A novel cycling assay for nicotinic acid–adenine dinucleotide phosphate with nanomolar sensitivity

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Nicotinic acid–adenine dinucleotide phosphate (NAADP) is a novel nucleotide derived from NADP that has now been shown to be active in releasing Ca^{2+} from intracellular stores in a wide variety of cells ranging from plant to human. Despite the obvious importance of monitoring its cellular levels under various physiological conditions, no assay has been reported for NAADP to date. In the present study, a widely applicable assay for NAADP with high sensitivity is described. NAADP was first dephosphorylated to nicotinic acid–adenine dinucleotide by treatment with alkaline phosphatase. The conversion was shown to be stoichiometric. NMN-adenylyltransferase was then used to convert nicotinic acid–adenine dinucleotide into NAD in the presence of high concentrations of NMN. The resultant NAD was amplified by a cycling assay involving alcohol dehydrogenase and diaphorase. Each time NAD cycled through these coupled reactions, a molecule of highly fluorescent resorufin was generated. The reaction could be performed for hours, resulting in more than a 1000-fold amplification. Concentrations of NAADP over the 10–20 nM range could be routinely measured. This novel cycling assay was combined with an enzymic treatment to provide the necessary specificity for the assay. NAADP was found to be resistant to NADase and apyrase. Pretreatment of samples with a combination of the hydrolytic enzymes completely eliminated the interference from common nucleotides. The versatility of the cycling assay can also be extended to measure nicotinic acid, which is a substrate in the synthesis of NAADP catalysed by ADP-ribosyl cyclase, over the micromolar range. All the necessary reagents for the cycling assay are widely available and it can be performed using a multi-well fluorescence plate reader, providing a high-throughput method. This is the first assay reported for NAADP and nicotinic acid, which should be valuable in elucidating the messenger functions of NAADP.

Key words: Ca^{2+} signalling, NAD, NADP, nicotinamide–adenine dinucleotide, NMN-adenylyltransferase.

INTRODUCTION

The Ca^{2+}-releasing activity of nicotinic acid–adenine dinucleotide phosphate (NAADP) was first discovered in sea urchin egg homogenates [1,2]. The Ca^{2+} storage it mobilizes is separate from the endoplasmic reticulum, which responds to the two other known Ca^{2+} messengers, cyclic ADP-ribose (cADPR) and inositol trisphosphate [1–3]. Despite their widely different structures and mechanisms of action, both NAADP and cADPR are synthesized by a single enzyme, ADP-ribosyl cyclase, and its mammalian homologue, CD38 [4]. The crystal structure of the cyclase has been solved and its active site has been characterized by X-ray crystallography and site-directed mutagenesis [5,6]. A catalytic mechanism of how the cyclase can use two different substrates, NAD and NADP, and synthesize two structurally different Ca^{2+} messengers has been described previously [7,8].

Since its discovery, NAADP has been shown to be active in mobilizing intracellular Ca^{2+} stores in a wide variety of cells ranging from plant to human (reviewed in [9–11]). Despite the general relevance of NAADP in Ca^{2+} signalling in cells, no assay has yet been reported. In principle, sea urchin egg homogenates can be used as a bioassay for NAADP. Indeed, the homogenates are responsive to nanomolar concentrations of NAADP and the response is highly specific [2]. The main disadvantage is that egg homogenates are not readily available, preventing a general adoption of the bioassay. We have recently developed [12] a novel cycling assay for cADPR by reversing the cyclase reaction to produce NAD stoichiometrically from cADPR. NAD is then amplified by coupling with an enzyme-cycling system that employs diaphorase to produce a fluorescent product [12]. A similar strategy is used in the present study. NAADP is first converted into nicotinic acid–adenine dinucleotide (NAAD) using alkaline phosphatase. NMN-adenylyltransferase (NMN-AT) is then used to convert NAAD into NAD, which is amplified by the same diaphorase-cycling system as described above for cADPR.

MATERIALS AND METHODS

Materials

Alcohol dehydrogenase from yeast (suitable for cycling assays), apyrase from potato, activated charcoal, diaphorase from *Clostridium kluyveri*, resazurin, Tris, NAD^+, nicotinamide, nucleotide pyrophosphatase from *Crotalus atrox* venom, NADase from *Neurospora crassa*, tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane were obtained from Sigma. Alkaline phosphatase from calf intestine was from Boehringer. Immobilon-P filters were from Millipore (Bedford, MA, U.S.A.).

