. We have not included the hydride transfer between NAD^+ and NADH, because this is involved in a huge number of redox reactions. Moreover, these reactions are irrelevant for the buildup and degradation of NAD^+ and the associated signalling processes. The detected EFMs comprise synthetic routes not only for NAD^+, but also for relevant NAD^+ precursors such as N-ribosylnicotinamide (NR). From the 113 EFMs computed for the generic network, 100 correspond to yeast, 50 correspond to humans of which 40 are common to both organisms, and 3 do not occur in any of the two organisms. The higher abundance of pathways in yeast is surprising, because of the more diverse ways of NAD^+-related signalling in humans. Moreover, a general tendency is that the human network is primarily based on pathways involving the amidated forms of the NAD^+ derivatives while yeast uses preferentially the deamidated forms.

Several of the obtained EFMs can be assigned to well-known pathways. The Preiss-Handler pathway [11, 12] produces NAD^+ from nicotinic acid (NA), which humans must take up in their diet. To what extent intestinal flora produces NA as NAD^+ precursor remains to be determined [14]. One specific observation is that Na is produced from Nam by deamidation by bacterial nicotinamidase in the gut (cf. [14]).

The kynurenine pathway has also been found in the form of an EFM. It provides quinolinic acid (QA) as NAD^+ precursor, which originates from tryptophan, Moreover, we have identified the EFMs for the Preiss-Handler independent NAD^+ synthesis routes present in fungi and humans, which start from NR. They use either Nrk [16] or the more recently described Nrk-independent pathway [39]. NR was identified in cow milk [16]. Thus, it can be speculated that it is used as a precursor for NAD^+ by infants and animal sucklings, since milk is low in NA [49]. Moreover, it is interesting that the most economical pathways in terms of ATP consumption are those salvaging the amidated NAD^+ precursors, NR and nicotinamide (Nam). Another interesting nutritional fact is that corn has only very limited contents of NA without being enriched by other NAD^+ precursors. Thus, a predominant consumption of corn can cause pellagra [49].

Many of the above-mentioned biosynthesis/salvage pathways are likely to occur in the liver, kidney and small intestine (cf. [15, 38, 50]). Thus, these organs would be responsible for the levelling of Nam in the serum and, consequently, mainly pathways producing Nam are operative therein, while other organs may preferentially use Nam for NAD^+ generation. Our analyses indicate that, in yeast, nicotinate D-ribonucleoside (NAR) can be made from NR, Nam, NA and QA, while in humans, it can only be produced from NA and QA. The tissue distribution of the various pathways in humans could thus reveal interesting interdependencies between different organs with regard to NAD^+ supply.

We found two EFMs that do not involve net formation or use of NAD^+ (apart from the futile cycles). One of them is present in yeast and the other in humans (Fig. 2g,h). Both transform ribose-phosphate and ATP into an ADP-ribosyl moiety and proceed via Nam. This indicates that NAD^+-dependent signalling can proceed without net consumption of NAD^+ and that NAD^+ signaling activity can be investigated by measuring the Nam pool. Thus, at steady state, signalling need not involve a loss in the NAD^+/NADH pool. Whether these pathways are indeed at steady state and physiologically relevant depends on kinetics and regulation, which we have not considered here. It was recently found [42, 43] that the NAD^+ salvage pathway shown in Fig. 2h is under control of the circadian clock. Both the expression of NAMPT and the liver NAD^+ levels oscillate in a clock-dependent manner. These experimental findings highlight the importance of Nam utilization by NAMPRT in mammals, as they point to a strong link between the circadian clock and NAD^+ metabolism.

Pathway and flux analyses in the NAD^+ network are of interest in the framework of research related to ageing. Several hypotheses on the role of the positive effect of calorie restriction on longevity and its relation to NAD^+ metabolism have been put forward (cf. [41, 51]). One of them states that calorie restriction increases NAD^+ turnover without altering steady-state NAD^+ levels [41, 52]. This suggestion would correspond to the cyclic modes consuming ribose phosphate and producing ADP-ribosyl-X. However, it appears questionable whether increased consumption of ribose for NAD^+ turnover is compatible with calorie restriction.

