ATP for the DNA Ligation Step in Base Excision Repair Is Generated from Poly(ADP-ribose)*

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In mammalian cells, the base excision repair (BER) pathway is the main route to counteract the mutagenic effects of DNA lesions. DNA nicks induce, among others, DNA polymerase activities and the synthesis of poly(ADP-ribose). It is shown here that poly(ADP-ribose) serves as an energy source for the final and rate-limiting step of BER, DNA ligation. This conclusion was drawn from experiments in which the fate of [32P]poly(ADP-ribose) or [32P]NAD added to HeLa nuclear extracts was systematically followed. ATP was synthesized from poly(ADP-ribose) in a pathway that strictly depended on nick-induced DNA synthesis. NAD was used for the synthesis of poly(ADP-ribose), which, in turn, was converted to ATP by pyrophosphorylcleavage utilizing the pyrophosphate generated from dNTPs during DNA synthesis. The adenyllylation of DNA ligase III, from which it was transferred to the 5' side of the nicked DNA. Finally, ligation to the 3'-OH end resulted in the release of AMP. When using NAD, but not poly(ADP-ribose), in the presence of 3-aminobenzamide, the entire process was blocked, confirming poly(ADP-ribose)lation to be the essential initial step. Thus, poly(ADP-ribose) polymerase-1, DNA polymerase β, and ligase III interact with x-ray repair cross-complementing protein-1 within the BER complex, which ensures that ATP is generated and specifically used for DNA ligation.

The maintenance of an intact genome is crucial to each individual. Therefore, DNA damages need to be efficiently removed, which is accomplished by complex DNA repair mechanisms (reviewed in Ref. 1). The major pathway, BER, is initiated by DNA glycosylases that cleave the base-deoxyribose glycosyl bond of a damaged nucleotide residue. Then, endonucleases are recruited that cleave the chain on the 5' side of the abasic site. As a result, nicked DNA intermediates occur. Nicked DNA, in turn, triggers the catalytic activities of DNA polymerase β (Pol β) and poly(ADP-ribose) polymerase-1 (PARP-1) (2–4).

Several different enzymes with poly(ADP-ribose)ylation activity (EC 2.4.2.30) have been described recently, but the major cellular pathway of NAD catabolism in response to the appearance of DNA lesions has been ascribed to the catalytic activity of the 116-kDa protein PARP-1 (Ref. 4; reviewed in Ref. 5). Besides a potential participation of PARP-1 in transcription (6, 7), recombination, apoptosis, and necrosis (5), a large number of molecular and genetic studies have clearly implicated PARP-1 activity in positively regulating BER (8, 9). Originally, it was suggested that poly(ADP-ribose)ylation may activate a DNA ligase required for DNA repair in mammalian cells (10). Several further investigations confirmed a positive influence of PARP-1 activity on DNA repair, especially on DNA ligation (Ref. 11–13; reviewed in Ref. 9). However, the actual function of PARP-1 in the BER process and the mechanism whereby poly(ADP-ribose) synthesis stimulates ligation (14) have still remained obscure (reviewed in Ref. 9). X-ray repair cross-complementing protein-1 (XRCC1) was the first human gene product isolated that mediates the cellular response to ionizing radiation (15). This protein is apparently essential and required for the BER pathway (16). Recent investigations demonstrated specific interactions of XRCC1 with Pol β, DNA ligase III (Lig III), and PARP-1 (13, 17–20). Therefore, the complex of these proteins is supposed to control BER (1, 9, 19).

Most situations requiring highly efficient DNA repair are accompanied by a dramatic decrease of the cellular ATP concentration. Considering that ligation represents the rate-limiting, ATP-dependent step in BER, it appears reasonable to expect a compensatory mechanism that would enable efficient DNA repair even in situations of energy deprivation. In the present report, it is demonstrated that poly(ADP-ribose) synthesized by PARP-1 may serve as a source of ATP that is specifically used for ligation. This finding provides a molecular mechanism for previous observations, in vivo and in vitro, demonstrating the specific stimulation of ligation by poly(ADP-ribose)ylation during BER.

