Regulation of a NAD⁺ Kinase Activity Isolated from Asynchronous Cultures of the Achlorophyllous ZC Mutant of *Euglena gracilis*

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NAD⁺ kinase was isolated by chromatography steps from asynchronous cultures of the achlorophyllous ZC mutant of *Euglena gracilis*. A non Ca²⁺-calmodulin dependent form, whose activity was stimulated by EGTA, was selected for its large quantity and high specific activity. Studies of the kinetic parameters revealed two kinds of NAD⁺ binding site, depending on NAD⁺ concentrations, and changes induced by EGTA, Ca²⁺ and Ca²⁺-calmodulin. The search for effectors, soluble (S) and membrane-bound (P), in *Euglena gracilis* synchronously grown (in a light-dark regime of 12h:12h), and collected at circadian times (CT) – corresponding to the maximum, CT 17, and to the trough, CT 09, of the circadian rhythm of NAD⁺ kinase activity – was also undertaken by testing the modulations of the kinetic parameters of the prepared NAD⁺ kinase. The results suggest: (i) structural changes of NAD⁺ binding sites depending on NAD⁺ concentrations; (ii) possible binding of the $Mg\text{-}ATP^{-2}$ (or $Ca\text{-}ATP^{-2}$) on the NAD⁺ sites, because of their common ADP motif; and (iii) different and specific modulations of the kinetic parameters of the two types of NAD⁺ binding site by the Ca²⁺-calmodulin complex. In addition, the results indicate, in pelletable fractions isolated at CT 09 and CT 17, the presence of two kinds of effector: (i) the first one, possibly Ca²⁺, which increases the Vmax's while decreasing the binding of NAD⁺; (ii) the second one, possibly the Ca²⁺-calmodulin complex, which provokes a complete reverse effect. Each of these two effectors seems to be, alternatively and rhythmically (eight circadian hours apart), partially released from the membranes.

Introduction

NAD⁺ kinase (EC 2.7.1.23), also called ATP:NAD⁺2'-phosphotransferase, is an ubiquitous enzyme playing an essential role in the cellular metabolism of almost all organisms. NAD⁺ kinase catalyses the only known biochemical reaction leading to the production of NADP⁺ from ATP and NAD⁺. NADH (abundantly produced by glycolysis and citric acid pathways) and NADPH (generated either by the oxidative branch of pen-

tose phosphate pathway, or by many dehydrogenases, or by the energy linked nicotinamide nucleotide transhydrogenase (Enander and Rydström, 1982)) play different but complementary roles in the cell. While oxidation of NADH mainly promotes the ATP production in mitochondria, oxidation of NADPH supplies electrons and protons in most of the reduction steps necessary for the biosynthesis pathways. The NAD$^+$ kinase is therefore a key enzyme controlling the relative importance of anabolic and catabolic pathways in the cell. The total enzyme activity of NAD$^+$ kinase as well as the relative percentages of different molecular forms have already been shown to vary: in fungi (Afanasieva *et al.*, 1982), in green plants (Allan and Trewavas, 1985) and in the silkworm in which the enzyme activity of different forms was observed to be related to the regulation of ontogeny (Filippovich *et al.*, 1990). Moreover, in plants, two types of NAD$^+$ kinase were shown to be either calmodulin-dependent or directly activated by Ca^{2+} (Marmé, 1985; Roberts *et al.*, 1985; Zhang *et al.*, 1994). In corn, the calmodulin-dependent enzyme has been located in the cytoplasm (Marmé and Dieter, 1983), in the outer membrane of mitochondria (Dieter and Marmé, 1984), and also in the chloroplast envelope (Simon *et al.*, 1984) while a calmodulin-independent form was located in the stroma (Simon *et al.*, 1984). This last form may catalyze the light-induced conversion of NAD$^+$ to NADP$^+$ using the ATP produced by photophosphorylation (Muto and Miyachi, 1986). In *Saccharomyces cerevisiae*, the NAD$^+$ kinase was found distributed in the microsomal and cytosolic fractions, but the mitochondrial kinase exhibited a unique specificity to the NADH substrate (Iwahashi *et al.*, 1989).