It is of interest to compare our results with mutation studies. For example, the mutant strain Δ*nrk1*Δ*urh1*Δ*pnp1* of *S. cerevisiae* was studied and it was found that it accumulates Nam when NA is supplied in the growth medium [41]. When deleting the enzymes Nrk1, Urh1 and Pnp1, 23 EFMs remain, including a cyclic mode corresponding to steady-state signalling. Five out of these EFMs consume NAD^+ and three produce NAD^+ from QA, NA and Nam. From the former, there are two pathways producing NR; the remaining three produce one of the following precursors: NA, Nam and NAR. NR is also an important precursor for NAD^+ synthesis in other mutant strains. This allows cross-feeding between the wildtype of *S. cerevisiae*, which can secrete NR, and mutants that take it up [53].

We have made a distinction between futile cycles in the strict and in the broader sense. The overall transformation of the former is a hydrolysis of ATP. The latter hydrolyse ribose-phosphate or deamidate glutamine. Thus, the definition of futile cycles depends on what is

considered as a cofactor. Moreover, there are cyclic routes producing ADP-ribosyl-X. These play a role in signalling and, thus, form a bridge between metabolism and regulation.

We have not considered compartmentation in the present model, as this would have significantly increased complexity. Moreover, the subcellular distribution of NAD^+ biosynthetic enzymes as well as the presence of relevant metabolite carriers in organellar membranes still need to be conclusively determined. The present EFM analysis in yeast and humans is in agreement with experimental observations and thus provides a valuable tool for similar analyses of bacteria (e.g. enterobacteria, relevant for humans) and other clades. The generic model includes all possible conversions presently known and can thus readily be adopted to the specific subsets in other organisms.

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Tables and Figures

ATP	ADP	ribose-P	EFMs
−2	1	0	21
−4	3	0	22, 29
−4	3	−1	19
−5	4	0	30
−5	4	−1	26
−6	5	0	23, 27
−6	5	−1	20, 25
−7	6	0	24, 28

Table 1. Overall stoichiometry of ATP and ribose phosphate in NAD^+ synthesis pathways. Negative and positive values correspond to consumption and production, respectively. AMP production as well as the production of ribose were converted to extra dephosphorylation of ATP.