EXPERIMENTAL PROCEDURES

In Vitro Assays—All reactions were carried out with nuclear HeLa cell extracts purchased from Promega or prepared (21). In standard reactions (10 mM Hepes, pH 7.9, 10% glycerol, 7 mM MgCl2, 50 mM KCl, 100 mM NaCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 10 μM/ml aminophipholin), 8 μM of nucleoprotein were incubated with 200 ng of nicked plasmid DNA. Further additions are indicated in the legends to the figures. The reaction volume was 10 μl, and incubations were conducted at 30 °C for the time periods indicated. All data presented are representative of at least three independent experiments. Nicked plasmid was obtained by controlled incubation of an empty vector plasmid, pUC 9, with DNase I and subsequent purification of nicked plasmids by CsCl ethidium bromide centrifugation. Poly(ADP-ribose) was synthesized with 10 μM/ml purified recombinant PARP-1 from 100 μM NAD as described before (22). Synthesized polymers were freed of residual amounts of DNA by DNase treatment followed by phenol/chloroform extraction and precipitation.

Thin Layer Chromatography—Reactions were stopped by precipitation with 10 volumes of acetone. Precipitated nucleotides were redissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. Samples containing equal amounts of radioactivity (as estimated by Cerenkov counting) were subjected to cellulose-coated plates (Machery-Nagel). Chromatog-
**RESULTS**

First, the metabolism of $\alpha^{32}$P-labeled deproteinized poly(ADP-ribose) (PAR) incubated in the presence of nicked DNA was analyzed in HeLa nuclear extracts. The major product of PAR degradation is ADP-ribose (Fig. 1A, lane 1) arising from the activity of poly(ADP-ribose) glycohydrolase activity present in nuclear extracts (24, 25). In the presence of 1 mM deoxynucleotides (dNTPs), which allowed nick-induced DNA synthesis, in addition to radiolabeled ADP-ribose and AMP, labeled ATP was detected (Fig. 1A, lane 2). Addition of only one or two unlabeled dNTPs failed to support the generation of labeled ATP. A mixture of at least three dNTPs enabled the synthesis of labeled ATP from labeled PAR (not shown). On the other hand, addition of 1 mM sodium pyrophosphate instead of dNTPs did not give rise to the generation of significant amounts of ATP (Fig. 1A, lane 3). Thus, synthesis of ATP strictly depended on DNA synthesis. Moreover, the requirement for dNTPs indicated that the pyrophosphate released during DNA synthesis is specifically channeled to the ATP-forming activity, because added pyrophosphate was inefficient. It should be pointed out that the only feasible direct pathway for the generation of ATP from poly(ADP-ribose) includes the cleavage of the phosphodiester bond of ADP-ribose by a pyrophosphorylase, yielding ATP and ribose-5-phosphate (26). If radioactively labeled ADP-ribose was used in similar reactions, synthesis of ATP was only detected at very high concentrations (10 mM) of pyrophosphate (not shown).

The dependence of ATP generation on the presence of ADP-ribose polymers was further supported by incubations of nuclear extracts with dNTPs and $\alpha^{32}$P-labeled NAD (Fig. 1B,
labeled ATP from labeled NAD was not detected, if the reaction was performed in the presence of the inhibitor of poly(ADP-ribosyl)ation, 3-aminobenzoamide (3-AB) (Fig. 1B, lane 5). However, the presence of 3-AB did not prevent the synthesis of radiolabeled ATP from labeled PAR (Fig. 1B, lane 10). Consequently, dNTP-dependent formation of ATP exhibited an absolute requirement for PAR, either added directly or synthesized by endogenous PARP activity. ATP generation from labeled NAD or labeled PAR (Fig. 1B) attained a maximum after about 20 min of incubation (compare in Fig. 1B, lanes 3 and 8 versus lanes 4 and 9). If an amount of \([\alpha-\text{}^{32}\text{P}]\text{ATP}\) equivalent to that of NAD or ADP-ribose units in PAR was used instead, it was almost entirely metabolized to ADP. Also, in this case, only a very little AMP (<1%) was formed (Fig. 1C, right lane). In contrast, ATP synthesized from PAR coupled to DNA synthesis was apparently not degraded to a maximum after about 20 min of incubation (compare in Fig. 1B, lanes 3 and 8 versus lanes 4 and 9). This suggests that the PAR-derived ATP is inaccessible to ATPases, such as topoisomerases, helicases, or kinases, but is accessible only to enzymes metabolizing ATP to AMP. Thus, the pathway of ATP synthesis using PAR as intermediate product appeared to provide ATP for a specific reaction.