Purification of NMN-AT

The enzyme was partially purified from pig liver as described by published methods [13]. Briefly, the liver was cut into small pieces and homogenized in buffer A [0.1 M potassium phosphate (pH 7.4)/1 mM dithiotreitol/1 mM $MgCl_2$/0.5 mM EDTA/

Abbreviations used: (c)ADPR, cyclic ADP-ribose; NAAD, nicotinic acid–adenine dinucleotide; NAADP, nicotinic acid–adenine dinucleotide phosphate; NMN-AT, NMN-adenylyltransferase.

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1 mM PMSF]. After centrifugation at 13000 *g* for 20 min, the supernatant was adjusted to pH 5.0 with dropwise addition of 1 M acetic acid. The precipitated protein was collected by centrifugation and dissolved in buffer A. The protein was loaded on to a phenyl-Sepharose column equilibrated with 3 M KCl in 0.1 M potassium phosphate (pH 7.4). After washing with 2 M KCl in 0.1 M potassium phosphate (pH 7.4), the NMN-AT was eluted by a linear gradient of 2–0 M KCl in 0.1 M potassium phosphate (pH 7.4). The enzyme could be used for the cycling assay at this step. Further purification resulted in loss of activity. NMN-AT was stable when stored at 4 °C in 1 M KCl at a protein concentration of 3.2 mg/ml.

Cycling assay for NAADP

Samples were incubated with alkaline phosphatase (10 units/ml) overnight at 37 °C. This resulted in stoichiometric conversion of NAADP into NAAD. The alkaline phosphatase was removed by filtration with Immobilon-P and the filtrate was recovered.

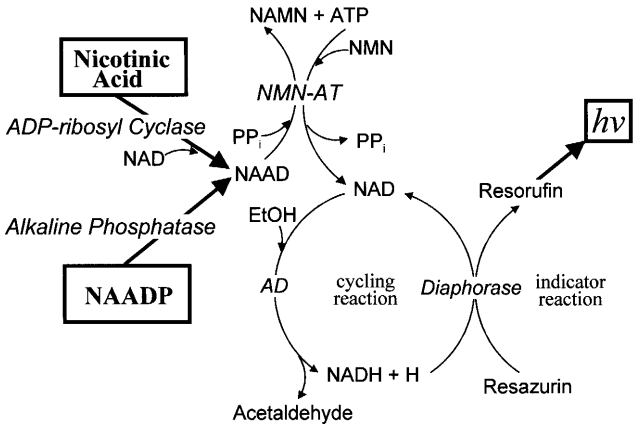
Subsequent reactions were conducted in 96- or 384-well plates. In 384-well plates, three additions of 30 μl were made. To 30 μl of sample, 30 μl of reagent 1 was added, which contained 0.2 mg/ml NMN-AT, 0.2 mM NMN, 0.2 mM PP_i, 10 mM nicotinamide, 2 mM NaF, 2 mM $MgCl_2$ and 100 mM Tris/HCl (pH 8). This initiated the conversion of NAAD in the samples to NAD. The conversion was allowed to proceed for 15 min at room temperature (25 °C). The cycling reagent (30 μl) was then added, which contained 2% ethanol, 100 μg/ml alcohol dehydrogenase, 10 μM resazurin, 10 μg/ml diaphorase, 10 μM flavin mononucleotide, and 100 mM sodium phosphate (pH 8). The cycling reaction was allowed to proceed for 4–10 h and the increase in the resorufin fluorescence (with excitation at 544 nm and emission at 590 nm) was measured periodically using a fluorescence plate reader. The results shown are means ± S.D. for at least three independent measurements.

The commercial diaphorase was treated with charcoal to remove bound NAD^+. Typically, the freeze-dried powder was diluted to 12 mg of protein/ml with water. Just before preparing the cycling reagent, 20 μl of enzyme solution was diluted with 60 μl of a 2% charcoal suspension in 20 mM phosphate (pH 7), and the enzyme/charcoal suspension incubated for 30 min at 37 °C. The charcoal was removed by centrifugation for 5 min in a microfuge and the enzyme recovered from the supernatant. Alcohol dehydrogenase as suitable for enzyme cycling was purchased, and it was used without charcoal treatment.