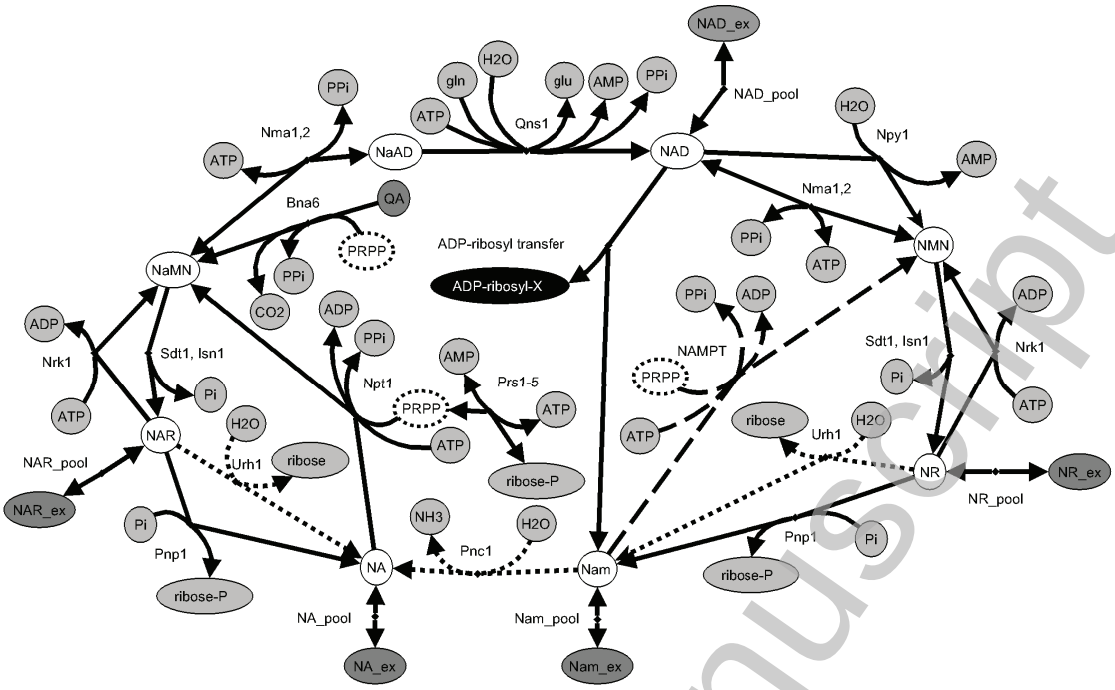

Fig.1. Overview of NAD$^+$ metabolism as considered here for modelling. See List of Abbreviations for names of metabolites and enzymes. Irreversible and reversible reactions are indicated by unidirectional and bidirectional arrows, respectively. White ellipses, internal metabolites; black ellipse, ADP-ribosyl-X designates the formation of ADP-ribose derivatives from NAD$^+$ in various signalling reactions; dark grey ellipses, external metabolites representing the pool of NAD$^+$ and its precursors; light grey ellipses, other external metabolites. Gene name abbreviations correspond to yeast except for the reaction catalyzed by nicotinamide phosphoribosyltransferase (NAMPT) which is present in human, but not yeast. Dashed arrow, reaction specific to humans; dotted arrows, reactions specific to yeast. The graph was drawn with yEd software package.

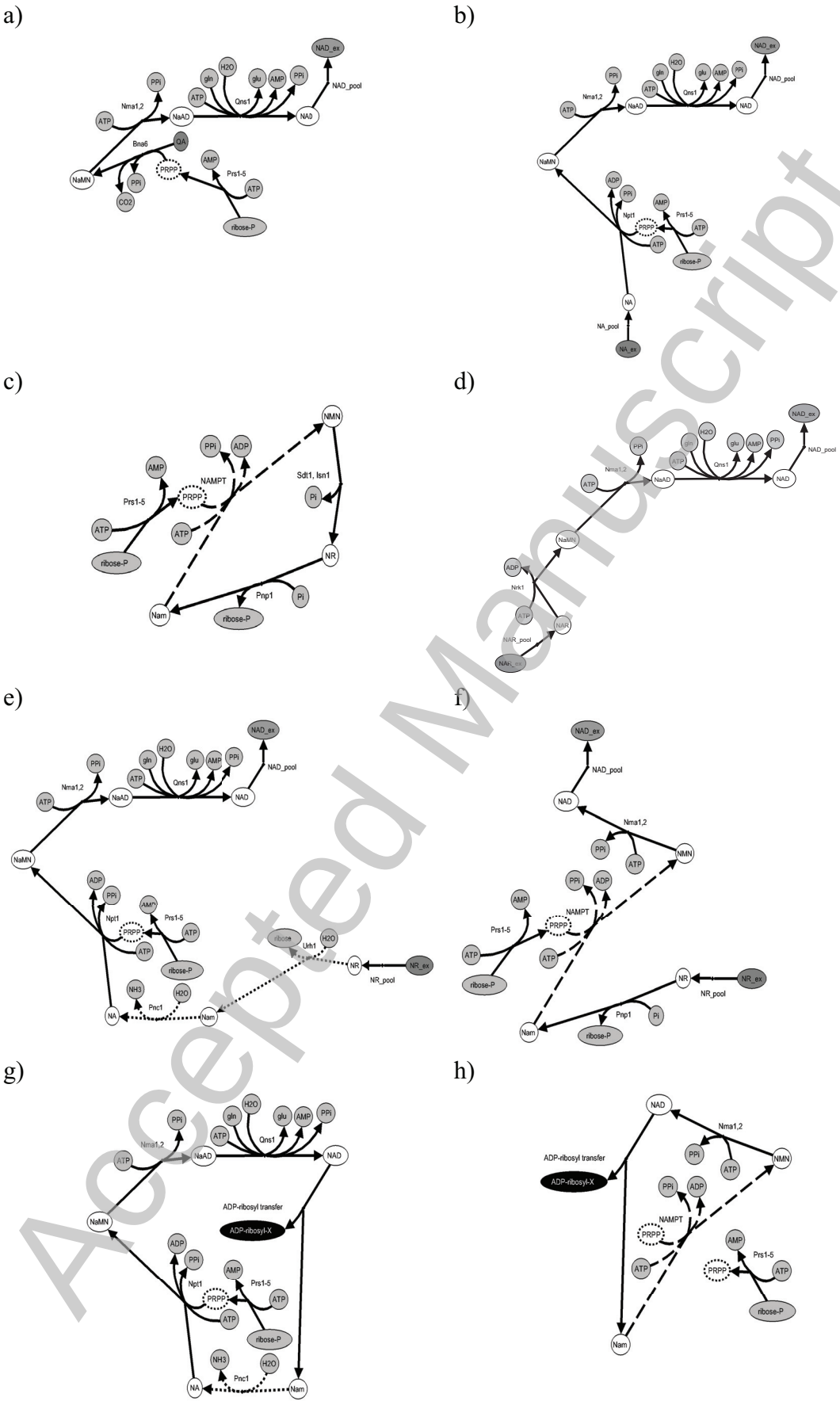

Fig. 2. Visualization of elementary flux modes in NAD$^+$ metabolism. (a) *de novo* biosynthesis of NAD$^+$ from QA, which is generated from tryptophan in the kynurenine