In the first step of DNA ligation, ATP is used to form an adenylylated ligase intermediate. In subsequent steps, the adenylyl group is transferred to the 5'-phosphoryl donor, and eventually, AMP is released when the 5'-phosphoryl and the 3'-OH ends are joined. Owing to the fact that both PARP-1 and Lig III are constituents of the BER complex (17–20), it appeared to be a likely possibility that Lig III (molecular weight, 103,000) is specifically using ATP generated from PAR as described above. To test this possibility, nuclear extracts were incubated in the presence of nicked DNA, dNTPs, and \([\alpha-\text{}^{32}\text{P}]\text{NAD}\). As expected, sev-

The subsequent step after adenylation of DNA ligases is the activation of the donor DNA by transferring the adenylyl group to the phosphorylated 5’-end of a DNA nick. The resulting DNA-AMP complex then reacts with the 3’-OH acceptor group, leading to the ligation of the phosphorylated 5’-end with the 3’-OH under release of AMP. The intermediate DNA-AMP complex can be trapped, if the final joining step of the ligation is prevented. This was accomplished by using 2’-3’-dideoxy NTPs. The specific synthesis of ATP from PAR (see Fig. 1) was still significant, if a mixture of ddATP, ddCTP, ddGTP, and ddTTP was used instead of dNTPs (not shown). Therefore, during nick-induced DNA synthesis, the incorporation of ddAMP or ddCMP into nicked plasmids would lead, in part, to DNA-AMP intermediates of nicked plasmids with 2’-3’-dideoxy-ends adjacent to an adenylylated 5’-end. This possibility was verified in ligation reactions in nuclear extracts using nicked DNA, \([\alpha-\text{}^{32}\text{P}]\text{NAD}\), and unlabeled ddATP, ddCTP, ddGTP, and ddTTP (Fig. 1F). The formation of labeled DNA-[\(\alpha-\text{}^{32}\text{P}\)]AMP complexes was clearly detectable, but only in the absence of 3-AB (Fig. 1F), that is, only if poly(\([\alpha-\text{}^{32}\text{P}]\text{ADP-ribosyl})ation was allowed to occur. These observations provide direct evidence for the conclusion that the adenylylated moieties of poly(ADP-ribose) may be used to activate the 5’-phosphoryl ends of nicked DNA. In the NAD-dependent pathway, the activities of poly(ADP-ribose)ylation, DNA synthesis, and ligation contribute directly to the ligation of nicked DNA only when combined together.

All the enzymes implicated in this mechanism (PARP-1, Pol β, and Lig III) interact with the scaffold protein XRCC1 within the BER complex. Heterodimerization of XRCC1 and Lig III leads to enhanced ligation activity (20). On the other hand, interaction of XRCC1 with either PARP-1 or Pol β results in a down-regulation of the respective catalytic activity (13, 18). It was confirmed in this study that in cultured HeLa cells during BER Pol β and PARP-1 activities are indeed similarly regulated (Fig. 2A). Treatment of HeLa cells with 100 μM MNNG, a well known inducer of the BER pathway, led to transient activation of both PARP-1 and Pol β activities followed by a sharp decline after about 30 min. The assay of DNA synthesis used in this study was restricted to Pol β activity, because other known DNA polymerases were inhibited by aphidicolin (29). As tested for PARP-1 in quantitative Western blot analyses (not shown), the modulation of activity was not caused by changes of protein expression. This holds true also for the reactivation after about 3 h following the MNNG treatment. These in vivo experiments support the conclusion of previous studies that during DNA repair both PARP-1 and Pol β activities are regulated in concert by their interaction with XRCC1. The time course of the activation of PARP-1 and Pol β also paralleled the enhanced occurrence of poly(ADP-ribose) and depletion of the cellular NAD, ATP, and dNTP pools reported in earlier studies (30, 31). The onset of the final step of BER, ligation, coincides with these events. The ligation step is most important for successful DNA repair; it is rate-limiting and ATP-dependent.