To remove all contaminating nucleotides, a mixture containing hydrolytic enzymes was added to the samples with the following final concentrations: 2.5 units/ml apyrase, 0.125 unit/ml NADase, 2 mM $MgCl_2$, 1 mM NaF, 0.1 mM PP_i, 0.16 mg/ml NMN-AT and 20 mM sodium phosphate (pH 8.0). The incubation proceeded overnight at 37 °C. The enzyme mixture hydrolysed all nucleotides, including NAD, in the samples, except NAADP, which is resistant to these enzymes. Enzymes were removed by filtration with Immobilon-P and samples were recovered in the filtrate with a Millipore filtration apparatus. After the hydrolytic treatment, alkaline phosphatase was added to convert the remaining NAADP into NAAD as described above.

RESULTS

The rationale of the cycling assay for NAADP is shown in Scheme 1. Both the cycling and indicator portions of the reaction are the same as those used previously in the cycling assay for cADPR and have previously been characterized and optimized [12]. The crucial enzyme in the cycling assay for NAADP is



Scheme 1 The cycling assay for NAADP

AD, alcohol dehydrogenase; NAMN, nicotinic acid mononucleotide; *hν*, fluorescent light.

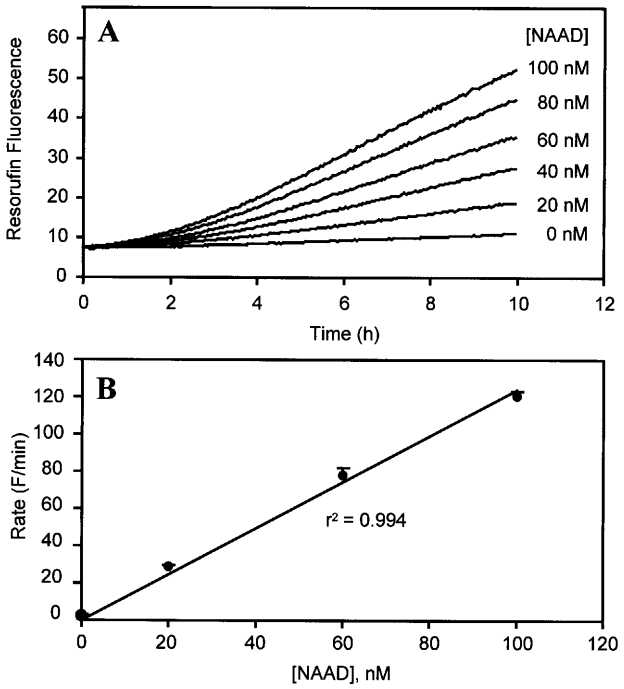


Figure 1 Time course of the cycling assay

(**A**) The reactions were started by the addition of the indicated concentrations of NAAD in 20 mM sodium phosphate (pH 8) to a mixture containing NMN-AT, alcohol dehydrogenase, diaphorase, resazurin, NMN and PP_i, as described in the Materials and methods section. The resultant increase in fluorescence due to the production of resorufin was monitored in a multi-well plate reader. (**B**) After a steady state was reached (4–10 h), the rates of resorufin fluorescence increase (Rate) were obtained by linear regression analyses of fluorescence time courses and plotted against NAAD concentration.

NMN-AT, which normally catalyses the synthesis of NAD from ATP and NMN, as shown on the right-hand side of the NMN-AT reaction in Scheme 1 [13]. A by-product of this reaction is PP_i. NMN-AT is a reversible enzyme and can catalyse the opposite reaction in the presence of high concentrations of PP_i. The enzyme can also use NAAD instead of NAD and, in the reversed mode, produces nicotinic acid mononucleotide and ATP [14], as shown in the left-hand side of the NMN-AT reaction in Scheme

