Extremophiles (2001) 5:393–398
Digital Object Identifier (DOI) 10.1007/s007920100216


ORIGINAL PAPER

**Guangliang Pan · Marc F.J.M. Verhagen**
**Michael W.W. Adams**

# Characterization of pyridine nucleotide coenzymes in the hyperthermophilic archaeon *Pyrococcus furiosus*

Received: January 24, 2001 / Accepted: May 12, 2001 / Published online: October 16, 2001

**Abstract** Pyridine-type nucleotides were identified in cell-free extracts of the hyperthermophilic archaeon *Pyrococcus furiosus* by their ability to replace authentic nicotinamide adenine dinucleotide (phosphate) [NAD(P)] in assays using pure *P. furiosus* enzymes. The nucleotides were purified using a combination of ion-exchange and reverse-phase chromatography. They were identified as NAD and NADP by analyses using liquid chromatography–mass spectrometry and high performance liquid chromatography. Their intracellular concentrations were measured in *P. furiosus* grown using maltose and peptides as the carbon sources. The concentrations decreased during the lag phase but remained constant during the exponential phase at approximately 0.17 and 0.13 mM, respectively. The amount of NAD was significantly lower (more than four-fold lower) than that in mesophilic bacteria, although the NADP concentration was comparable. The internal concentrations of NADH and NADPH in *P. furiosus* were determined to be 0.14 mM and 0.04 mM, respectively. The overall cellular concentration of NAD(P)(H) in *P. furiosus* (0.48 mM) is about half the value in the mesophiles. The NAD(H)/NADP(H) ratio in *P. furiosus* is consistent with the preferred use of NADP by several catabolic enzymes that have been purified from this organism. The mechanisms by which hyperthermophiles stabilize these thermally labile nicotinamide nucleotides are not known.

**Key words** Archaea · *Pyrococcus* · Nicotinamide · Thermostability · Hyperthermophile

Communicated by G. Antranikian

G. Pan · M.F.J.M. Verhagen · M.W.W. Adams (✉)
Department of Biochemistry and Molecular Biology and Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602, USA
Tel. +1-706-5422060; Fax +1-706-5420229
e-mail: adams@bmb.uga.edu

Presented at the Third International Congress on Extremophiles, Hamburg, 2000

## Introduction

The pyridine nucleotides (nicotinamide adenosine dinucleotide), NAD(H) and NADP(H), function as electron carriers in virtually all biological systems. Typically, NAD is involved in catabolic processes, such as glycolysis and the citric acid cycle, whereas NADP is used in assimilatory pathways, such as the biosynthesis of nucleotides and fatty acids. The nucleotides are usually considered to be ubiquitous, but their utilization might be limited by their lack of stability under extreme conditions. For example, at neutral pH, the half-life of NAD decreases from several hours at 60°C to approximately 13 min near 100°C, and NADP is even more unstable at these temperatures (Lowry et al. 1961; Robb et al. 1992; Daniel 1998). This finding raises the question of whether these cofactors are actually present in microorganisms that have optimum growth temperatures near, and in some cases above, 100°C, the so-called hyperthermophiles (Huber et al. 2000). For example, it is possible that these organisms could possess slightly modified forms of the standard pyridine nucleotides (NAD or NADP). Covalent modification can increase the stability of nucleotides in RNA, and it has been suggested that many, if not all, hyperthermophiles have taken advantage of this to stabilize their tRNAs (Kowalak et al. 1994). Additional evidence for the modification of conventional cofactors in such organisms comes from *Pyrococcus furiosus*, a fermentative hyperthermophile that grows optimally at 100°C (Fiala and Stetter 1986). *P. furiosus* contains folates with appended *N*-acetyl glucosamine residues. As yet, the effect of these modifications on the thermostability of the cofactors has not been determined (White 1993).