The data presented so far suggested that synthesis of poly(ADP-ribose) may serve as an emergency device to complete DNA repair under conditions of cellular ATP depletion. It would be expected then that availability of sufficient ATP should suppress such a pathway. This hypothesis was tested by adding 5 mM ATP to incubations of HeLa nuclear extracts in the presence of nicked DNA, dNTPs, and \([\alpha-\text{}^{32}\text{P}]\text{NAD}\). Surprisingly, under these conditions, modification of nuclear proteins other than PARP-1 or its fragments was not just inhibited but virtually absent (Fig. 2B, left panel, lane 2), as opposed to the poly(ADP-ribosyl)ation observed in the absence of ATP (lane 1, cf. also Fig. 1D). Moreover, \([\alpha-\text{}^{32}\text{P}]\) adenylation of Lig III was also not detectable. Analysis of radiolabeled nucleotides confirmed that in the presence of 5 mM ATP, hardly any of the added \([\alpha-\text{}^{32}\text{P}]\text{NAD}\) was metabolized (Fig. 2B, right panel, lane 2). Addition of other NTPs, dNTPs, ADP, or AMP at the
DNA was extracted, precipitated, and separated in an agarose gel. The gel was dried and subjected to autoradiography. As demonstrated in the experiment shown in Fig. 2, in the absence of any added ATP or an ATP regenerating system, cells were treated with 100 μM MNNG for 5 min, washed twice, and further cultured in RPMI medium. After the time intervals indicated, nuclear extracts were prepared from the cultured cells. Endogenous poly(ADP-ribose)ylation (●) and nick-induced DNA synthesis (x) activities were determined (see “Experimental Procedures”) and related to the values obtained for untreated cells (100%). B, nuclear HeLa extracts were incubated with nicked DNA, 300 μM dNTPs, and 100 nM [α-32P]NAD in the absence (lanes 1) or presence (lanes 2 and 3) of 5 mM unlabeled ATP. In the reaction represented by lanes 3, 500 ng of PARP automodification domain (auto) was also added. After 20 min of incubation, aliquots were subjected to SDS-PAGE (left panel) or thin layer chromatography (right panel) followed by autoradiography (see under “Experimental Procedures”). The asterisks indicate fragments of PARP-1 as detected by Western blot analysis using anti-PARP-1 antibodies. C, nuclear HeLa extracts were incubated with nicked plasmid; 300 μM each of dATP, dGTP, and dTTP; and 1 nM [α-32P]dCTP in the absence (lanes 1) or presence (lanes 2 and 3) of 5 mM unlabeled ATP. For the reaction represented by lanes 3, 500 ng of PARP automodification domain (auto) was also supplied. After 20 min of incubation, DNA was extracted, precipitated, separated in an agarose gel, and stained with ethidium bromide (left panel). The corresponding autoradiogram is shown in the right panel. Closed circles indicate completed ligation. D, nicked plasmids were preincubated with nuclear extracts (see “Experimental Procedures”) and 1 nM [α-32P]dCTP for 20 min at 30 °C. The reactions were then supplemented with unlabeled dNTPs (1 mM), NAD (1 mM), deproteinized PAR (50 μM), or ADP-ribose (ADPR, 1 mM) as indicated, and incubation was continued for further 20 min. Thereafter, DNA was extracted, precipitated, and separated in an agarose gel. The gel was dried and subjected to autoradiography.