An interesting feature of NAD$^+$ kinase is to be a putative element of the circadian oscillator, or to play a role upstream of the clock to regulate its function. A model for a biochemical feedback loop has been proposed (Goto *et al.*, 1985) that would underline circadian rhythmicities in the wild type photosynthetic Z strain of *Euglena gracilis*. NAD$^+$ kinase had already been shown to display circadian rhythmicity in *Lemna* (Goto, 1984). Cultures of the achlorophyllous ZC mutant of *Euglena gracilis* (Calvayrac and Ledoigt, 1976) that exhibited circadian rhythms of cell division (Carre *et al.*, 1989) have also been shown to exhibit circadian variations in NAD$^+$ kinase activity, which persisted in stationary phase although cells had stopped dividing (Laval-Martin *et al.*, 1990a). These oscillations of the enzyme activity, measured in soluble and membrane-bound fractions, were associated with changes in the affinity of the enzyme for the substrate NAD$^+$ (Laval-Martin *et al.*, 1990b). Such changes may be caused by the expression of different types of isoenzymes; alternatively, they may reflect the interaction of NAD$^+$ kinase with cellular effectors, which levels might be controlled by the circadian oscillator. For this reason, the effects of possible effectors (Ca^{2+} and calmodulin), susceptible to vary as a function of the circadian time, and able to modify the binding of the substrate NAD$^+$ (apparent K_m) and/or the maximum velocity (V_{max}) of the NAD$^+$ kinase activity, have been tested in this study.

The enzyme was isolated from asynchronous cultures of achlorophyllous ZC mutant of *Euglena gracilis*, grown under constant light at 25 °C, conditions in which they did not exhibit any circadian rhythmicity (Carre *et al.*, 1989). This present paper reports (i) the isolation of a NAD$^+$ kinase from endogenous effectors and (ii) the modulation of its enzyme activity by effectors commonly used in physiological and biochemical studies: calcium (Ca^{2+}), calcium and calmodulin (Ca^{2+}-CaM), or EGTA. This prepared enzyme was also used for the search for possible effectors, that circadianly may modulate the binding of the substrate NAD$^+$ (apparent K_m) and/or the maximal velocity (V_{max}) of the enzyme activity. For that purpose, the kinetic parameters of the prepared NAD$^+$ kinase were analysed after mixing the enzyme with the effectors possibly present in the soluble (S) and pelletable (P) fractions, which were separated from synchronized cells collected at CT09 (i.e. 9 hours after the onset of light) and CT17, respectively corresponding to the minimum and the maximum values of the entrained circadian rhythm of NAD$^+$ kinase activity. Finally, these observed effects on the prepared enzyme were compared to those induced by EGTA, Ca^{2+}, and Ca^{2+}-calmodulin.

Materials and Methods

Culture Conditions

For the isolation of the prepared NAD⁺ kinase

Achlorophyllous ZC mutant of *Euglena gracilis* were grown for 7 days under constant light, LL (illumination of 20 μE m^{-2} sec^{-1} provided by cool white fluorescent bulbs), at 25 °C in a medium supplemented with vitamins B_1, B_{12}, with 10^{-4} M of cysteine and methionine (Edmunds and Funch, 1969), and containing 0.1 percent of ethanol as the sole carbon source (Carre *et al.*, 1989).

For the preparation of cell extracts at different circadian times

Cells were grown at 16.5 °C, up to the density of 10^5 cells·ml^{-1}, in LD: 12,12 (same illumination of 20 μE m^{-2} sec$^{-1)}$, conditions in which they exhibited entrained circadian rhythms of NAD⁺ kinase activity (Laval-Martin *et al.*, 1990a). At times corresponding to the trough, CT09, and to the maximum, CT17, of the enzyme activity, the cells were harvested (1,500 x *g*, 10 min), resuspended in 50 mM Tris pH 7.5 (at a density of 10^6 cells·ml^{-1}), separated in aliquots stored in liquid nitrogen.

On the day of the assays, 1 mM PMSF and 1 μg/ml leupeptin were added prior to sonication (3 times 15 sec at 0 °C, separated by 30 sec intervals). The resulting supernatants (39,000 x *g*, 10 min) constituted the S fractions, S09 or S17; the corresponding pellets, resuspended in an equivalent volume of 50 mM Tris pH 7.5, 1 mM PMSF and 1 μg/ml leupeptin, then homogenized by a 15 sec sonication constituted the P fractions, P09 or P17.

Isolation of NAD⁺ kinase

The procedure was performed in the cold, and generated reproducible results.