The presence of structural analogues of the pyridine nucleotides is also suggested by the fact that several oxidoreductase-type enzymes purified from *P. furiosus* and related organisms, including hydrogenases, ferredoxin:NAD(P) oxidoreductase, and glutamate dehydrogenase, have very low affinities or activities with NAD, and that they preferentially, and in some cases exclusively, utilize NADP (Ma and Adams 1994, 1999; Ma et al. 1994; Robb et al. 1992). This result is unexpected because these

enzymes are involved in the catabolism of growth substrates such as sugars or peptides and, as already noted, NADP is typically used as the electron carrier in biosynthetic pathways. Moreover, in these catabolic pathways there appears to be discrimination against NAD(P) in favor of the extremely thermostable redox protein ferredoxin (Daniel and Danson 1995; Daniel 1998). A good example of this discrimination occurs in the glycolytic pathway of *P. furiosus,* wherein the expected NAD-dependent glyceraldehyde-3-phosphate dehydrogenase is replaced by the novel ferredoxin-dependent enzyme, glyceraldehyde-3-phosphate oxidoreductase (Mukund and Adams 1995). Pyruvate oxidation is also coupled to ferredoxin reduction in *P. furiosus* (Blamey and Adams 1993), as is typically the case in anaerobic microorganisms.

In addition, there is only indirect evidence that hyperthermophiles contain the traditional pyridine nucleotides. Various dehydrogenases have been purified from such organisms and that these, by definition, oxidize or reduce NAD(P)/H in in vitro assays (Daniel and Danson 1995; Ma and Adams 1994, 1999; Ma et al. 1994, 2000; Robb et al. 1992; Aoshima et al. 1996; Higuchi et al. 1997; Aalen et al. 1997; Rahman et al. 1998; Antoine et al. 1999; Kujo et al. 1999). Although these enzymes show high sequence similarity to their mesophilic counterparts, this would be expected even if the hyperthermophilic enzymes were using slightly modified versions of NAD(P). Obviously, if modified nucleotides were being used, it is extremely important to elucidate the modifications of NAD(P) that lead to increased thermal stability.

Consequently, we sought to address two issues. First, do microorganisms that can grow near and even above 100°C contain the conventional pyridine nucleotides, or do they actually utilize (minor) derivatives of them? Second, how do the cellular concentrations of such nucleotides, whether novel or not, compare with those usually found in mesophilic organisms? We report here on the purification of the redox-active pyridine nucleotides from the hyperthermophilic archaeon *P. furiosus.*

## Materials and methods

### Growth of *Pyrococcus furiosus*

*Pyrococcus furiosus* (DSM 3638; Fiala and Stetter 1986) was grown at 90°C in a 600-l fermenter under pH-controlled conditions with maltose (Sigma, St Louis, MO, USA), yeast extract, and tryptone (both from United States Biochemical, Cleveland, OH, USA) as the carbon sources, each at a concentration of 5 g/l as described earlier (Bryant and Adams 1989). *Escherichia coli* (strain RK4353) was grown at 37°C in Luria-Bertani (LB) medium and harvested at stationary phase.

### Enzymes and reagents

All chemicals (high performance liquid chromatography [HPLC] grade), analytical grade NAD(H) and NADP(H), and the yeast enzymes hexose kinase, glucose-6-phosphate dehydrogenase, and alcohol dehydrogenase, were purchased from Sigma. Glutamate dehydrogenase (GDH; Robb et al. 1992) and NADPH : rubredoxin oxidoreductase (NROR; Ma and Adams 1999), purified from *P. furiosus*, were provided by Dr. Kesen Ma of the University of Georgia.