**FIG. 2.** Joint regulation of poly(ADP-ribosyl)ation, nick-induced DNA synthesis, and DNA ligation during BER. A, cultured HeLa cells were treated with 100 μM MNNG for 5 min, washed twice, and further cultured in RPMI medium. After the time intervals indicated, nuclear extracts were prepared from the cultured cells. Endogenous poly(ADP-ribose)ylation (●) and nick-induced DNA synthesis (x) activities were determined (see “Experimental Procedures”) and related to the values obtained for untreated cells (100%). B, nuclear HeLa extracts were incubated with nicked DNA, 300 μM dNTPs, and 100 nM [α-32P]NAD in the absence (lanes 1) or presence (lanes 2 and 3) of 5 mM unlabeled ATP. In the reaction represented by lanes 3, 500 ng of PARP automodification domain (auto) was also added. After 20 min of incubation, aliquots were subjected to SDS-PAGE (left panel) or thin layer chromatography (right panel) followed by autoradiography (see under “Experimental Procedures”). The asterisks indicate fragments of PARP-1 as detected by Western blot analysis using anti-PARP-1 antibodies. C, nuclear HeLa extracts were incubated with nicked plasmid; 300 μM each of dATP, dGTP, and dTTP; and 1 nM [α-32P]dCTP in the absence (lanes 1) or presence (lanes 2 and 3) of 5 mM unlabeled ATP. For the reaction represented by lanes 3, 500 ng of PARP automodification domain (auto) was also supplied. After 20 min of incubation, DNA was extracted, precipitated, separated in an agarose gel, and stained with ethidium bromide (left panel). The corresponding autoradiogram is shown in the right panel. Closed circles indicate completed ligation. D, nicked plasmids were preincubated with nuclear extracts (see “Experimental Procedures”) and 1 nM [α-32P]dCTP for 20 min at 30 °C. The reactions were then supplemented with unlabeled dNTPs (1 mM), NAD (1 mM), deproteinized PAR (50 μM), or ADP-ribose (ADPR, 1 mM) as indicated, and incubation was continued for further 20 min. Thereafter, DNA was extracted, precipitated, and separated in an agarose gel. The gel was dried and subjected to autoradiography.

The suggested effect of the added automodification domain of PARP-1 was further supported by analysis of nick-induced DNA synthesis and ligation catalyzed by the nuclear extracts. Whereas DNA synthesis, but no ligation, was observed in the presence of ATP (Fig. 2B, lanes 3), 1 nM [α-32P]dCTP resulted in the partial recovery of poly(ADP-ribosyl)ation (Fig. 2C, lanes 3). However, adenylation of Lig III was still not detectable. The partial recovery of poly(ADP-ribosyl)ation in the presence of ATP (Fig. 2C, lanes 3) resulted in the partial recovery of poly(ADP-ribosyl)ation (Fig. 2C, lanes 3). However, adenylation of Lig III was still not detectable. The automodification domain of PARP-1 is known to mediate specific interaction with partner proteins (22), in particular XRCC1 (18). Therefore, it appears that excess of this domain affects the binding of the endogenous PARP-1 to XRCC1, and thus to the BER complex. Consequently, PARP-1 inhibition was abolished, but the BER complex was disabled to synthesize ATP.

The same concentration (5 mM) did not cause any comparable effect. A direct effect of ATP on PARP-1 was also excluded, because the catalytic activity of the isolated enzyme did not exhibit such a sensitivity toward ATP (not shown). Moreover, addition of isolated recombinant automodification domain of PARP-1 (amino acids 337–573) resulted in the partial recovery of poly(ADP-ribosyl)ation in the presence of ATP (Fig. 2B, lanes 3). However, adenylation of Lig III was still not detectable. The automodification domain of PARP-1 is known to mediate specific interaction with partner proteins (22), in particular XRCC1 (18). Therefore, it appears that excess of this domain affected the binding of the endogenous PARP-1 to XRCC1, and thus to the BER complex. Consequently, PARP-1 inhibition was abolished, but the BER complex was disabled to synthesize ATP.

The suggested effect of the added automodification domain of PARP-1 was further supported by analysis of nick-induced DNA synthesis and ligation catalyzed by the nuclear extracts. Whereas DNA synthesis, but no ligation, was observed in the absence of ATP (Fig. 2C, lanes 1), the presence of 5 mM ATP led to ligation but suppressed nick-induced DNA synthesis (Fig. 2C, lanes 2). Addition of the automodification domain of PARP-1 restored nick-induced DNA synthesis in the presence of ATP. Still, the ligated plasmid (closed circle) did not contain radiolabel of the added dNTPs (Fig. 2C, lanes 3). It is well known that DNA repair processes need a high level of ATP. Therefore, in vitro assays are usually conducted in the presence of about 2 mM ATP and a regenerating system (32). It was demonstrated in the experiment shown in Fig. 2D that in the absence of any added ATP or an ATP-regenerating system nicked plasmids can be rejoined by HeLa nuclear extracts. However, rejoicing occurs only if the conditions established above for the ATP synthesis from PAR are met. That is, conversion of nicked circles to closed circles was only observed if PAR (either directly added or formed from NAD by endogenous PARP-1) and dNTPs were present (Fig. 2D, lanes 5 and 6). As mentioned before, added ADP-ribose did not serve as a substitute for PAR.

