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Longevity of major coenzymes allows minimal *de novo* synthesis in microorganisms

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Abstract

Coenzymes are vital for cellular metabolism, and act on the full spectrum of enzymatic reactions. Intrinsic chemical reactivity, enzyme promiscuity and high flux through their catalytic cycles make coenzymes prone to damage. To counteract such compromising factors and ensure stable levels of functional coenzymes, cells use a complex interplay between *de novo* synthesis, salvage, repair, and degradation. However, the relative contribution of these factors is currently unknown, and so is the overall stability of coenzymes in the cell. Here we use dynamic ^{13}C -labelling experiments to determine the half-life of major coenzymes of *Escherichia coli*. We find that coenzymes, such as PLP, flavins, NAD(P) and coenzyme A are remarkably stable *in vivo*, and allow biosynthesis close to the minimal necessary rate. In consequence, they are essentially produced to compensate for dilution by growth and passed on over generations of cells. Exceptions are antioxidants, which are short-lived, suggesting an inherent requirement for increased renewal. While the growth-driven turnover of stable coenzymes is apparently subject to highly efficient end-product homeostasis, we exemplify that coenzyme pools are propagated in excess in relation to actual growth requirements. Additional testing of *Bacillus subtilis* and *Saccharomyces cerevisiae* suggest that coenzyme longevity is a conserved feature in biology.

Keywords

vitamin; half-life; dynamic labelling experiment; homeostasis; salvage

Introduction

A large proportion of enzymes depend on organic cofactors to enlarge the biochemical potential of cellular metabolism that can be generated by proteins alone¹. In fact, all enzyme classes harbour members that depend on organic cofactors, with oxidoreductases being the most prominent representative as more than 80% of these enzymes require coenzymes such as NAD¹. Unlike most metabolites, coenzymes act mainly catalytically and their

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Data availability. Data obtained from LC-MS measurements of long-term dynamic labelling experiments generated as part of this manuscript are provided in Supplementary Data Files 1-5. The raw data from short-term labelling experiments used for Fig. 2a can be obtained from MetaboLights59 (accession number MTBLS229). Further data that support the findings of this study are available from the corresponding author upon request.

Author contributions. J.H., P.K., F.M. and J.A.V. planned the project and designed the experiments. J.H. and F.M. performed the experiments. J.H. and P.K. set-up LC-MS methods and data analysis. J.H. performed measurements and data analysis. J.H. and J.A.V. wrote the manuscript with input from all authors.

configuration is reverted back to its original state usually by a second enzymatic reaction (e.g. in redox reactions). This is referred as catalytic turnover and typically occurs at high rate. For instance, based on the activity² and abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)³ alone, the complete pool of NAD⁴ can be estimated to undergo a whole redox cycle on a (sub-) second timescale in a growing culture of the Gram negative model bacterium *Escherichia coli*.

Coupled with the intrinsic but necessary reactivity of organic coenzymes⁵, such large catalytic turnover comes with an elevated risk of imperfection or damage. First, enzymes are promiscuous ("sloppy")⁶, which can result in chemical corruption of coenzymes such as the hydration of NADH by GAPDH⁷. Second, purely based on their chemistry, metabolites undergo spontaneous reactions inside cells such as oxidation, hydro- or photolysis, elimination or condensation^{8,9} - augmented through the presence of reactive molecules^{8,10}, or other distinguishing conditions inside cells (e.g. respective redox status and pH). It has been argued that especially coenzymes are among the intracellular metabolites most prone to damage *in vivo*^{5,8,11–14} and are thus overrepresented in the group of core cellular metabolites that are particularly exposed to compromising reactions⁸. However, instability would be detrimental and lead to loss of available coenzymes. Consequently, the cell must increase turnover, here, dispose of compromised coenzymes and rebuild them or use means to counteract damage. Strategies to protect coenzymes include repair mechanisms and pre-emption of coenzyme damage (and other metabolites), which are subject to intensive investigations^{5,12–15}, also due to their emerging role in context of disease^{5,11,15,16}. Indeed, coenzymes are prominent among these metabolite proofreading systems and are found across all kingdoms of life⁵.

Despite the pivotal role of coenzymes in metabolism, their actual *in vivo* stability at the cellular level remained unknown. Although intimately linked to it, similarly little is known about the actual turnover of the organic (carbon-) backbone of coenzymes in the cell, here defined as pool turnover (opposed to the catalytic turnover), i.e. the rate at which coenzymes are made to replenish pools during growth and replace defective species. Curiously, early nutritional studies on vitamin requirements in mammalian systems suggest relatively low exchange rates^{17,18}. However, actual pool turnover and propagation of organic coenzymes on a cellular level in a well-controlled setup that allows untangling the factors influencing coenzyme homeostasis such as synthesis rate, damage, and decay - or stability (Fig. 1), remained elusive.

To address this knowledge gap, we performed metabolic tracer analysis (MTA), a powerful method for investigation of cellular metabolism, as the rate of isotope enrichment into metabolites can be used to determine pathway activity and turnover of metabolites¹⁹. Here, we conducted ¹³C dynamic labelling switch experiments and employed liquid chromatography high resolution mass spectrometry (LC-HRMS) in order to measure all possible isotopologues related to ¹³C incorporation and ¹²C depletion into the backbone of coenzymes with a variety of physicochemical properties and function. The resulting isotopologue distributions were used to determine pool turnover based on the decay rate of unlabelled (M₀) coenzymes over time and the corresponding half-life T₅₀ (i.e. the time required to exchange half of the unlabelled pool). While this carbon-based labelling

approach is blind to potential (repair-) reactions that do not affect carbon-carbon bonds, it effectively determines propagation rates and hence the outcome of coenzyme homeostasis. Because coenzymes such as NAD or coenzyme A (CoA) are also substrate for non-catalytic reactions where they are partially consumed but reassembled by salvage, we developed and extended a tandem-HRMS to obtain positional label information to correct for such reactions. In addition to switch experiments from ^{12}C to ^{13}C , we also reversed the label and excluded the potential effect of ^{13}C isotopes on cellular metabolism and demonstrated that our results are highly reproducible (Supplementary Fig. 1).