Extraction

Cells were harvested at the end of the exponential phase of growth (2.5 x 10^6 cells·ml^{-1}) by centrifugation (1,500 x *g*, 10 min). They were resuspended in buffer A (62.5 mM Tris-HCl pH 7.5, 7 mM MgCl$_2$, 0.6 M KCl, 10 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT and 3.5 μg/ml pepstatin), then sonicated at 0 °C for five intervals of 15 sec separated by 30 sec rest periods for cooling

(Branson Sonifier Model 250, set on 12 W). The crude extract (CE) was centrifuged (105,000 x *g*, 60 min) to generate the supernatant (S105).

Desalting by Sephadex G-75 chromatography

To remove phenolic and low molecular weight compounds, the S105 was applied on a Sephadex G-75 column (2.5 x 50 cm), previously equilibrated with the buffer B (62.5 mM Tris-HCl pH 7.5, 7 mM MgCl$_2$, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and 3.5 μg/ml pepstatin). Fractions (5.0 ml) were collected (flow rate, 15 ml per hour) and analyzed for the determination of protein concentration and NAD⁺ kinase activity. The column calibration was performed with: dextran blue (1,000 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), ATP (551 Da).

Exclusion chromatography on Sephacryl S-300

The active fractions from Sephadex G-75 column were pooled, concentrated by centrifugation (1,000 x *g*, 60 min) on Centriflo-Amicon Membrane CF 25 (fractionation range 25,000), and then loaded on a Sephacryl S-300 column (1.6 x 70 cm) previously equilibrated with a buffer C (62.5 mM Tris-HCl pH 7.5, 7 mM MgCl$_2$, 1 mM DTT). Fractions (2 ml) were automatically collected (elution performed with buffer C, flow rate 5 ml per hour) and assayed for the determination of protein concentration and NAD⁺ kinase activity. The active fractions were either immediately analyzed, or aliquoted and stored in liquid nitrogen after addition of 20% glycerol (v/v) for later use. Molecular weights of proteins contained in the active fractions were determined using: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA, carbonic anhydrase and cytochrome c as above.

Determination of NAD⁺ kinase activity

Assay for the prepared enzyme

The NAD⁺ kinase activity was routinely assayed at 25 °C in a medium containing 50 mM Tris-HCl pH 7.5, 7 mM MgCl$_2$, 6 mM nicotinamide, 0.55 mM NAD⁺, 2.5 mM ATP. The reaction, initiated by the addition of 25 μl of the sample (2 mg of protein per ml) to a total final volume of 250 μl, was either immediately stopped by a two min. heating of the assay mixture in a bath of boiling water (t_0 sam-

ple), or performed at 25 °C for 10 min. and then stopped as described (t_{10} sample). In these conditions, the initial velocities were linear for one hour.

The NAD⁺ kinase activity was also tested in the presence of either 2 mM $CaCl_2$ (Ca^{2+}), or 2 mM $CaCl_2$ plus 6 nM of calmodulin from bovine brain (Ca^{2+}-CaM), or 2.5 mM EGTA, added to the control assay.

Assays for the cell fractions S09, P09, S17 and P17

The reaction was initiated by the addition of 150 μl of the cell fraction (5 mg of protein per ml) and performed in the same conditions than those described above.

Assays after mixing the prepared enzyme with the cell fractions

For these mixing assays, 150 μl of cell fraction were added to 5 μl of the prepared enzyme, and tested as above.

NADP⁺ measurements

For the NADP⁺ measurements (Goto, 1984), a microassay was used. In each microplate well, 75 μl of the NAD⁺ kinase assay mixture were added to 175 μl of a medium containing 100 mM bicine-NaOH pH 8.0, 5 mM G6-P, 1 mM PES, 0.42 mM MTT, 1% PVP. A reaction was initiated by the addition of 0.25 unit of G6-PDH. The kinetics of reduction of the MTT were followed at 570 nm using a EL 340 Biokinetics reader (BioTek instruments INC) coupled with a computer. The results were automatically quantified by comparison with those obtained in the presence of known amounts of NADP⁺ ranging from 0 to 75 pmol. The values of the t_0 samples corresponding to the endogenous NADP⁺ contents of the initial extracts, were subtracted from the results of the t_{10} samples. Furthermore, in order to detect eventual inhibition of MTT reduction, 5 pmol of NADP⁺ were added into each well after about twenty readings. The ensuing change in slope was compared to the standard slope corresponding to 5 pmol of NADP⁺, and corrective factors (which ranged from 1.0 to 1.2 for S fractions and from 1.0 to 1.6 for P fractions and the prepared enzyme) were applied when necessary.