### Enzymatic analysis of pyridine nucleotides

Anaerobic cuvettes containing 2 ml 50 mM *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid (EPPS) buffer (pH 8.4) and 1 mM benzyl viologen were preheated to 85°C in a thermostatically controlled cuvette holder before addition of 1 mM glutamate, 1 µl GDH (5 mg/ml), and 1 µl NROR (5 mg/ml). The reaction was initiated by adding samples of potential redox cofactors with authentic NAD and NADP as controls. Reduction of benzyl viologen was measured at 595 nm using a molar absorbance of 7,800 $M^{-1}$ $cm^{-1}$. One unit of activity is defined as the reduction of 1 µmol benzyl viologen/min under these conditions. Yeast alcohol dehydrogenase activity was measured in a 2-ml reaction mixture containing 0.1 M Tris-HCl, 0.2 M ethanol, 0.65 mM dithiothreitol, and 0.05 M semicarbazide-HCl, pH 6.5 (Wykes et al. 1972). After adding NAD or a potential NAD analogue, the reaction was initiated by adding 1 U alcohol dehydrogenase. After 30 min the mixture was analyzed by reverse-phase chromatography, as described below. NADP and potential NADP analogues were measured using yeast glucose-6-phosphate dehydrogenase. The reaction was carried out in 2.5 ml triethanolamine (50 mM, pH 7.6) containing 0.22 M glucose, 8 mM $MgCl_2$, 0.64 mM ATP, 1.5 U yeast glucose kinase, and 1.5 U yeast glucose-6-phosphate dehydrogenase (Bergmeyer et al. 1983). The reaction was initiated by adding the redox cofactor, and after 30 min the mixture was analyzed using reverse-phase chromatography.

### Purification of redox-active nucleotides from *Pyrococcus furiosus*

Frozen *P. furiosus* cells (180 g, wet weight) were suspended in 540 ml Tris-HCl buffer (50 mM, pH 8.0, containing 4 mM dithionite) and disrupted by sonication for 30 min. The extract was centrifuged at 100,000 *g* for 1.5 h, and the pH of the supernatant was adjusted to 1.5 using 6 N HCl. The precipitate was removed by centrifugation at 100,000 *g* for 1 h, and the pH of the supernatant was adjusted to 4.5 using 6 N NaOH; this was passed through an Amicon (Beverly, MA, USA) ultrafiltration unit equipped with a YM-3 membrane. The flow-through was concentrated to approximately 100 ml by lyophilization and loaded at 4 ml/min onto a column (5 × 31 cm) containing Dowex-1 resin (Bio-Rad, Richmond, CA, USA) with formate as the counterion. The column was washed with two bed volumes of water, and the bound cofactors were eluted with formic acid using the following nonlinear gradient: 0–1 M (1,800 ml), 1 M (300 ml), 1–4 M (300 ml), and 4 M (300 ml). The column eluent was monitored at 254 nm.

The two peaks containing a redox cofactor (F1 and F2) from the Dowex-1 column were pooled separately, concentrated by lyophilization, and loaded onto a C-18 column (2.6 × 20 cm) after addition of tetrabutylammonium phosphate (4 mM) as the ion-pairing agent. The column was prepared using material from 9 Sep-Pak Vac 35 cc C-18 cartridges (Waters, Milford, MA, USA) and was equilibrated with 200 ml $H_2O$ after activation with 50% (v/v) methanol. The bound cofactors were eluted using a linear gradient (300 ml) from 0% to 50% (v/v) methanol. The cofactor in fraction F1 eluted when 35% methanol was applied to the column whereas the compound present in fraction F2 eluted at 40% methanol. The methanol in the two samples was removed by rotary evaporation (Brinkmann, Westbury, NY, USA) at 45°C. Each sample was further concentrated by lyophilization and further purified by anion-exchange chromatography using a Mono Q column (Pharmacia, Piscataway, NJ, USA) by modifying a previously described method (Orr and Blanchard 1984). The column was equilibrated with 20 mM Tris-HCl, pH 7.7 (buffer A) at a flow rate of 1 ml/min. The concentrated cofactor samples were separately applied to the column and were eluted using a nonlinear KCl gradient in buffer A as follows: 0–200 mM (28 ml), 200–1,000 mM (5 ml), and 1,000 mM (3 ml). Active fractions from each sample were concentrated by lyophilization. The putative cofactor present in F1 eluted at 5 mM KCl and was termed PfNAD; that present in F2 eluted at 130 mM KCl and was termed PfNADP.

### HPLC analysis of *Pyrococcus furiosus* pyridine nucleotides

A Sep-Pak C-18 column (3.9 × 150 mm; particle size, 4 μm) coupled to a Waters 2690 HPLC was used to analyze the cofactors both in cell-free extracts of *P. furiosus* and in the different column fractions. Tetrabutylammonium phosphate (4 mM) was added to each sample as an ion-pairing agent. The column was equilibrated with 15 mM $NH_4H_2PO_4$ buffer, pH 5.4, containing 2 mM tetrabutylammonium phosphate (buffer C) at a flow rate of 1 ml/min. The bound nucleotides were eluted with a linear gradient (45 ml) from buffer C to 30% (v/v) acetonitrile in water. Authentic NAD(H) and NADP(H) were used as the standards.