We found that a structurally, functionally, and chemically diverse set of organic coenzymes are produced only to compensate for dilution by cell division without active turnover. This highlights the effectiveness of the cellular machinery to control coenzyme chemistry and reduce or reverse potential off-target effects. Moreover, we imply a high degree of end-product homeostasis in coenzyme metabolism, which is effectively sensing concentration changes during growth, maximizes coenzyme propagation over generations, while concomitantly enabling enzyme saturation.

Results

Global metabolite pool turnover by dynamic labelling experiments and LC-HRMS

Initially, we used the model bacterium *E. coli* that synthesizes all organic compounds *de novo* from the supplied carbon source (here glucose). We then performed ^{13}C labelling experiments coupled with LC-HRMS and applied a recently developed untargeted metabolome analysis strategy²⁰ to determine pool turnover of small molecules in the cell. The LC-HRMS method and sample preparation employed were designed to capture a large proportion of organic coenzymes. Strikingly, label incorporation into measured coenzymes occurred at low rate with exchange rates close to the doubling time (DT, here 59 min) (± 0.5 DTs) (Fig. 2a, red dots), indicating that mainly *de novo* synthesis countering dilution by cell division dictates pool turnover. Such reduced conversion rates are, however, not a common feature of metabolites in the cell (Fig. 2a, grey dots) as expected²¹ and reflect well that many small molecules are pathway-intermediates that are rapidly turned over.

Pyridoxal 5-phosphate and flavins are stable *in vivo*

Next, we performed a targeted analysis and compared the decay of unlabelled coenzymes (Figs. 2c, 3, Supplementary Figs 1-2 black solid lines) with the null hypothesis, which is that *de novo* synthesis compensates for enzymatic dilution as a consequence of growth (Figs. 2c, 3, Supplementary Figs 1-2 red dashed lines). First, we analysed the turnover of pyridoxal 5-phosphate (PLP), a coenzyme that acts purely catalytically, but is prone to damage^{5,8,22}. Most notably, the labelling kinetics of PLP matched the first-order kinetic model based on the observed growth rate (Fig. 2b and c; T_{50} of 54 ± 2 min). Thus, PLP synthesis accounts only for dilution by growth, and a combination of chemical stability *in vivo* (either intrinsic, or promoted by repair systems against e.g. half-transaminations^{5,22}), together with a high degree of end-product homeostasis, keeps *de novo* synthesis at the minimum necessary to maintain stable PLP pools.

Additionally, we found that flavin mononucleotide (FMN), despite the proposed chemical instability and potential for irreversible binding^{5,23}, showed similar labelling kinetics (Fig. 2c; T_{50} of 56 ± 3 min). Note, however, that turnover of FAD was moderately faster with a T_{50} of 40 ± 2 min.

The catalytic carbon backbones of CoA and NAD undergo minimal exchange

In agreement with their additional substrate function in non-catalytic reactions, CoA and NAD turned over more quickly (Fig. 3a and e, T_{50} of 29.9 ± 1 , 28.3 ± 1 min). Indeed, we noted a build-up of distinct isotopologues for CoA and NAD (Fig. 3a, e, red arrows). In the case of CoA, the isotopologues (M11/12; M2 shift originates from acetylation) indicate a rapid exchange of the nucleotide moiety of CoA, which is congruent with a role of CoA to provide its 4-phosphopantetheine moiety to apo-acyl-carrier-proteins (apo-ACP), forming the active holoenzyme (Fig. 3b). In *E. coli*, a phosphodiesterase is able to cleave holo-ACP, thereby releasing 4-phosphopantetheine, which together with ATP is reassembled into CoA²⁴. We developed a tandem HRMS approach to determine the turnover of the catalytic core of CoA (i.e. 4-phosphopantetheine) and found that it matched the generation time of *E. coli* (T_{50} of 60 ± 3 min, Fig. 3d, Supplementary Table 3) thus revealing that *de novo* synthesis is at minimum.

Similarly, NAD is not exclusively used as a key redox coenzyme but also consumed in metabolic reactions by cleavage to NMN and recycling via a salvage pathway^{25–27} (Fig. 3f). We demonstrated that the build-up of the observed isotopologues M9/10 originates solely from AMP exchange (Supplementary Fig. 3g and h), allowing us to correct for label incorporation into these mass traces. We found that the pool turnover of the catalytic niacin moiety in NAD propagation closely coincides with the generation time of *E. coli* (Fig. 3h, T_{50} of 50 ± 1 min), thus highlighting its stability and the efficiency of salvage reactions. While the labelling dynamics and isotopologue distribution (ID) of NADP was similar to NAD (Supplementary Fig. 2a), we observed a delay of label incorporation. This finding is in-line with NAD being the precursor of NADP, but suggests low exchange rates between NAD/NADP pools. Overall, we show that dilution by growth explains turnover of key coenzymes in the cell, which demands exceptional stability under *in vivo* conditions.

Minimal pool turnover is not an intrinsic feature of all coenzymes

Minimum pool turnover of coenzymes is not a general rule in *E. coli*: The antioxidant glutathione was far more short-lived, as the unlabelled glutathione pool turned over more than twice every doubling (Supplementary Fig. 2c), suggesting that growth-independent factors dominate exchange. Although we can only speculate about the origin and necessity for such high turnover of glutathione, its anti-oxidant role, and thus challenging (oxidative) microenvironment, makes chemical damage a likely candidate. Indeed, it has been shown in yeast that glutathione is continuously degraded and reassembled²⁸.