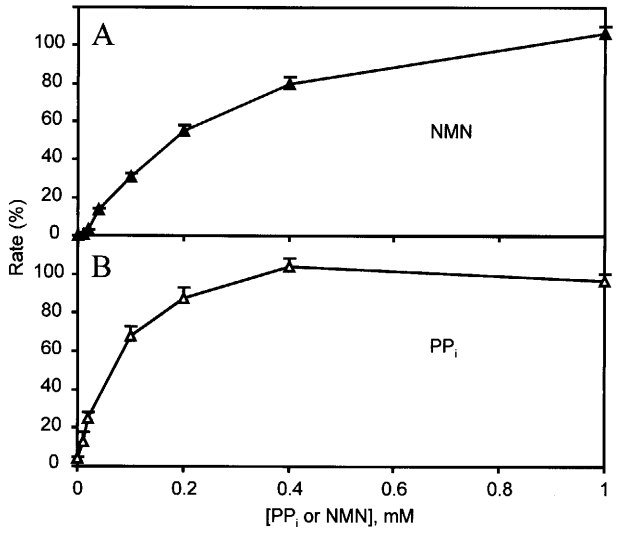


Figure 2 Optimization of the cycling assay

(**A**) Concentration of NMN, a substrate for the cycling reaction, was varied while the other substrates NAAD (100 nM) and PP_i (1 mM) were kept constant. (**B**) Concentration of PP_i was varied while NAAD (100 nM) and NMN (1 mM) were kept constant. The cycling reaction required both NMN and PP_i. In the absence of either, the rate of resorufin fluorescence increase (Rate) was close to zero.

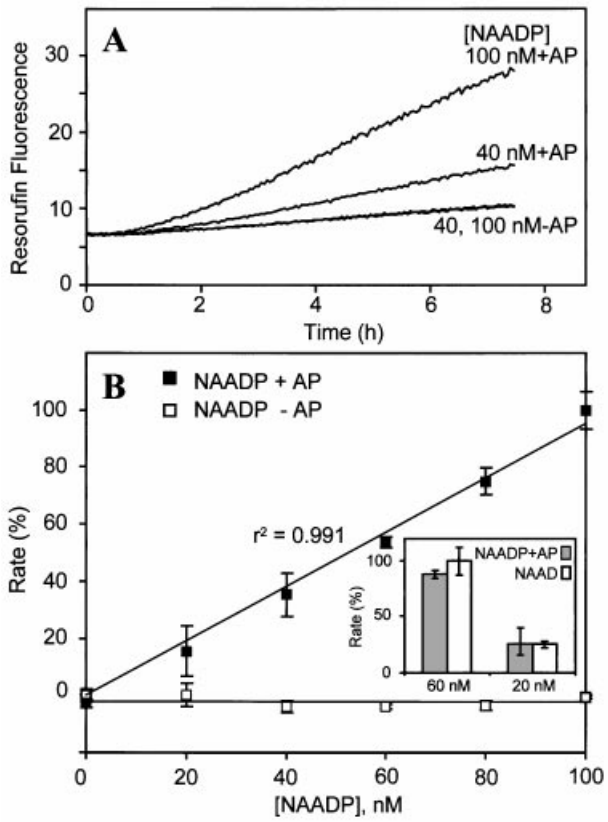


Figure 3 The cycling assay for NAADP requires pretreatment of the samples with alkaline phosphatase

(**A**) Addition of 40 and 100 nM of NAADP alone to the cycling assay mixture produced only background fluorescence change, indicating NAADP itself was not a substrate for the cycling reaction. Treatment of NAADP with alkaline phosphatase (AP) converted it into NAAD and resulted in a concentration-dependent increase in resorufin fluorescence. Details of the alkaline phosphatase treatment are given in the Materials and methods section. (**B**) The rate of fluorescence increase (Rate) in the linear phase of NAADP samples after the alkaline phosphatase treatment (■) is linearly related to the NAADP concentration, with a r^2 value of 0.991. Without the alkaline phosphatase treatment (□), no fluorescence increase above the background was seen. The inset shows that the conversion was stoichiometric. After the conversion, NAADP produced essentially the same rate of resorufin fluorescence increase as NAAD.

1. In other words, NAAD can be enzymically converted into NAD in the presence of excess PP_i and NMN. NAD is then amplified by the cycling reaction, which couples alcohol dehydrogenase with diaphorase. In each cycle, a molecule of highly fluorescent resorufin is produced.

The time course of the reaction could be followed fluorimetrically and continuously, as shown in Figure 1(A). The reaction mixture contained all the enzymes, NMN-AT, alcohol dehydrogenase and diaphorase, as well as the substrates, excess PP_i and NMN. The reaction was started by the addition of various concentrations of NAAD. There was an initial lag period of approx. 2 h, during which a steady state was reached between all the reactions catalysed by the three enzymes. Thereafter, the production of resorufin increased linearly with time. The rate of fluorescence increase (Rate) in this linear phase from 4 to 10 h was directly proportional to the concentration of NAAD as shown in Figure 1(B), and the regression line has a regression coefficient r^2 of 0.994.