### Liquid chromatography–mass spectrometry

The cofactors purified from *P. furiosus* were applied to an AsaHipak ODP C-18 column (1 × 250 mm ID; particle size, 5 μm) equilibrated with 0.25 M ammonium acetate, pH 6.6, at a flow rate of 30 μl/min, and were eluted using a step gradient of 30% (v/v) acetonitrile in water. The elution profile was monitored at 254 nm and peaks were analyzed using negative ion mass spectrometry by injection into an API$^{plus}$ mass spectrometer (Perkin-Elmer, Norwalk, CT, USA) set with the following parameters: scan range, *m/z* 180–780; step size, 0.2 U; dwell time, 2.0 ms.

### Determination of the intracellular concentration of NAD(H) and NADP(H)

*Pyrococcus furiosus* was grown under pH-controlled conditions in a 600-l fermenter; 10-l samples were removed periodically. The samples were rapidly cooled using a glass-cooling coil submerged in a water/ice mixture, and the cells were harvested by centrifugation at 6,000 *g* for 10 min. The cell pellets were collected, frozen in liquid nitrogen, and stored at –80°C. For analyses of NAD and NADP, cell-free extracts were prepared and analyzed by HPLC as already described. Intracellular concentrations were calculated using a cell volume of 4.5 μl/mg protein (Martins and Santos 1995). To determine the amounts of the reduced forms of the pyridine nucleotides in *P. furiosus*, a separate alkaline extraction method was used. Cells were suspended in 0.2 M KOH and vortexed vigorously until they lysed. The extract was then centrifuged at 100,000 *g* for 30 min and the pH of the supernatant was adjusted to 7.5. The sample was subjected to Centricon centrifugation (Amicon), and the pass-through was then concentrated using a SpeedVac (Savant, Farmingdale, NY, USA). The amount of reduced pyridine dinucleotides was determined by HPLC as previously described.

### Thermal stability assays

To prepare a cell-free extract, 2 g (wet weight) of *P. furiosus* cells was suspended in 2 ml buffer (25 mM Tris-HCl, pH 7.0) and disrupted by sonication for 5 min. After centrifugation at 100,000 *g* for 1.5 h, the supernatant was concentrated using Centricon centrifugation (Amicon) and the pass-through was then concentrated by three-fold using a Speed-Vac. Samples of authentic NAD, NADP, NADH, or NADPH were added (1 mM, final concentration) either to the cell-free extract or to 25 mM Tris/HCl, pH 7.0 (measured pH at 90°C was 5.8). All samples were then heated at 70°, 80°, or 90°C for 10 min, and the amount of each nucleotide remaining was determined by HPLC.

## Results and discussion

### Purification of redox cofactors from *Pyrococcus furiosus*

The GDH and NROR of *P. furiosus* were chosen as the model enzymes for identifying the relevant redox cofactors in cell-free extracts of the organism. Both enzymes, in their purified states, have been shown to use both NADP and NAD as electron carriers (Robb et al. 1992; Ma and Adams 1999) in in vitro assays at 95°C. The assay system utilized here to investigate the cofactor(s) in cell-free extracts of *P. furiosus* consisted of the two enzymes with glutamate as a substrate and benzyl viologen (BV) as an artificial electron carrier. Under these conditions, GDH oxidizes glutamate and reduces the redox cofactor(s) in the extract, which in turn is oxidized by NROR, and NROR then reduces benzyl viologen. Hence, reduction of benzyl viologen occurs only

in the presence of a redox cofactor in the extract that can transfer electrons between GDH and NROR. For example, using this assay system, activity was readily detected in the supernatant fraction obtained after acidification, centrifugation, and neutralization of a cell-free extract of *P. furiosus* (the supernatant contained 2.1 U/mg protein in the original cell-free extract from which it was derived). Chromatography of the supernatant on an anion-exchange resin yielded four peaks with significant absorption at 254 nm. Only two of the four, which eluted at 0.6 and 4.0 M formic acid, were able to couple electron flow between GDH and NROR, and these were termed F1 and F2, respectively. These compounds were separately purified further by reverse phase (C-18) and anion-exchange chromatography to give cofactors PfNAD and PfNADP, respectively.