Protein content

Proteins were detected in the column eluates by monitoring absorbency at 280 nm. Protein concentration was determined using Biorad reagent and BSA as standard (Bradford, 1976).

Results

Characterization of the prepared NAD⁺ kinase

After extraction in the buffer A, the cytosoluble proteins of the supernatant (S105), represented 34% of total proteins in the crude extract, CE (Table I). Nevertheless, in S105 the total and specific NAD⁺ kinase activities, were respectively amplified by factors 1.7 and 4.9 compared with those in the CE. This clearly illustrates the inhibitory interactions between membrane effectors and soluble NAD⁺ kinase previously hypothesized (Laval-Martin *et al.*, 1990a).

Most of the total NAD⁺ kinase activity of S105 was eluted from Sephadex G-75 column in the range 56 to 250 kDa, with an optimum around 70 kDa. Two peaks of specific activity were respectively and reproducibly observed (pattern not shown) : one within the void volume, MW greater than 250 kDa, and the other at 63 kDa. The total active pooled fractions displayed a specific activity

Table I. Summary of the procedure for the isolation of a NAD⁺ kinase from achlorophyllous ZC mutant of *Euglena gracilis*.

Steps	Total protein [mg]	Specific activity (nmol NADP⁺/h x mg of protein)	Purification (fold)	Recovery (%)
Crude extract	670	12.9	1.0	100.0
S105	231	63.9	4.9	170.4
Sephadex G-75	92	129.2	10.0	136.8
– after concentration	92	27.2	2.1	29.0
Sephacryl S-300	38	85.2	6.6	37.5

of 129 nmol of $NADP^+$ per hour per mg of proteins. This chromatography step resulted in a 10-fold increase of the initial specific activity of CE (Table I). However, after the subsequent concentration of the pooled fractions using "Centriflo-Amicon Membrane CF 25," considerable loss of specific activity was observed. This could be due to the elimination of factors of MW smaller than 25 kDa during the concentration step, and/or to structural changes provoked by aggregation of proteins when concentrated, modifying the activity. The lost activity was not restored after the mixing of the concentrated and "eliminated" fractions, which therefore favored the hypothesis of an inactivation by aggregation effect.

The concentrated NAD^+ kinase submitted to Sephacryl S-300 column, was eluted as a single peak (called fraction T), corresponding to an average MW of 227 kDa (Fig. 1). A purification factor of 6.6 could be noticed after this step (Table I), and five peaks of specific activity (A to E) could be distinguished (Fig. 2A): two within the void volume (A and B of MW upper than 200 kDa, respectively eluted with 29 and 35 ml), and the three others ones being C, 170 kDa; D, 95 kDa;
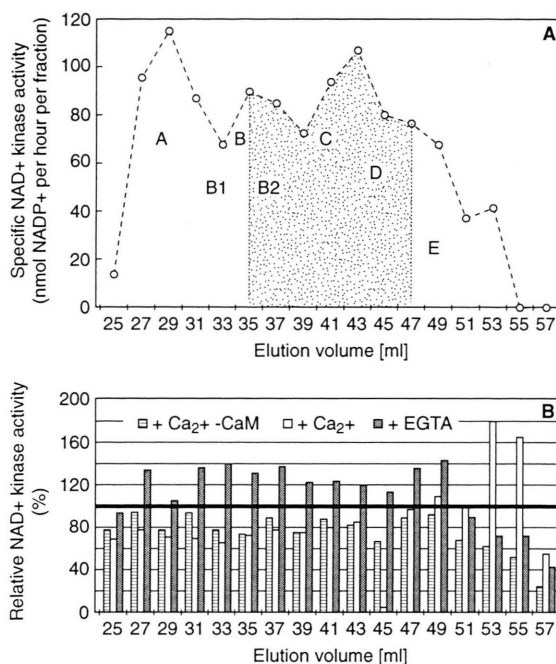

Fig. 1. Elution pattern of NAD^+ kinase from Sephacryl S-300 column (size of the column : 1.6 x 70 cm; flow rate: 5 ml per hour). The column was equilibrated with buffer C (62.5 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 1 mM DTT) and elution performed with the same buffer. The volume of each fraction was 2 ml. NAD^+ kinase activity (nmol $NADP^+$ formed per hour) was measured in each fraction, each point representing the mean of 3 assays. This column was calibrated with marker proteins symbolized by arrows: (1) β-amylase (200 kDa), (2) alcohol dehydrogenase (150 kDa), (3) bovine serum albumin (66 kDa), (4) carbonic anhydrase (29 kDa), (5) cytochrome c (12.4 kDa). NAD^+ kinase was eluted as a single peak, called total fraction T, symbolized by shaded area.