pathway, mode 26; (b) Preiss-Handler pathway, mode 25; (c) futile cycle in human, mode 47; (d) Nrk-dependent pathway for NAR salvage (Tempel *et al.* 2007), mode 22; (e) Nrk-independent NAD^+ pathway of NR utilization in yeast (Belenky et al. 2007b), mode 28; f) Nrk-independent NAD^+ salvage pathway in humans, mode 29; (g) steady-state signalling pathway in yeast, mode 51; (h) steady-state signalling pathway in human, mode 52. Dashed arrow, reaction specific to humans; dotted arrows, reactions specific to yeast.

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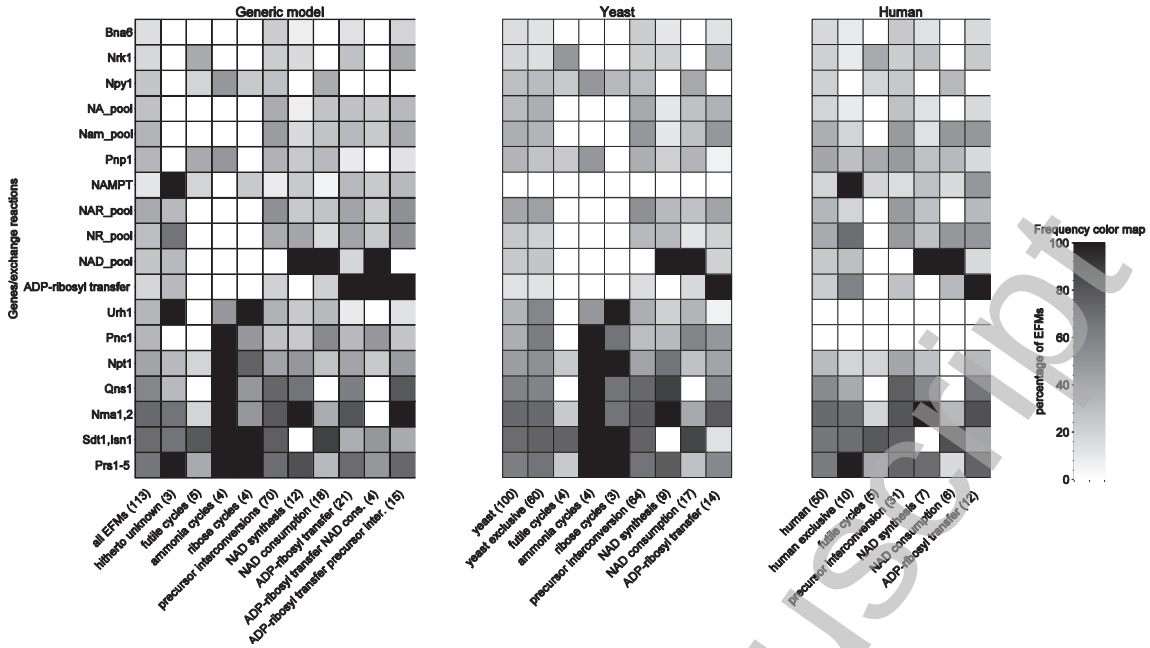

Fig. 3. Presence of enzymes/exchange reactions in each EFM subset. The numbers in brackets correspond to the number of EFMs present in the subset. Only futile cycles in the "strict sense" (see text) are shown here. Futile cycles in the broader sense are here classified amidating/deamidating cycles (termed "ammonia cycles") and cyclic pathways consuming ribose-P and releasing ribose (termed "ribose cycles"). All the pathways termed "ADP-ribosyl transfer" involve the reaction with the same name. The pathways additionally specified as "NAD cons." or "precursor inter." involve NAD^+ consumption or precursor interconversion, respectively. The colour code continuously ranges from black (enzyme is absent from all EFMs) to white (present in all EFMs).