## Identification of redox cofactors

Electron spray ionization–mass spectrometric analysis of PfNADP indicated the presence of a single compound with an *m/z* ratio of 741.8, a value identical to authentic NADP (Fig. 1). The fraction containing PfNAD was only partially purified but it consisted mainly (>80%) of a compound with an *m/z* ratio of 662, which is identical to authentic NAD (data not shown). These findings were confirmed with ion-pair HPLC chromatography. The retention times of PfNAD and PfNADP were identical to those of authentic NAD and NADP, respectively (data not shown). Furthermore, HPLC analysis of PfNAD before and after incubation with mesophilic alcohol dehydrogenase indicated the formation of a peak with a retention time identical to that of NADH. Similarly, the product formed after reduction of PfNADP with mesophilic glucose-6-phosphate dehydrogenase had a retention time identical to that of NADPH. These results demonstrate that the redox cofactors PfNAD and PfNADP purified from *P. furiosus* are, indeed, NAD and NADP, respectively. In a separate experiment, known amounts of authentic NAD or NADP were added to a cell-free extract of *P. furiosus.* Using them as internal standards, it was estimated that at least 90% of nucleotides were recovered after the purification procedure.

## Cellular concentrations of redox cofactors

The intracellular concentrations of NAD and NADP were measured throughout the growth phase of *P. furiosus* when it was grown in a 600-l fermenter. Both nucleotides decreased slightly in concentration during the transition from the lag phase to the exponential phase (Fig. 2). The average concentrations of NAD and NADP in the exponential phase were 0.17 and 0.13 mM, respectively. Using the same techniques that were applied to *P. furiosus*, the intracellular concentrations of NAD and NADP in *E. coli* were estimated at 1.0 mM and 0.2 mM, respectively, which are in excellent agreement with the published values (London and Knight 1966). For *Salmonella typhimurium*, the intracellular amounts of NAD and NADP were reported to be 0.80 and 0.06 mM, respectively (Bochner and Ames 1982). Thus, the amount of NAD in *P. furiosus* is significantly lower (more than four-fold) than that in the two mesophilic organisms, although the NADP concentration is comparable. On the other hand, the internal concentrations of NADH and NADPH in *P. furiosus* were determined to be 0.14 and 0.04 mM, respectively. Thus, the total values for NAD(H) and for NADP(H) in *P. furiosus* are 0.31 and 0.17 mM, respectively, with an overall cellular concentration of 0.48 mM for NAD(P)(H). For comparison, the concentrations of NAD(H) and NADPH in *Salmonella typhimurium* are 0.81 and 0.2 mM, respectively, for an overall NAD(P)(H) concentration of 1.01 mM (Bochner and Ames 1982), more than twice that in *P. furiosus*.

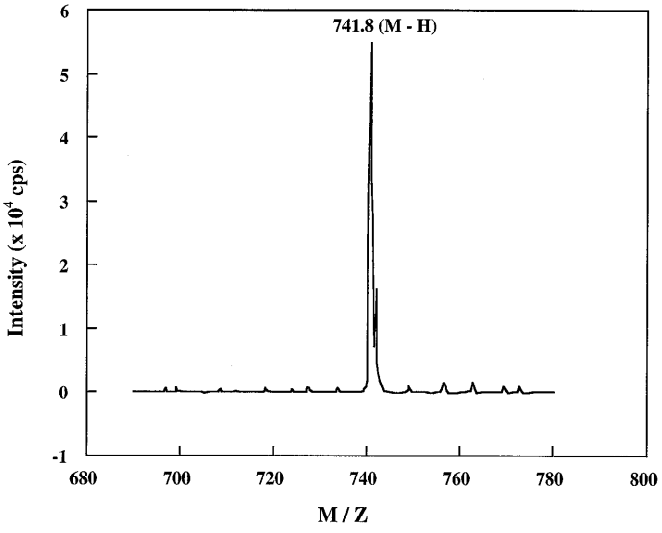


**Fig. 1.** Electrospray mass spectrum of PfNADP (Pf-nicotinamide adenine dinucleotide phosphate) purified from *Pyrococcus furiosus*. The observed $M_r$ at *m/z* 741.8 represents the deprotonated nucleotide (*M-H*)