Fig. 2. Specific NAD^+ kinase activity of active Sephacryl S-300 fractions and effects of Ca^{2+}, or Ca^{2+} and calmodulin, or EGTA.
A. Specific NAD^+ kinase activity pattern (expressed as nmol $NADP^+$ formed per hour per mg of protein) of active fractions eluted from Sephacryl S-300 column (shaded area of the Fig. 1).
B. Effects of either 2.5 mM Ca^{2+}, or 2.5 mM Ca^{2+} plus 1 unit of calmodulin, or 2.5 mM EGTA. The specific NAD^+ kinase activity of a given fraction tested in the presence of an effector was expressed as the percentage of that of the same fraction tested without effector.
The shaded area indicates the fractions pooled for further kinetic studies.

and E, 40 kDa. The specific NAD^+ kinase activities of A to D peaks were: 115, 89, 107, and 76 nmol of $NADP^+$ per hour per mg of proteins, while E peak presented a lower specific activity (41 nmol $NADP^+$ per hour).

Despite the weak purification factor of 6.6 (mainly due to the concentration step), the NAD^+ kinase activity, devoid of all small effectors, was considered satisfying for the physiological study undertaken.

In the case of most of the eluates constituting A to D peaks (Fig. 2B), EGTA (known as specific Ca^{2+} chelator) surprisingly induced a significant stimulation of the NAD^+ kinase activity (140% compared to the control taken as 100%), while the
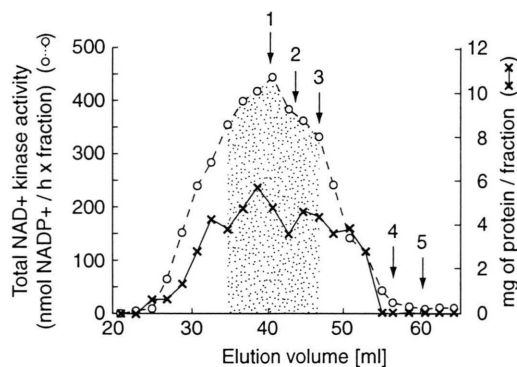
addition of Ca^{2+}, or of Ca^{2+}-CaM resulted in about a 20% inhibition.

Conversely, in eluates of the E peak, Ca^{2+} alone strongly increased the NAD$^+$ kinase activity by about 70%, while EGTA reduced it by 30%. The effect of Ca^{2+}-CaM on these E peak eluates was a reduction in enzyme activity, as it was the case with eluates of A to D peaks, but twice as stronger.

Determination of kinetic parameters

For kinetic studies requiring NAD$^+$ kinase in quantity, the fractions of B2 to C peaks (characterized by great amounts of proteins, molecular weights about 250 to 100 kDa, high specific activities and comparable sensitivities to effectors) were pooled together, and referred as prepared NAD$^+$ kinase.