Although longer-lived compared to glutathione, pool turnover of tetrahydrofolate (THF) still exceeded the requirement explained by growth alone (Supplementary Fig. 2a). THF decayed with a biphasic kinetics, indicating additional factors contributing to carbon-exchange. Potential explanations include the susceptibility of THF-derivatives to (photo-) oxidative²⁹

or enzymatic cleavage³⁰ or incomplete clearance of damaged folates such as 5-formyl-THF, despite observed recycling³¹. It should also be noted that the extensive polyglutamination of THF in *E. coli*³² (we detected up to 7 glutamates, Supplementary Fig. 4b) might explain additional sources of label incorporation leading to accelerated depletion of the unlabelled fraction.

Lastly, as expected, the carbon backbone of ATP and GTP had high exchange rates (T_{50} of 4.7 ± 0.3 ; 4.6 ± 0.3 min, Supplementary Fig. 2a, Supplementary Table 1). This can be explained by most of the nucleotides being incorporated into nucleic acids and does not indicate reduced stability of these building blocks.

Dilution by growth dictates coenzyme pool turnover

Because the half-lives of most coenzymes closely coincided with the doubling time for *E. coli* grown on glucose, we next investigated whether a lower imposed growth rate would translate to a lower coenzyme turnover. We employed a variety of carbon substrates supporting reduced generation times compared to glucose, conducted $^{12}\text{C}/^{13}\text{C}$ switch experiments and sampled after 60 min of labelling and after completion of one doubling, i.e. after ~60 min (glucose), ~81 min (fructose), ~82 min (glycerol), ~145 min (pyruvate). We found that the reduction of label incorporation strongly correlated with the respective reduction in growth-rate (Fig. 4a, c), and dilution by growth was directly correlated to renewal of long-lived coenzymes. Likewise, when sampled after one generation, label incorporation into the identified stable coenzymes (or their respective catalytic backbone), i.e. PLP, FMN, FAD, CoA and NAD was still close to minimal (Fig. 4b). As expected, THF and especially glutathione (GSH) turnover was overall dominated by growth-independent factors, as with increased doubling time only little unlabelled coenzyme remained after one doubling (Fig. 4b).

To ensure that an altered growth-rate rather than the nature of the substrate determine coenzyme pool turnover, we used an *E. coli* strain with a deletion in *fdx*, which grows markedly slower (generation time ~ 92 min on glucose) compared to wild-type due to defects in iron-sulphur-cluster assembly³³. Indeed, essentially all investigated coenzymes had reduced exchange rates (Supplementary Fig. 5). Consequently, *E. coli* possesses the regulatory capacity to effectively adjust *de novo* coenzyme synthesis to counteract varying dilution rates independent of the mechanism of growth reduction.

Excess propagation of coenzyme pools prevents growth limitation

The observed minimal *de novo* synthesis rate of essential coenzymes provokes the question of how the coenzyme pools relate to actual growth requirements, whether they potentially limit growth or, alternatively, are propagated in excess. To address these issues, we used a set of auxotrophic *E. coli* mutants with defects in coenzyme biosynthesis. After withdrawal of the required vitamin supplement, the corresponding coenzyme pool will deplete over time (Fig. 5a). As long as the coenzyme concentration is in excess, continued exponential growth will be supported until a limiting concentration is reached and growth ceases (Fig. 5a red filled line). Alternatively, in case the coenzyme pool is propagated at its limit, impaired growth will be observed immediately as rate-determining reactions pace the population e.g.

to linear growth (Fig. 5a, red dashed line)³⁴. When testing seven auxotrophic mutants involved PLP, NAD(P), CoA and THF biosynthesis, the strains initially maintained exponential growth without supplementation (Fig. 5b), congruent with a previous study³⁵. Similarly, oxygen consumption rates during coenzyme depletion experiments initially remained undistinguishable from supplemented controls (Supplementary Fig. 6a). The observed residual exponential growth can be explained by excess levels of coenzymes (either inherent, or induced by supplementation), or by replenishment via remaining precursors (Fig. 5a). In order to distinguish these processes, we assessed the relative pool size of PLP, NAD and NADP in wild-type and auxotrophic mutants (*pdxA*, *nadB*) during steady state, and after removal of required vitamins. In wild-type and *pdxA*, pyridoxal addition had no effect on levels of PLP (Fig. 5c). Instead, supplementation with nicotinic acid led to doubling of NAD and NADP pools in both wild-type and *nadB* (Fig. 5d). As propagation rates of PLP (Fig. 2c) and NAD (Fig. 3h) are determined by growth (note, *nadB* maintains salvage ability), we anticipated that the respective intracellular pools would decline inversely with the growth after removal of vitamins. This was tested and confirmed experimentally for both PLP and NAD (Fig. 5c, d), demonstrating that no major additional sources (precursors) are tapped, thus providing independent confirmation for the above described stability of NAD and PLP by an orthogonal method.

Finally, we determined the relative pool size when exponential growth was no longer supported. These data indicated that the pool sizes of both PLP and NAD exceed the requirement for the actual demand, roughly two-fold (Fig. 5c, d). NADP levels decreased more slowly (Fig. 5d, right panel), which might be due to fuelling from the more than 20-fold larger NAD pool⁴. Overall, while coenzyme turnover is minimized, an impact on growth is prevented by excess propagation of functional coenzymes or respective precursor pools.