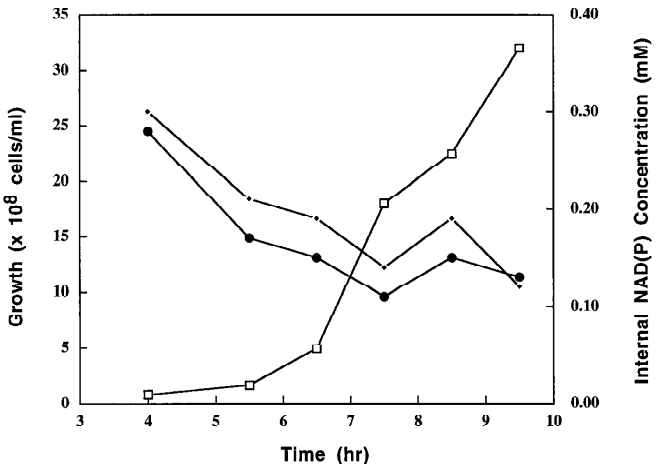


**Fig. 2.** Effect of growth phase of *P. furiosus* on intracellular concentrations of nicotinamide adenine dinucleotide (NAD) (*closed squares*) and NADP (*closed circles*). The other curve (*open squares*) indicates cell density

The intracellular concentrations of the pyridine nucleotide coenzymes in *P. furiosus* appear to be in accordance with the fact that the enzymes that utilize these nucleotides which have been characterized from *P. furiosus* typically have much higher catalytic efficiencies with NADP; these include enzymes such as hydrogenase (I), ferredoxin : NADP oxidoreductase, and glutamate dehydrogenase (Robb et al. 1992; Ma and Adams 1994; Ma et al. 1994). There are exceptions, however; although hydrogenase I of *P. furiosus* is virtually inactive with NAD, hydrogenase II utilizes the two nucleotides with comparable efficiency (Ma et al. 2000). Although a preference for NADP is in general agreement with the lower NAD : NADP ratio (compared to *E. coli* and *S. typhimurium*), the reason for this remains unknown. Similarly, why the overall concentration of NAD(H) in *P. furiosus* is much lower than that in the mesophilic organisms is also unclear. Even if, in general terms, redox-active enzymes in *P. furiosus* discriminate against NAD(P) in favor of ferredoxin (Daniel and Danson 1995; Daniel 1998), this finding does not correlate with lower nucleotide concentrations. The latter would require that enzymes that use such cofactors have higher affinities for them than do the comparable mesophilic enzymes. As yet, insufficient data have been accumulated to decide whether this is generally the case.

In mesophilic organisms, pyridine nucleotides can be synthesized de novo or can be recovered via specific salvage pathways for degradation products of NAD metabolism (Moat and Foster 1987). All these pathways contain a common step involving the conversion of nicotinamide mononucleotide (NMN) to NAD, a reaction catalyzed by the enzyme nicotinamide mononucleotide adenyltransferase (Raffaelli et al. 1997). Sequence analysis of the genome from *P. furiosus* (http://comb5-156.umbi.umd.edu/genemate/) shows that it contains a gene with high similarity (64%, 50% identity) to the nicotinamide mononucleotide adenyltransferase from the hyperthermophilic archaeon *Methanococcus jannaschii* (Bult et al. 1996). In addition, *P. furiosus* contains genes with similarity to the enzymes aspartate oxidase and quinolinate synthetase, both of which are thought to be involved in the de novo synthesis of NAD in mesophilic organisms (Moat and Foster 1987). The presence of these synthesis pathways combined with the unambiguous identification of these two nucleotides in *P. furiosus* indicates that, in spite of their instability at high temperature, both NAD and NADP play important roles in the metabolism of *P. furiosus* and presumably in other hyperthermophiles.