The fact that the fluorescence increase was indeed due to the conversion of NAAD into NAD catalysed by NMN-AT was shown by the dependence of the reaction on the substrates PP_i and NMN. In the absence of either substrate, very little fluorescence increase was seen. The residual fluorescence was probably due to contamination of some of the reagents with minute amounts of NAD or ATP. Increasing the concentration of NMN while maintaining PP_i constant increased the rate of fluorescence increase, reaching a saturating value at approx. 1 mM NMN as shown in Figure 2(A). A double-reciprocal plot indicated the half-maximal concentration of NMN was approx. 500 μM. A similar dependence on concentration was also seen with increasing concentrations of PP_i while NMN was kept constant, as shown in Figure 2(B). The half-maximal concentration of PP_i was approx. 69 μM. In all subsequent experiments, 0.2 mM was used for each of the two substrates. The saturating concentration of 1 mM for NMN was not used because of the slight contamination of commercial NMN with NAD.

After optimizing the substrate concentrations, the cycling reaction was applied to NAADP. Figure 3(A) shows that NAADP was not a substrate of NMN-AT and only background fluorescence increase was seen. We have previously shown that alkaline phosphatase can specifically remove the 2′-phosphate of NAADP and convert it into NAAD [11,15]. Indeed, after treatment of NAADP with alkaline phosphatase, a fluorescence increase could now be readily detected. The time course showed an initial lag followed by a linear phase, essentially the same as observed with NAAD itself (Figure 2). The rate of fluorescence increase in the linear phase was directly proportional to the concentration of NAADP, as shown in Figure 3(B, ■). The regression line has a r^2 value of 0.991, close to perfect correlation. The conversion of NAADP into NAAD was essentially stoichiometric, as shown in the inset of Figure 3(B). The rate of fluorescence increase for the same concentration of NAADP following the enzyme treatment was essentially the same as NAAD.

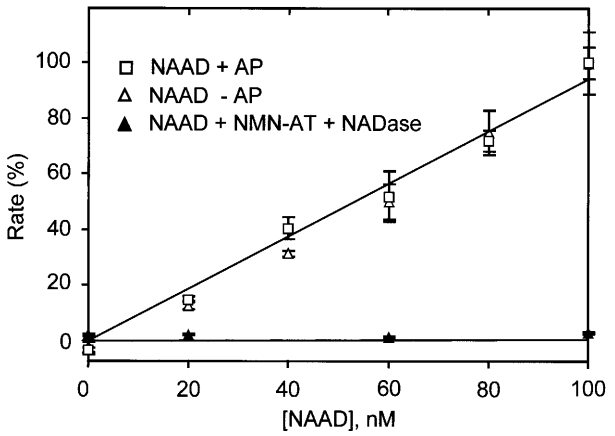


Figure 4 Enzymic removal of NAAD

NAAD itself was a substrate of the cycling reaction, and the rate of fluorescence increase (Rate) was the same both before (△) and after (□) the alkaline phosphatase treatment. Pretreatment of NAAD with an enzyme mixture containing NMN-AT and NADase (▲) completely hydrolysed NAAD and eliminated the resorufin fluorescence increase. Details of the enzyme treatment are given in the Materials and methods section.

Table 1 Removal of nucleotides interfering with the NAADP assay by enzyme pretreatment

AP, alkaline phosphatase; Enz, enzyme; NGD, nicotinamide–guanine dinucleotide; NHD, nicotinamide–hypoxanthine dinucleotide.

	Rate of resorufin fluorescence increase		
Nucleotide (100 nM)	A (−Enz−AP)	B (+Enz*+AP†)	C (+NAADP‡+Enz*+AP†)
NAADP	0±0.5	40±5	–
NAAD	37±1	0.1±0.1	43±4
NAD	523±35	0.6±0.2	45±1
ATP	117±6	0.2±0.2	43±3
ADP	100±2	0.4±0.3	44±2
NGD	41±1	0.7±0.3	41±3
NHD	40±1	0.2±0.2	42±2
GTP	11±2	0.3±0.2	42±1
cADPR	6±1	0.7±0.1	46±2
NADP	4±1	0.6±0.3	42±3
AMP	3±1	0.8±0.4	44±3
GMP	2±1	0.4±0.3	43±3
ADPR	2±1	0±0.7	49±0.2

* Samples were pretreated with an enzyme mixture containing NMN-AT, NADase and apyrase as described in the Materials and methods section. These enzymes were subsequently removed by filtering through Immobilon-P.

† Alkaline phosphatase was added to convert NAADP, if present, into NAAD. It was subsequently removed by filtering through Immobilon-P.