In the presence of 2.5 mM ATP-7 mM MgCl$_2$, NAD$^+$ concentrations were tested over a range of 0–2.5 mM. The ratios initial substrate concentration [NAD$^+$]$_o$/initial velocity (v_o) were plotted versus [NAD$^+$]$_o$, (Fig. 3), according to Hanes-Woolf equation, $[S]/v_o = K_m/V_{max} + [S]/V_{max}$, (Henderson, 1986). The assays, repeated three times and considered as control in Table II, consistently generated bimodal curves indicating two distinct kinetic behaviors of the enzyme, depending on the range of NAD$^+$ concentrations (low concentrations, 0–0.12 mM and high concentrations, 0.15–2.5 mM). An apparent K_m value of 0.16 mM for NAD$^+$, with a V_{max} value of 101 nmol of NADP$^+$
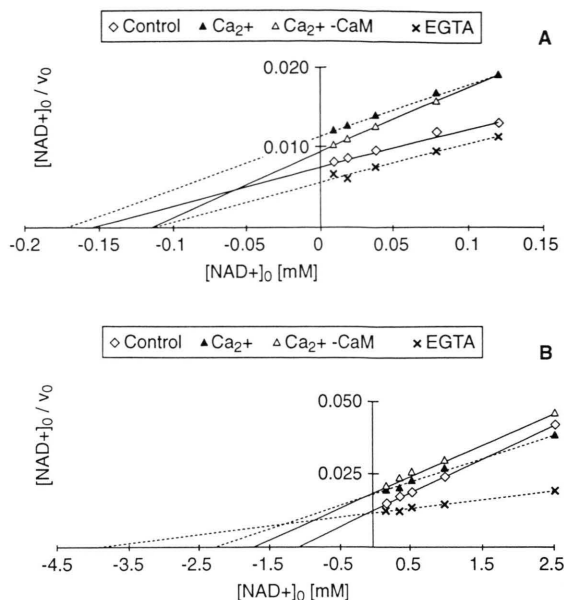

Fig. 3. Influence of either 2.5 mM Ca^{2+}, or 2.5 mM Ca^{2+} plus 6 nM of calmodulin, or 2.5 mM EGTA on the reaction rate of NAD$^+$ kinase tested in two NAD$^+$ concentration ranges: low range of NAD$^+$ concentrations, 0 to 0.15 mM, **(A)**; high range of NAD$^+$ concentrations, 0.15 to 2.5 mM, **(B)**. The initial velocity was expressed as pmol of NADP$^+$ formed per hour per assay and the ratio: initial substrate concentration [NAD$^+$]$_o$ / initial velocity (v_o), was plotted versus [NAD$^+$]$_o$. Equations of the linear regression curves as well as the correlation coefficients (R^2) are:

NAD$^+$ CONCENTRATION RANGE : 0 to 0.15 mM
Control	y = 0.0494x + 0.0077 (0.95)
Ca^{2+}	y = 0.0677x + 0.0114 (0.99)
Ca^{2+}-CaM	y = 0.0829x + 0.0095 (0.99)
EGTA	y = 0.0506x + 0.0057 (0.95)

NAD$^+$ CONCENTRATION RANGE : 0.15 to 2.5 mM
Control	y = 0.0117x + 0.0133 (0.99)
Ca^{2+}	y = 0.0085x + 0.0197 (0.84)
Ca^{2+}-CaM	y = 0.0108x + 0.0192 (0.98)
EGTA	y = 0.0031x + 0.0127 (0.99)

Table II. Kinetic parameters, apparent K_m (app. K_m) and V_{max}, of the prepared NAD$^+$ kinase for the two ranges of NAD$^+$ concentrations (0–0.15 mM and 0.15–2.5 mM). Modulations of these parameters by either 2.5 mM Ca^{2+}, or 2.5 mM Ca^{2+} plus 6 nM of calmodulin (Ca^{2+}-CaM), or 2.5 mM EGTA. The values were computed from the data presented in Fig. 3A and 3B.

RANGE OF NAD$^+$ CONCENTRATIONS	app. K_m [mM]	V_{max} (nmol NADP$^+$/ h x ml)
0 to 0.15 mM		
Control	0.16	101
with Ca^{2+}	0.17	74
with Ca^{2+} and CaM	0.11	60
with EGTA	0.11	99
0.15 to 2.5 mM		
Control	1.14	427
with Ca^{2+}	1.78	463
with Ca^{2+} and CaM	2.32	588
with EGTA	4.10	1613

per hour per ml, were calculated in the lowest range of NAD$^+$ concentrations; while within the highest range of NAD$^+$ concentrations, the apparent K_m (1.14 mM for NAD$^+$) and V_{max} (427 nmol of NADP$^+$ per hour per ml) were considerably higher. Thus, the apparent affinity for the lowest NAD$^+$ concentrations was 7.3-fold than that for the highest, while the V_{max} was 4.2-fold depressed.

Such a bimodal behavior could suggest either two kinds of NAD$^+$ binding sites presenting different kinetic parameters, or one unique kind of

binding sites on which the NAD$^+$ would cooperatively link.