Coenzyme propagation in other microorganisms

Lastly, we asked if the uncovered congruence of growth rate and *de novo* coenzyme biosynthesis applies to other organisms, including the Gram-positive bacterium *Bacillus subtilis* and the eukaryote *Saccharomyces cerevisiae* (note, less coenzymes can be analysed due to vitamin auxotrophies). Overall, coenzyme turnover proved to be strikingly conserved (Table 1, Supplementary Figs. 7, 8). In agreement with early labelling studies^{26,36}, we noted that the half-life of NAD is substantially shorter in *S. cerevisiae* in relation to its doubling time (Supplementary Fig. 8), and this can be attributed to the major roles of NAD in AMP and ADP-ribose transfer reactions compared with bacteria^{25,27}. Notably, when correcting for salvage, turnover was again at the minimum. In *B. subtilis*, the magnitude of NAD turnover and salvage was comparable to *E. coli*, although recycling apparently also proceeds via nicotinamide (Supplementary Fig. 7). Unlike *E. coli* (Supplementary Fig. 2a), we did not observe delayed labelling of NADP, suggesting that the pools of NAD/NADP undergo substantially more exchange. Interestingly, THF turnover was minimized in *B. subtilis* (Supplementary Fig. 7a), demonstrating that *in vivo* stability of folates can be achieved. Curiously, this correlated with shorter polyglutamate chains of THF compared to *E. coli* (Supplementary Fig. 4b and c). In contrast, a major anti-oxidant of *B.*

subtilis, bacillithiol37, had remarkably high exchange rates (Supplementary Fig. 7a, T_{50} of 5.6 ± 0.1 min).

Discussion

Understanding metabolism necessitates knowledge on the topology of metabolic networks, metabolic pool sizes and their turnover. Beyond doubt, organic coenzymes are among the most crucial metabolites in the cell; however, in contrast to comprehensive information on their catalytic turnover, insights on pool renewal was essentially lacking. Here we found that *de novo* biosynthesis of major coenzymes closely matches dilution in consequence of growth. Curiously, this shows that macromolecules like enzymes can actually be outlived by their substrate. The longevity of coenzymes becomes possible due to their catalytic nature, minimal propagation rates, and requires *in vivo* stability of these core metabolites. This stability is achieved despite the very large number of catalytic cycles each coenzyme undergoes. The extended life of coenzymes beyond the here observed conservation of carbon-carbon bonds might well be metabolically enabled, and the contribution of emerging metabolite repair systems⁵, remains to be shown.

To ensure coenzyme homeostasis, highly effective regulatory systems are required. For coenzymes, this precise tuning is achieved in spite of the apparently low flux through *de novo* synthesis pathways, e.g. by riboswitches³⁸ and enables dissemination of coenzymes over the maximal number of generations.

Although the susceptibility of coenzymes to chemical and metabolic detriment has been argued to necessitate damage control systems⁵, their emergence might not only enable coenzyme longevity but also be a consequence for their requirement. Coenzymes are usually metabolic end-products, and in agreement with our data not generally converted by chemical flux or other means of exchange. Thus the probability of damage compared to many other molecules is largely increased based on their extended half-life and repeated re-usage in the cell. The finding that major coenzymes are mainly renewed by cell division implies that the prevention of damage to coenzymes becomes increasingly important the slower cells divide and might additionally explain why knock-outs of metabolic proofreading or damage control systems, which often act on coenzymes, can have severe phenotypic effects in mammals, but not microorganisms^{5,15,16}. In the latter, however, their importance can be context-dependent as challenging conditions may lead to an accumulation of damage products³¹.

The longevity of coenzymes allows interesting parallels to protein turnover where high propagation rates have already been demonstrated^{39–42} and especially in microorganisms, dilution by growth seems to be the key determinant for turnover. Such a low rate of renewal harbours the danger of accumulating damaged proteins over time, and has i.e. contributed to their role as potential "senescence factors"⁴³. A similar role can be attributed to metabolites, which may act as both damaged and damaging agents^{5,44}.

It has been previously shown that coenzymes such as NAD, THF, or CoA are metabolic replicators in that they are autocatalytic (i.e. required for their own production) across all domains of life^{45,46}. Thus, the respective metabolic networks are unable to start or proceed

without their presence. In that context, the here demonstrated combination of *in vivo* stability of coenzymes and the excess propagation of their pools provide an effective way of maintaining an active metabolism.

Finally, the here shown extended life of organic coenzymes opens a number of questions, including the degree to which metabolite proofreading systems act on organic coenzymes *in vivo*, the metabolic flexibility to adapt coenzyme pools to changing environmental conditions and potential links to cell cycle control, as well as coenzyme homeostasis in higher (multicellular) eukaryotes.

Methods

Strains, media and reagents

[U13C]-D-glucose (99%), [U13C]-glycerol (99%), [U13C]-D-fructose (99%) and [U13C]-sodium-pyruvate (99%) were purchased from Cambridge Isotope Laboratories. All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich whereby analytical standards were obtained in the highest available purity.

All strains used in this study are listed in Supplementary Table 2. Briefly, *E. coli* K12 BW2511347, *B. subtilis* 168 (*trp*-), and *S. cerevisiae* BY4741 with restored prototrophy48 were used throughout this study. All *E. coli* knockouts were obtained from the Keio collection47.