‡ Nucleotide samples were spiked with 100 nM NAADP.

The probability that NAADP requires the alkaline phosphatase treatment before it can produce a signal in the cycling assay provides a means to distinguish it easily from NAAD. As shown in Figure 4, NAAD produced the same rate of fluorescence increase in the presence or absence of the alkaline phosphatase treatment. By exploiting the differences in sensitivity of NAADP and NAAD towards various enzymes, the versatility of the cycling assay can in fact be extended. For example, to be able to measure NAADP even in samples containing NAAD, a strategy was devised to remove NAAD from the samples first. NAAD, like NAADP, is resistant to NADase, but unlike NAADP, it is a substrate for NMN-AT. Thus its removal could be achieved by first converting it into NAD with NMN-AT, which can then be hydrolysed by NADase included also in the reaction mixture. As shown in Figure 4 (▲), this treatment indeed totally removed NAAD. When the samples were subsequently measured with the complete cycling assay, no fluorescence was produced.

This strategy was further tested by spiking samples of NAAD with NAADP. The results are summarized in Table 1. When NAADP itself was measured with the complete cycling assay, no fluorescence increase was detected (Table 1, column A, row 1). After the alkaline phosphatase treatment (Table 1, column B, row 1), the rate of fluorescence increase was about the same as that produced by the same concentration of NAAD (Table 1, column A, row 2). If NAAD was first pretreated with NMN-AT and NADase, the signal was totally eliminated (Table 1, column B, row 2). When samples containing both NAAD (100 nM) and NAADP (100 nM) were pretreated with NMN-AT and NADase, only NAAD was removed but not NAADP, since the latter is not a substrate for either NMN-AT or NADase (see Figure 3A). Indeed, subsequent measurement of the samples following alkaline phosphatase treatment produced the same signal as NAAD itself without enzyme pretreatment (Table 1, column C, row 2). These results indicate that the pretreatment can effectively remove NAAD but leave NAADP in the same samples untouched. This strategy required effective removal of the enzymes after the pretreatment. This could be done easily by filtering the samples through Immobilon-P, which has high protein-binding capacity. The same procedure can also be used to remove alkaline phosphatase before the complete cycling mixture was added. The use of a filtration apparatus designed for use with a multi-well plate made these steps simple to perform.

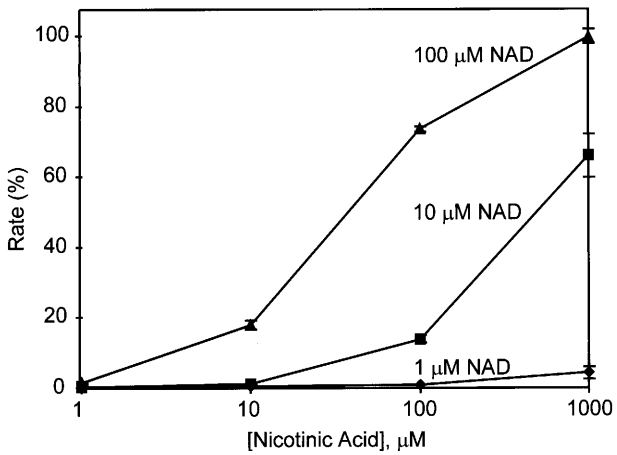


Figure 5 A cycling assay for nicotinic acid

Various concentrations of nicotinic acid were converted into NAAD in the presence of the indicated concentrations of NAD by incubating overnight with 0.1 μg/ml *Aplysia* ADP-ribosyl cyclase at pH 4 in 100 mM sodium acetate. The base-exchange reaction was terminated by adjusting to pH 8 using a solution containing 1 M Tris base, 100 mM nicotinamide and 10 mM $MgCl_2$. The remaining NAD was removed by adding 0.05 unit/ml NADase and incubating overnight at 37 °C. The enzymes were removed by filtration through Immobilon-P plates. The samples were assayed for NAAD using the same conditions as described in the Materials and methods section.