Effects of Ca^{2+}, Ca^{2+}-CaM, or EGTA on the prepared NAD$^+$ kinase activity

The ratios initial substrate concentration [NAD$^+$]$_o$/initial velocity (v_o) were plotted versus [NAD$^+$]$_o$ for each of the three conditions, Ca^{2+}, Ca^{2+}-CaM, or EGTA (Fig. 3). Here again, the two distinct kinetic behaviors of the enzyme depending on the NAD$^+$ concentration ranges tested (0–0.12 mM and 0.15–2.5 mM) were evidenced. The apparent K_m and V_{max} values in these two NAD$^+$ ranges are reported in Table II.

In the lowest range of NAD$^+$ concentrations (Fig. 3A, Table II), it could be observed that: (i) in the presence of EGTA, the apparent K_m value decreased by 28% while the V_{max} was not significantly affected; moreover, similar slopes (1/V_{max}) between the EGTA and control curves could be noticed – thus EGTA acted as an activator nullifying the effect of a putative competitive inhibitor of the NAD$^+$ kinase; (ii) curiously, Ca^{2+} did not provoke an opposite effect to that obtained in the presence of EGTA, but it decreased the V_{max} by 26% without significantly changing the apparent K_m value – the Ca^{2+} then proceeded as a noncompetitive inhibitor; and (iii) interestingly, the Ca^{2+}-CaM complex reduced both the apparent K_m and the V_{max} values by 26% and 40% respectively, while identical apparent K_m values were derived from both the Ca^{2+}-CaM and EGTA curves – thus, the Ca^{2+}-CaM played the role of a noncompetitive inhibitor towards either the enzyme alone, or the EGTA-activated enzyme, as did the Ca^{2+} alone.

Considering the highest range of NAD$^+$ concentrations (Fig. 3B), apparent K_m and V_{max} values were increased by EGTA (about 270%), and EGTA and control curves displayed identical intercepts (apparent K_m /V_{max}) – thus, EGTA seemed to eliminate an uncompetitive inhibitor, and acted again as an activator. Secondly, the role of Ca^{2+} could not be clearly defined from the comparison of Ca^{2+} and control curves, however there was an increase by 56% of the apparent K_m value (Table II) – here again, the Ca^{2+} and the EGTA did not induce contrary effects. Thirdly, the Ca^{2+}-CaM complex doubled the apparent K_m and am-

plified the V_{max} value by 38%, and similar slopes (1/V_{max}) were observed between the Ca^{2+}-CaM and control curves. In this case, in spite of the lack of structural analogy with the NAD$^+$, the Ca^{2+}-CaM complex seemed to play the role of a competitive inhibitor. Furthermore, identical intercept values on the ordinate axis (apparent K_m/V_{max}) of the Ca^{2+}-CaM and Ca^{2+} curves, suggested an uncompetitive effect of the CaM on the enzyme inhibited by the Ca^{2+}.

Modulation of the prepared NAD$^+$ kinase activity by soluble and membrane-bound effectors present at different circadian times

Kinetic parameters of the prepared NAD$^+$ kinase and of the four fractions S09, P09, S17 and P17

All samples (the prepared NAD$^+$ kinase, and the cell fractions collected at CT09 and CT17) had been frozen and kept in liquid nitrogen until the day of the assays. The kinetic parameters of the prepared NAD$^+$ kinase, as well as those of the NAD$^+$ kinase present in S09, P09, S17, and P17 had been determined according to Hanes-Woolf equation (Henderson, 1986), prior to the mixing assays. Depending on the NAD$^+$ concentration range, the data values from triplicate assays generated trimodal curves indicating three distinct kinetic behaviors of the enzyme, instead of two when the assays were performed on the freshly prepared NAD$^+$ kinase. These results could suggest association and disassociation of a multi-subunit enzyme.

The kinetic parameters, Table III, exhibited consistent ratios of 10 to 20 between the apparent K_m's determined for the middle range and for the lowest range of NAD$^+$ concentrations (except a value of only 3 for P17). For four of the six samples tested, the ratios between the apparent K_m's - determined for the highest range and for the middle range of NAD$^+$ concentrations – varied only from 2 to 5.3; nevertheless, they reached 15 for the S09 and 16 for the P17. Table III also indicated that the V_{max} values characterizing the two pellets were similar, but smaller than the ones of the supernatants (respectively by factors 4.5 in S17 and 24 in S09). The V_{max}'s of the prepared NAD$^+$ kinase were consistently the strongest.