E. coli was cultivated in M9 minimal medium containing (g per litre): 6.78 Na₂HPO₄, 3 KH₂PO₄, 0.5 NaCl, 1 NH₄Cl, 0.049 MgSO₄·7H₂O, 0.0015 CaCl₂·2H₂O and trace elements (mg per litre) 0.5 FeCl₃·6H₂O, 0.09 ZnSO₄·7H₂O, 0.088 CuSO₄·5H₂O, 0.045 MnCl₂, 0.09 CoCl₂·6H₂O. Carbon source was 20 mM D-glucose unless otherwise stated. *B. subtilis* was cultured in M9 minimal medium with (g per litre) 5.64 Na₂HPO₄, 3 KH₂PO₄, 0.5 NaCl, 1 NH₄Cl, 0.246 MgSO₄·7H₂O, 0.012 CaCl₂·2H₂O and trace elements (mg per litre) 15.98 FeSO₄·7H₂O, 0.77 MnCl₂, 3.59 ZnSO₄·7H₂O, 0.63 CuSO₄·5H₂O, 0.6 CoCl₂·6H₂O and 0.6 Na₂MoO₄·2H₂O49. Carbon source was 20 mM D-glucose. Media was additionally supplemented with 0.12 mM L-tryptophan. *S. cerevisiae* was grown in F1 minimal medium containing (g per litre) 3.13 (NH₄)₂SO₄, 2 KH₂PO₄, 0.1 NaCl, 0.55 MgSO₄·7H₂O, 0.09 CaCl₂·2H₂O and trace elements (mg per litre) 62 myo-inositol, 14 thiamine-HCl, 4 pyridoxine-HCl, 4 Ca-pantothenate, 0.3 biotin, 0.07 ZnSO₄·7H₂O, 0.01 CuSO₄·5H₂O, 0.01 H₃BO₃, 0.01 KI and 0.05 FeCl₃·6H₂O50. As carbon source 27 mM D-glucose was added.

Growth assays

Turbidity of cultures was determined by measuring the optical density at 595 nm ("OD₆₀₀") in semi-micro cuvettes (Bio-Greiner) employing a biophotometer plus (Eppendorf). Appropriate dilutions were prepared to keep the determined OD₆₀₀ in the linear range of the instrument.

Cell culture

Unless otherwise stated, all cells were grown as batch cultures in baffled (*E. coli*) or non-baffled (*B. subtilis*, *S. cerevisiae*) shake flasks in an Minitron incubator (Infors HT) shaking

at 120 rpm (*E. coli*, *S. cerevisiae*) or 200 rpm (*B. subtilis*). Cultivation temperatures were 37°C for bacteria and 30°C for yeast. Bacteria were streaked and grown from glycerol stocks (kept at -80°C) on LB-agar plates (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl, 20 g/l agar). Single colonies were used to inoculate overnight (o/n) cultures of 4 ml M9 minimal media in Falcon® round bottom tubes. Cultures typically reached an OD₆₀₀ of ~2.0. These o/n cultures were then used to inoculate fresh pre-cultures of 20 ml M9 minimal medium in 100 ml flasks (dilution 1:10 or adjusted to OD₆₀₀ of 0.2). After > 2 doublings (OD₆₀₀ of ~1.0) exponential steady-state growth was reproducibly reached and respective experiments were started. Yeast was grown similarly, but streaked on YPD-agar plates (20 g/l glucose, 10 g/l yeast extract, 10 g/l peptone, 20 g/l agar). O/n cultures were made in flasks with 20 ml F1 minimal medium and inoculated with various dilutions to ensure exponential growth in the morning (OD₆₀₀ of ~1.0). This culture was then used to inoculate fresh pre-cultures (adjusted to OD₆₀₀ of 0.4 by centrifugation and re-suspension in fresh media) and grown for > 1 doubling (OD₆₀₀ of ~1.0) until labelling experiments were started.

Vitamin depletion experiments

Auxotrophic *E. coli* strains were cultivated in M9 minimal medium with respective required supplements (10 µM 4-aminobenzoic acid, 10 µM pyridoxal-HCl, 20 µM nicotinic acid, 20 µM calcium D-pantothenate). Exponentially growing cells were harvested (2 min, 10.000 g at 25°C), the supernatant was discarded, and the pellet was washed with 20 ml pre-warmed (37°C) medium without (w/o) required supplements. This marked the starting points (t=0 min) of the experiments. Cells were again pelleted (2 min, 10.000 g at 25°C), the supernatant was discarded and the pellet quickly re-suspended in pre-warmed (37°C) minimal medium w/o supplements, the OD₆₀₀ was determined, and culture aliquoted to flasks agitated in a shaking water bath (37°C). Cultures were then quickly reconstituted with the required supplements (by addition of supplement from a 1000x stock. The control cultures were "mock" treated with dH₂O. Growth was resumed and OD₆₀₀ was monitored. The time required from the first addition of medium w/o supplement (t=0 min) to the initial OD₆₀₀ measurement was 5.5 min.

Sample preparation for LC-MS analysis

Bacterial samples (see above) were quenched and extracted by the fast-filtration method similarly as previously described^{51,52}. Briefly, 0.2 - 5 ml culture (corresponding to roughly 125 µg cell dry weight; 0.5 ml of OD₆₀₀ ~1.0) were applied to a polyethersulfone filter (PESU, 0.2 µm, Sartorius Setim, pre-washed with > 20 ml 50°C dH₂O) situated on a vacuum filtration setup. Cells were quickly separated from the medium (< 1 s), washed with 10 ml dH₂O, and the filter was immediately transferred to 8 ml pre-cooled (-20°C) organic acidified quenching and extraction solution (60:20:20 acetonitrile/methanol/water with 0.5 M formic acid (0.1 M final concentration), adapted from⁵³). After an incubation period of 10 min on ice and repeated sonification, the filter was removed, the extract was freeze-dried in liquid nitrogen, and lyophilized at -40°C. Subsequently, the dried extract was re-suspended in dH₂O and kept at -80°C until analysis.