We have also found that NAADP is resistant to hydrolysis by apyrase. Inclusion of this additional hydrolytic enzyme in the pretreatment mixture allowed removal of all common nucleotides

in the samples. As shown in Table 1, there were a number of interfering nucleotides. The most obvious ones were NAD and ATP (Table 1, column A, rows 3 and 4), since they are components of the cycling assay (see Scheme 1). Both were effectively hydrolysed by the NADase and apyrase present in the pretreatment mixture (Table 1, column B, rows 3 and 4). In fact, the pretreatment was capable of removing the signals produced by ADP, nicotinamide–hypoxanthine dinucleotide, nicotinamide–guanine dinucleotide and GTP, as well as those mildly interfering nucleotides, such as cADPR, NADP, AMP, GMP and ADPR (Table 1, compare columns A and B for rows labelled with the nucleotides). Some of these nucleotides may not be interfering by themselves, but instead may be contaminated by small amounts of either ATP or NAD. Without the pretreatment, the contamination would be greatly amplified by the cycling assay. In all cases, NAADP spiked into the samples was not affected by the pretreatment and produced a similar signal as NAAD itself without the pretreatment (Table 1, column C). In summary, treatment of samples with an enzyme mixture containing NMN-AT, NADase, apyrase and alkaline phosphatase readily removes interference of all the nucleotides listed in Table 1 (column B), and only NAADP can produce a signal under this condition (Table 1, column C). This strategy provided the cycling assay with a high degree of specificity.

The versatility of the cycling assay can be easily extended to measure nicotinic acid as well. Nicotinic acid is a substrate for the synthesis of NAADP via the base-exchange reaction catalysed by ADP-ribosyl cyclase and CD38 [4]. As far as we know, no biochemical assay has been described for nicotinic acid. Nicotinic acid can be converted into NAAD using the base-exchange reaction in the presence of high concentrations of NAD (see Scheme 1). The samples can then be treated with NADase to hydrolyse the remaining NAD. NAAD produced by the base-exchange reaction is not a substrate of NADase and would not be affected. After removal of the NADase, NAAD can then be assayed by the cycling reaction. The results are shown in Figure 5. In the presence of 0.1 mM NAD, the assay is capable of detecting nicotinic acid in the micromolar range. The concentration dependence was not linear, which is probably a reflection of the substrate dependence of the base-exchange reaction.

DISCUSSION

The cycling assay described in the present study can readily detect 10–20 nM NAADP, as shown in Figures 1 and 3(A). The sensitivity can be easily extended by increasing the cycling time. In this respect, the limitation would be the purity of the reagents used, since any contamination with NAD would also be amplified by the assay. We have found that treatment of some of the enzymes used in the assay with activated charcoal indeed helps in reducing the background fluorescence increase. Although not used in the present study, pretreatment of the nucleotide substrate NMN with NADase to remove contaminating NAD should also help.

NAADP is an unusual nucleotide that is resistant to NADase and apyrase. Taking advantage of this property, an enzyme pretreatment strategy is devised, which provides a high degree of specificity for the assay. After the enzyme pretreatment, none of the 12 related nucleotides exhibit any interference (Table 1). NAADP itself is also not a substrate for NMN-AT and thus in the absence of the alkaline phosphatase treatment produces no fluorescence increase. Therefore the difference in signals in the presence and absence of the alkaline phosphatase treatment provides a valuable diagnostic test that further ensures that the measured signals are indeed from NAADP (Figure 3A). In this respect, withholding one of the required substrates of the NMN-AT, NMN or PP_i also would be a convenient way to obtain background measurements (cf. Figure 2). Additionally, nucleotide pyrophosphatase can be used, which we have previously shown [11,15] to be able to hydrolyse NAADP effectively and thus eliminate the signal. The use of enzyme pretreatment to remove interference is a common practice in many assays and is also a necessary step even for the highly specific RIA for cADPR [16,17]. Compared with chromatographic purification, enzyme pretreatment is preferred, since chromatography is cumbersome, requiring exact measurement of sample recovery, and can only process one sample at a time. In contrast, the present assay is performed on a multi-well plate, allowing high-throughput measurement of a large number of samples simultaneously.

In principle, a bioassay for NAADP using sea urchin egg homogenates can be devised. Similarly, a radio-receptor assay making use of the specific and high-affinity binding of NAADP to the egg microsomes is also possible [18,19]. Both, however, require access to sea urchin eggs, which are not easily available. The radio-receptor assay also requires custom synthesis of radioactive NAADP with a high enough radiospecific activity; not a trivial procedure. The present cycling assay aims to circumvent these difficulties and uses widely available reagents, allowing easy and widespread adoption of the assay. Additionally, the remarkable linearity of the cycling assay is also much preferred to the cumbersome curvilinear calibration required by both the bioassay and radio-receptor assay.

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