The presence of NAD(P) in hyperthermophiles such as *P. furiosus* poses this question: how can such organisms maintain adequate concentrations of these compounds at physiological temperatures? A possible explanation is that their biosynthesis near 100°C occurs at much higher rates than in mesophilic organisms. Alternatively, specific proteins or organic compounds could be present in the cytoplasm of hyperthermophiles to protect the nicotinamide nucleotides against thermal denaturation. Many hyperthermophiles do indeed contain high concentrations of unusual solutes, typically phosphocompounds of various forms (Martins and Santos 1995; Martins et al. 1997; Ramakrishnan et al. 1997; Lamosa et al. 2000). However, this is not a universal phenomenon in the hyperthermophilic world, and none of the compounds that have been characterized has been reported to stabilize nucleotide cofactors. To investigate this possibility, we compared the thermal stability of NAD(P)(H) in a cell-free extract and in buffer at 90°C (pH 5.8), but there were no significant differences. For example, in both cases, the half-life of NAD was about 10 min, and more than 95% of the NADPH was destroyed after this same period. In any event, the unambiguous identification of the different pyridine nucleotides present in *P. furiosus* is the first step toward the elucidation of the precise mechanisms by which these organisms maintain and utilize them, albeit at reduced concentrations, under extreme conditions.

**Acknowledgments** This research was supported by grants from the National Science Foundation (MCB 9809060 and MCB 9904624).

## References

Aalen N, Steen IH, Birkeland NK, Lien T (1997) Purification and properties of an extremely thermostable $NADP^+$-specific glutamate dehydrogenase from *Archaeoglobus fulgidus*. Arch Microbiol 168: 536–539

Antoine E, Rolland JL, Raffin JP, Dietrich J (1999) Cloning and overexpression in *Escherichia coli* of the gene encoding NADPH group III alcohol dehydrogenase from *Thermococcus hydrothermalis*: characterization and comparison of the native and the recombinant enzymes. Eur J Biochem 264:880–889

Aoshima M, Yamagishi A, Oshima T (1996) Eubacteria-type isocitrate dehydrogenase from an archaeon: cloning, sequencing, and expression of a gene encoding isocitrate dehydrogenase from a hyperthermophilic archaebacterium, *Caldococcus noboribetus*. Arch Biochem Biophys 336:77–85

Bergmeyer HU, Graßl M, Walter HW (1983) Biochemical reagents for general use. In: Bergmeyer J, Graßl M (ed) Methods of enzymatic analysis, 3rd edn. Verlag Chemie, Weinheim, pp 222–223

Blamey JM, Adams MWW (1993) Purification and characterization of pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Biochim Biophys Acta 1161:19–27

Bochner BR, Ames BN (1982) Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. J Biol Chem 257:9759–9769

Bryant FO, Adams MWW (1989) Characterization of hydrogenase from the hyperthermophilic archaebacterium, *Pyrococcus furiosus*. J Biol Chem 264:5070–5079

Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann RD, Sutton GG, Blake JA, FitzGerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougherty BA, Tomb JF, Adams MD, Reich CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JL, Geoghagen NSM, Venter JC (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science 273:1058–1073

Daniel RM (1998) Primitive coenzymes and metabolites in archaeal/thermophilic metabolic pathways. In: Wiegel J, Adams MWW (eds) Thermophiles: the keys to molecular evolution and the origin of life? Taylor and Francis, Philadelphia, pp 299–310

Daniel RM, Danson MJ (1995) Did primitive microorganisms use nonheme iron proteins in place of NAD/P? J Mol Evol 40:559–563

Fiala G, Stetter KO (1986) *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch Microbiol 145:56–61

Higuchi S, Kobayashi T, Kimura K, Horikoshi K, Kudo T (1997) Molecular cloning, nucleotide sequence and expression in *Escherichia coli* of hyperthermophilic glutamate dehydrogenase gene from *Thermococcus profundus*. J Ferment Bioeng 83:405–411