Quenching and extraction of yeast cells was performed as described elsewhere⁵⁴. Briefly, cells were directly injected into methanol (maintaining a culture:methanol ratio of 1:3) pre-

chilled on dry ice. Cells were harvested by centrifugation (2 min, 4000 g, at 4°C), the supernatant was discarded and resulting pellets re-suspended in 200 µl 75:25 acetonitrile:methanol with 0.2% formic acid and incubated 20 min at 4°C. Cells were disrupted by vigorous shaking with glass beads by a FastPrep 120 (Thermo Savant, 3 cycles each for 20 sec). After centrifugation (5 min, 20.000 g at 4°C), the supernatant was taken off and kept on ice, the remaining pellet additionally extracted in dH₂O, centrifuged again, and resulting supernatants were pooled. Finally, pellets were dried with a SpeedVac (Eppendorf), re-suspended in dH₂O, and kept at -80°C until analysis.

Long term ¹³C dynamic labelling switch experiments

Prior to substrate switch, natural labelled sample of exponentially growing cells was obtained as reference. In ¹²C to ¹³C switch experiments, the same culture was then quickly harvested by centrifugation (1 min, 10.000 g), supernatant was discarded and the pellet was washed with 10 ml medium (pre-warmed to temperature of culture conditions) containing 20 mM (for bacteria) or 27 mM (for yeast) [U-¹³C]-D-glucose (99%, Cambridge Isotope Laboratories). This marked the starting point of the labelling experiment. The cultures were again pelleted (1 min, 10.000 g), the supernatant was discarded, and cells were re-suspended in 120 ml fresh medium containing [U-¹³C]-D-glucose, and transferred to 500 ml shake flasks sitting in a shaking water bath; growth was monitored (see Supplementary Fig. 9). After labelling switch, samples for LC-MS analysis were taken at 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 150 min, 180 min, 240 min, 300 min, 360 min and 420 min (only yeast). In-between sampling periods growth was resumed in a Minitron incubator (see above). For ¹³C to ¹²C switch experiments, o/n and pre-cultures were grown on [U-¹³C]-D-glucose and then switched to naturally labelled glucose. Respective data is provided in Supplementary Data 1-5. Additional labelling experiments upon growth with glucose, pyruvate, fructose and glycerol were performed as described above; cells were grown in 250 ml flasks with baffles containing 40 ml of respective labelled medium (20 mM substrate). Growth was monitored and samples taken after 60 min and when the OD₆₀₀ had doubled.

LC-HRMS and tandem LC-HRMS analysis

Microscale ultra-high-performance liquid chromatography high resolution mass spectrometry (UHPLC-HRMS) was performed using a Dionex UltiMate 3000 (Thermo Scientific) system hyphenated to a Q Exactive Plus (Thermo Scientific) instrument by a heated electrospray-ionization (HESI) probe. Metabolite separation was performed using a C18 column (Dr. Maisch Reprosil-Gold 120, 1.9 µm, 50 x 2 mm, Morvay Analytik) as stationary phase and Solvent A/B as a mobile phase. Solvent A consisted of the ion-pairing reagent tributylamine (TBA, 1.7 mM; TBA was first dissolved in 1.5 mM aqueous acetic acid; pH was adjusted to 9.0 with ammonium hydroxide). As an eluent methanol (Solvent B) was used with the following multi-step-gradient: 0 min, 3%; 9 min, 48%; 13 min, 90%; 15.3 min, 3%; 17.3 min, 3%. Sample injection volume was 10 µl, the flow rate was 500 µl/min. Mass acquisition was operating in negative Fourier transform mass spectrometry (FTMS) in full MS scan mode. Targeted MS experiments (Fig. 5c and d) were carried out by targeted-selective-ion-monitoring (t-SIM) with inclusion lists containing retention times and m/z values of the investigated molecules.

Targeted tandem MS experiments (by parent reaction monitoring; PRM) were performed in parallel to data independent MS scans with inclusion lists of pre-defined retention times and parent m/z values. In order to include all isotopologues of large parent molecules, but maintain a narrow isolation of corresponding isotopologue patterns multiplexing was applied setting isolation window width to 5.5 m/z , distributing parent m/z values over multiplexed windows accordingly (up to 5 windows per compound). Fragmentation was achieved by higher energy collisional dissociations (HCD) with a normalized stepped collision energy (nce, 25, 30, 35).

Nanoscale HPLC-HRMS was carried out with an EASY-nLC 1000 (Thermo Scientific) coupled to a Q Exactive Plus (Thermo Scientific) instrument applying the previously described method⁵⁵ which was slightly modified and adapted to the setup as follows: separation was achieved employing a C18 Column (Dr. Maisch Reprosil-Gold 120, 3.0 μm , 100 mm x 0.1 mm, Morvay Analytik) and Solvent A/B as described above. The gradient of the eluent (Solvent B) was: 0 min, 0%; 40 min, 90%; 50 min, 90%; 52 min, 0%; 53 min, 0%. Sample injection volume was 1 μl , the flow rate was 600 nl/min. Alternatively, nLC-ultra (Eksigent) was employed with Solvent B eluting with: 0 min, 3%; 27 min, 40%; 40 min, 90%; 45 min, 90%; 46 min, 3%; 56.5 min, 3%. Sample injection volume was 1 μl , flow rate was 400 nl/min. Data was acquired by negative FTMS operating in full MS mode. HESI source and mass acquisition settings of all LC-MS experiments are stated in Supplementary Table 3.

Metabolites were identified by their exact m/z values expected for respective adducts (mass tolerance of 3 milli mass units) and matching retention times by spiking analytical standards to ^{13}C bacterial extracts (see Supplementary Table 4). Due to high pH (9.0) of mobile phase most metabolites were identified in the oxidized form. Note, with the employed method the coenzymes listed in Supplementary Table 4 could be readily detected in cellular extracts, while other coenzymes such as thiamine, S-adenosylmethionine, or biotin could not be captured.