**DISCUSSION**

The results of the present study support the conclusion that poly(ADP-ribosyl)ation may directly contribute to the process of BER by providing a source of ATP for the ligation step. It is important to note that the favorable effects of NAD and PARP on ligation have long been known. Originally, the observed stimulation of strand break joining was suspected to be caused by poly(ADP-ribosyl)ation of a ligase (10). However, this suggestion has not been confirmed. In initial in vivo studies, antisense RNA expression was used to deplete cells of PARP-1. It was observed that the absence of PARP-1 resulted in a significant delay of DNA strand break rejoining (11). Moreover, analyses of cells derived from PARP-1−/− mice also revealed a substantial reduction of the DNA ligation activity (12). In the meantime, the BER complex has been well studied, and direct interactions of the proteins involved have been established, both in vitro and in vivo. It was clearly demonstrated that NAD, which provides the substrate for poly(ADP-ribosyl)ation, accelerates the ligation step without influencing, for example, Pol β activity (13). Considering the dramatic decrease of the cellular ATP con-
ATP and pyrophosphate are apparently not used by this complex, generation of ATP from PAR, and ligation, appears autoadenylation and subsequent activation of the DNA donor this ATP that is at least preferentially used by Lig III for its which is accompanied by the liberation of pyrophosphate. It is has been detected previously in HeLa cell extracts (26). product. An enzymatic activity catalyzing this kind of reaction the detected ATP synthesis includes pyrophosphorolytic cleav-

E. coli, PARP-1 synthesizes poly(ADP-ribose), yielding ribose phosphate as its second which is apparently directly channeled to the ligase. According to the data presented, the pathway of ATP synthesis does not necessarily include the cleavage of poly(ADP-ribose) to ADP-ribose. However, ADP-ribose may be generated within the tight complex of participating enzymes and may not be substituted for by added ADP-ribose, similar to the situation found for pyrophosphate (Fig. 1A, lane 3). ATP formation using pyrophosphate generated from dNTPs during nick-induced DNA synthesis is difficult to demonstrate directly, because the γ-phosphate group of only a single added \( \gamma^{32}\text{P} \text{dTTP} \) is readily detectable in ATP (not shown), presumably owing to transphosphorylation of ADP in the nuclear extracts. Nevertheless, the requirement of DNA synthesis to generate ATP from PAR strongly suggests the involvement of pyrophosphate. Furthermore, high concentrations (10 mM) of added pyrophosphate were effective to synthesize ATP from PAR in the absence of dNTPs (not shown). Therefore, it would appear that the detected ATP synthesis includes pyrophosphorolytic cleavage of ADP-ribose, yielding ribose phosphate as its second product. An enzymatic activity catalyzing this kind of reaction has been detected previously in HeLa cell extracts (26). It is of importance to the emerging model (Fig. 3) that the specific generation of ATP requires the synthesis of DNA, which is accompanied by the liberation of pyrophosphate. It is this ATP that is at least preferentially used by Lig III for its autoadenylation and subsequent activation of the DNA donor (5'-phosphorylated end) and, thus, mediates ligation. Moreover, the final phase of BER, including nick-induced DNA synthesis, generation of ATP from PAR, and ligation, appears to be accomplished by an autonomous complex. Bulk phase ATP and pyrophosphate are apparently not used by this complex. Rather, a high cellular energy state (that is, high ATP concentrations) may possibly serve as a signal that favors direct ligation of nicked DNA or execution of the Pol β-dependent short-patch repair pathway (2, 33).

An important observation (Fig. 2B) relates to the potential function of Lig III and PARP-1 as molecular nick sensors. Both proteins contain highly similar zinc-finger motifs that exhibit high affinity to single strand breaks (34). According to the data presented in Fig. 2, B and C, at high ATP concentrations, Lig III is readily adenylated. At the same time, poly(ADP-ribose)-ylation and DNA synthesis are strongly inhibited. A possible explanation would be that adenylation of Lig III within the BER complex enhances its affinity to DNA nicks or at least causes the BER complex to assume a conformation that prevents DNA binding of PARP-1 and thereby poly(ADP-ribose)-ylation. Such a mechanism would restrict the use of NAD as an energy source to emergency situations of ATP shortage.

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