Huber R, Huber H, Stetter KO (2000) Towards the ecology of hyperthermophiles: biotopes, new isolation strategies and novel metabolic properties. FEMS Microbiol Rev 24:615–623

Kowalak JA, Dalluge JJ, McCloskey JA, Stetter KO (1994) The role of posttranscriptional modification in stabilization of transfer RNA from hyperthermophiles. Biochemistry 33:7869–7876

Kujo C, Sakuraba H, Nunoura N, Ohshima T (1999) The NAD-dependent glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrobaculum islandicum*: cloning, sequencing, and expression of the enzyme gene. Biochim Biophys Acta 1434:365–371

Lamosa P, Burke A, Peist R, Huber R, Liu MY, Silva G, Rodrigues-Pousada C, LeGall J, Maycock C, Santos H (2000) Thermostabilization of proteins by diglycerol phosphate, a new compatible solute from the hyperthermophile *Archaeoglobus fulgidus*. Appl Environ Microbiol 66:1974–1979

London J, Knight M (1966) Concentration of nicotinamide nucleotide coenzymes in microorganisms. J Gen Microbiol 44:241–254

Lowry OH, Passonneau JV, Rock MK (1961) The stability of pyridine nucleotides. J Biol Chem 236:2756–2759

Ma K, Adams MWW (1994) Sulfide dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*: a new multifunctional enzyme involved in the reduction of elemental sulfur. J Bacteriol 176:6509–6517

Ma K, Adams MWW (1999) A hyperactive NAD(P)H-rubredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Bacteriol 181:5530–5533

Ma K, Zhou ZH, Adams MWW (1994) Hydrogen production from pyruvate by enzymes purified from the hyperthermophilic archaeon, *Pyrococcus furiosus*: a key role for NADPH. FEMS Microbiol Lett 122:263–266

Ma K, Weiss R, Adams MWW (2000) Characterization of hydrogenase II from the hyperthermophilic archaeon *Pyrococcus furiosus* and assessment of its role in sulfur reduction. J Bacteriol 182:1864–1871

Martins LO, Santos H (1995) Accumulation of mannosylglycerate and di-*myo*-inositol-phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Appl Environ Microbiol 61:3299–3303

Martins LO, Huber R, Huber H, Stetter, KO, Da Costa MS, Santos H (1997) Organic solutes in hyperthermophilic *Archaea*. J Bacteriol 63:896–902

Moat AG, Foster JW (1987) Biosynthesis and salvage pathways of pyridine nucleotides. In: Dolphin D, Poulson R, Avramovic O (eds) Pyridine nucleotide coenzymes, part B. Wiley, New York, pp 1–24

Mukund S, Adams MWW (1995) Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon *Pyrococcus furiosus*. J Biol Chem 270:8389–8392

Orr GA, Blanchard JS (1984) High-performance ion-exchange separation of oxidized and reduced nicotinamide adenine dinucleotides. Anal Biochem 142:232–234

Raffaelli N, Pisani FM Lorenzi R, Emanuelli M, Amici A, Ruggieri S, Magni G (1997) Characterization of nicotinamide mononucleotide adenyltransferase from thermophilic archaea. J Bacteriol 179:7718–7723

Rahman RNZA, Fujiwara S, Takagi M, Imanaka T (1998) Sequence analysis of glutamate dehydrogenase (GDH) from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1 and comparison of the enzymatic characteristics of native and recombinant GDHs. Mol Gen Genet 257:338–347

Ramakrishnan V, Verhagen MFJM, Adams MWW (1997) Characterization of di-*myo*-inositol-1,1′-phosphate in the hyperthermophilic bacterium *Thermotoga maritima*. Appl Environ Microbiol 63:347–350

Robb FT, Park JB, Adams MWW (1992) Characterization of an extremely thermostable glutamate dehydrogenase: a key enzyme in the primary metabolism of the hyperthermophilic archaebacterium, *Pyrococcus furiosus*. Biochim Biophys Acta 1120:267–272

White RH (1993) Structure of modified folates in the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Biochemistry 32:745–753

Wykes JR., Dunnill P, Lilly MD (1972) The preparation of soluble high molecular weight NAD derivative active as a cofactor. Biochim Biophys Acta 286:260–268