Relative pool size determination by isotope dilution

Relative pool sizes of metabolites were determined by the isotope dilution method⁵⁶. ^{13}C labelled extracts from *E. coli* serving as internal standards (IS) were prepared by extended growth (>15 generations) on [U13C]-D-glucose (99%) and stored at -80°C . These labelled extracts were then spiked into the quenching and extraction solution of the samples to be quantified. Relative pool size R of metabolites was determined as the fraction of the peak area of unlabelled (A_{M0}) over the fully-labelled (A_{MUL}) compound, normalized to the determined respective volume (V) and optical density (OD) of sample and IS:

$$R = \frac{A_{M0} \cdot V_{IS} \cdot OD_{IS}}{A_{MUL} \cdot V_{Sample} \cdot OD_{Sample}}$$

LC-MS data analysis

LC-MS level 1 and 2 data were analysed with the Python based framework eMZed²⁵⁷ (<http://emzed.ethz.ch/>). Retention time and m/z values of batch measurements obtained by

nanoscale LC-HRMS were aligned aided by a set of reference metabolites as previously described²⁰. Labelling fractions were determined by targeted peak integration of isotopic clusters, correction for natural labelled carbon (as explained previously^{20,58}), followed by calculation of isotopologue fractions:

$$f_{Mi} = \frac{A_{Mi}}{\sum_{j=0}^n A_{Mj}} \quad (\text{Eq.1})$$

where A_{Mi} is the area of isotopologue i , A_{Mj} the area of isotopologue j , and n the total number of carbon isotopes of the respective compound. Isotopologue fractions were plotted over time and fitted with non-linear regression employing the Python based SciPy (<https://www.scipy.org/>) optimization package. For selected models, calculation of half-life values and doublings and the applied correction for salvage reactions, see Supplementary Methods.

Additional data analysis and visualization

GraphPad Prism was used for plotting all bar plots in this manuscript and supplement, additional graphs in Figs 4 and 5 and Supplementary Figs 6 and 9 and spectra in Supplementary Fig. 3, as well as for the calculation of Pearson correlation coefficients and linear regression analysis.

Untargeted analysis of metabolite half-lives (T_{50})

T_{50} and number of carbon atoms were determined using DynaMet²⁰, a recently developed eMZed²⁵⁷ application, for untargeted extraction and characterization of metabolite labelling profiles. Short-term labelling data from *E. coli* ($T_{50} = 10$ min) was used as published²⁰ and long-term labelling data ($10 \text{ min} < T_{50} < 90 \text{ min}$) was originating from this study. The half-life of extracted compounds was estimated as the time needed to exchange half of the final ^{13}C labelling. Features with half-life values $\pm$ half a doubling time (i.e. ~ 30 min) were inspected and all features corresponding to coenzymes were manually annotated. Please note that due to the largely unsupervised untargeted approach, different adducts or in-source fragmentation products of grouped isotopologues might originate from the same compound and lead to partial redundancy of depicted data-points. Similarly, the unsupervised analysis can in some cases result in over- or underestimation of respective half-life values and carbon counts. For details see²⁰.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PLP	pyridoxal 5-phosphat
FMN	flavin mononucleotide
FAD	flavin adenine dinucleotide
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NMN	nicotinamide mononucleotide
PPi	diphosphate
(acetyl-) CoA	(acetyl) coenzyme A
PAP	phosphoadenosine phosphate
PRPP	phosphoribosyl disphosphate
R5P	ribose-5-phosphat
ADP-R	adenosine diphosphate ribose
THF	tetrahydrofolate
GSH	glutathione reduced
GSSG	glutathione oxidized
ATP	adenosine triphosphate
AMP	adenosine monophosphate
GTP	guanosine triphosphate
DLE	dynamic labelling experiment
ID	isotopologue distribution

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Figure 1.

Schematics of coenzyme homeostasis in prototrophic organisms. The pool of functional coenzymes (shown in green) is maintained by *de novo* synthesis from respective precursors (blue) to counteract factors diminishing the free coenzyme pool (corrupted/inaccessible coenzymes are indicated in red). These include damage, loss to extracellular space, dilution by growth and irreversible binding to other metabolites or macromolecules (grey). Repair systems correct common damage products; both damaged and functional coenzymes may undergo degradation. Lastly, coenzymes participate in non-catalytic reactions and are partially consumed, whereas the residual backbone can be recycled by salvage reactions.

Figure 2.

Turnover of organic coenzymes in relation to growth based on ^{13}C dynamic labelling in *E. coli*. **a**, Untargeted analysis estimating half-life of metabolites overall (grey) and coenzymes (red) plotted against the approximated carbon count of the respective molecules. **b**, Extracted ion chromatograms of the isotopologue distribution of PLP over time. Fraction of fully labelled and thus newly synthesized PLP is solely compensating dilution by growth ($\sim 1/2$ and $\sim 1/4$ after one, two doublings). **c**, Measured and predicted minimal labelling profiles of coenzymes (graphs) and corresponding isotopologue distribution (ID) shown as heatmaps (colour bar indicates scale). Open dots show decay of the unlabelled (M0) isotopologue fraction. Black solid lines illustrate corresponding fit obtained by non-linear regression. Red dashed lines represent first-order models predicting required minimal labelling incorporation based on dilution by growth. Corresponding half-life (T_{50}) values are shown in Table 1; data are from one DLE representative of three experiments. Assimilation of unlabelled bicarbonate contributes to incomplete labelling without affecting turnover (see Supplementary Fig. 1). For calculations, fitting and experimental information refer to Methods and Supplementary Methods. Additional information can be obtained from Supplementary Fig. 2.

Figure 3.

The catalytic core of CoA and NAD undergo minimal exchange. **a, e**, Measured and predicted minimal labelling profiles (graphs) and ID (heatmaps) for CoA (**a**) and NAD (**e**). For colour bar and further explanations see Fig. 2c. Red arrows indicate label incorporation independent of *de novo* synthesis leading to increased turnover. **b, f**, Schematics of PAP exchange in CoA as a result of acyl-carrier-protein (ACP) prosthetic group turnover (**b**) and the major NAD salvage route in *E. coli* (black) with simplified additional pathways of relevance to other organisms (grey) (**f**)25–27. Only reactions contributing to carbon-exchange are shown in detail. **c, d, g, h**, Kinetic labelling profiles in relation to minimal turnover of the indicated CoA (**c, d**) and NAD (**g**) fragments and of NAD upon correction for salvage reactions (**h**) (no niacin-related fragment was detected). Molecular structures shown in graphs correspond the respective parent (black) and fragments (coloured) molecules. Open dots show the indicated measured isotopologue fractions (Mi fraction) or the sum of multiple fractions. Acetyl-CoA was measured instead of CoA and the M2 isotopologue fraction was used to correct for acetyl-turnover. Refer to Supplementary Fig. 3, Supplementary Methods for explanations on MS2 data and correction for salvage reactions; data are from one DLE representative of two experiments; additional information can be obtained from Supplementary Fig. 2.

Figure 4.

Dilution by growth drives coenzyme turnover in *E. coli*. **a, b**, Labelling of coenzymes from cultures sampled after 60 min (**a**) or one generation (**b**) upon growth on various ¹³C labelled substrates. Bars show the indicated isotopologue fractions (Mi fractions; color code as indicated bar charts below). Although the time required to complete one doubling is substrate dependent (see graph in (**c**), bottom left), label incorporation and thus turnover correlated accordingly for long-lived coenzymes. While turnover of THF and GSH has a growth-dependent fraction (**a**), growth-independent factors dominate overall (**b**). **c**, Fractions

of residual unlabelled coenzymes after 60 min of growth on various ^{13}C labelled substrates (data from **(a)**) plotted against the predicted values assuming only dilution by growth. An "idealized" coenzyme, with minimal turnover on all applied substrates is indicated in black. Label incorporation of all measured coenzymes correlates with growth, and long-lived coenzymes retain close to minimal turnover over various doubling times. CoA and NAD were corrected for salvage reactions as described in Fig. 3; THF was measured as 5,10-CH₂-H₄Pte-Glu₃. The r^2 indicated in brackets are based on Pearson correlation coefficients; dotted lines show fits based on linear regression. For additional explanations ([†]) see Supplementary Fig. 2b-c. Mean values $\pm$ SD (n=3 biological replicates) are shown.

Figure 5.

Coenzyme pool propagation. **a**, Scheme illustrating growth of *E. coli* auxotrophic mutants after withdrawal of required precursor (red line) compared to exponential growth during continued supplementation (blue line) and a simulated linear growth model (red dashed line), as well as the relation to the corresponding coenzyme concentrations and factors influencing pool size. **b**, Vitamin-auxotrophic mutants initially maintain exponential growth in the absence of supplementation, indicating excess propagation of coenzyme pools (mean values $\pm$ SD of $n=4$ technical replicates per genotype) are shown; if SD was smaller than the depicted dot, no error bars are shown). Colour code like in (a). The blue solid line projects exponential growth with no limitation (doubling time 1h), the red dashed line simulates linear growth (i.e. the alternative hypothesis where growth is already limited and linear from $t=0$ min onwards). Note that x-axis differs for PLP. **c**, **d**, Relative pool size (full bars) as obtained by the isotope dilution method in wild-type, during steady-state and after removal of pyridoxal (PL) in *pdxA* (**c**) and nicotinic acid (NA) in *nadB* (**d**) at different time

points. Open bars show the expected pool size based on dilution by growth (mean OD₆₀₀) normalized to the first measurement after removal of respective vitamins. Mean values $\pm$ SD are shown (n=3 biological replicates). Red arrows highlight approximate pool size where exponential growth is not further supported; blue arrows mark wild-type levels. For additional details, error propagations, calculations and pool size determination see Supplementary Methods. Growth assessments based on oxygen consumption are shown in Supplementary Fig. 6.

Table 1
Half-life of organic coenzymes in exponentially growing cells

	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. cerevisiae</i>
Doubling time (min)	58.7 ± 0.5	73.4 ± 0.4	160.3 ± 6.8
Compound	Half-life* (T₅₀ in min)		
PLP	54.2 ± 1.8	55.4 ± 2	supp.
FMN	56.2 ± 2.8	55.6 ± 3	176.1 ± 40.3
FAD	40 ± 2.2	57 ± 1.8	110.4 ± 7.8
CoA	29.9 ± 1.2	57.2 ± 2.4	supp.
CoA (w/o salvage)	57.1 ± 3	n.q.	supp.
NAD	28.3 ± 1	28.3 ± 0.6	42 ± 1.9
NAD (w/o salvage)	49.5 ± 1.1	55.2 ± 0.9	165.6 ± 10.3
THF	43.6 ± 1.5	65.4 ± 3.4	n.q.
GSH	23.1 ± 1.8	n.d.	68.4 ± 2.3
Bacillithiol	n.d.	5.6 ± 0.1	n.d.

*Half-life (T₅₀) values are based on curves obtained from fitting decaying isotopologue fractions of respective coenzymes from dynamic labelling experiments (DLE). Models are based on single DLEs with 14 data points; data is available in Supplementary Data 1, 4, 5. The indicated ± SD are calculated from the standard deviations of the obtained parameters from curve fitting. Details on calculations, fittings are available in Supplementary Methods. For additional information, compounds see Supplementary Table 1. Abbreviations: n.d., not detected; n.q., not quantified; supp., supplemented with precursor.