The apparent K_m's of the prepared NAD$^+$ kinase used at first with the CT09, then with the

Table III. Kinetic parameters of the prepared NAD$^+$ kinase, and of NAD$^+$ kinase present in pellets (P) and supernatants (S) fractionated from crude extracts of ZC *Euglena gracilis* synchronously grown at 16.5 °C (by LD: 12,12)[1] and collected (density of 10^5 cells.ml^{-1}) at CT09[2], trough of NAD$^+$ kinase activity, and CT17[3], maximum of NAD$^+$ kinase activity. Apparent K_m (app. K_m) and V_{max} values are respectively expressed as mM of NAD$^+$, and pmol of NADP$^+$ formed per hour per ml of assay mixture[6] (cf. Material and Methods).

NAD$^+$ CONCENTRATION RANGES	0 to 0.05 mM NAD$^+$		0.067 to 0.4 mM NAD$^+$		0.5 to 2.5 mM NAD$^+$	
	app. K_m	V_{max}	app. K_m	V_{max}	app. K_m	V_{max}
NAD$^+$ kinase used for CT09 tests	0.016	9.8	0.33	79.7	0.85	194.2
NAD$^+$ kinase present in:						
S09[4]	0.025	4.3	0.22	15.7	3.31	108.8
P09[5]	0.020	0.71	0.19	2.4	0.37	4.5
NAD$^+$ kinase used for CT17 tests	0.037	10.3	0.34	47.1	1.2	78.4
NAD$^+$ kinase present in:						
S17[4]	0.016	2.2	0.32	11.7	1.7	14.2
P17[5]	0.032	0.13	0.10	0.19	1.6	3.0

Note:
1 – LD: 12,12: light dark cycles of 12 h of 20 µE m^{-2} sec^{-1} light, 12 h darkness;
2 – CT 09, circadian time – 9 hours after the onset of light in a LD:12,12;
3 – CT 17, circadian time – 17 hours after the onset of light in a LD:12,12;
4 – S09 and S17, supernatant fractions from cells collected respectively at CT 09 and CT 17;
5 – P09 and P17, pellet fractions from cells collected respectively at CT 09 and CT 17;
6 – The protein contents corresponding to 1 ml of assay mixture were respectively 40 µg for the prepared NAD$^+$ kinase and 750 µg for S09, P09, S17 and P17 fractions.

CT17 extracts, were comparable for each of the three NAD$^+$ concentration ranges, Table III. For the NAD$^+$ concentrations above 0.067 mM, the V_{max} values of the prepared NAD$^+$ kinase used for the tests with CT09 extracts, were about twice those of the prepared NAD$^+$ kinase used for the tests with CT17 extracts. Again, this clearly documented a loss of the prepared enzyme activity between the CT09 and the CT17 assays. A relative stability could be noticed with V_{max} values for S09 and S17 in the NAD$^+$ concentration ranges under 0.4 mM, while above 0.4 mM of NAD$^+$, a 10-fold diminution occurred between CT09 and CT17. Curiously, V_{max} values for P09 and P17 stayed stable above 0.4 mM of NAD$^+$, while only in the lowest range of NAD$^+$ concentrations, V_{max} values of P09 were higher than those of P17.

Modulation of the kinetic parameters of the prepared NAD$^+$ kinase mixed with each of the four fractions S09, P09, S17 and P17

In attempt to search for the either soluble (in S) or membrane-bound (in P) effectors, possibly present at different CT times, the kinetic parameters were determined at first, for each of the NAD$^+$ concentration ranges, in the prepared NAD$^+$ kinase and in the separated fractions S09, or S17, or P09, or P17. A second step consisted in mixing a given aliquot of the prepared NAD$^+$ kinase with each of the separated fractions (cf. Material and Methods), and re-examine the kinetic parameters of these four mixes. These last experimentally defined parameters were then compared with the theoretical parameters defined as follows. The v_o's experimentally determined for the prepared NAD$^+$ kinase and for either one of S09, S17, P09, or P17 tested alone, had been summed and the expected v_o's thus obtained plotted according to the Hanes-Woolf equation to generate the theoretical kinetic parameters. Finally, the ratios: (experimental apparent K_m/ theoretical apparent K_m) and (experimental V_{max}/ theoretical V_{max}) were then examined (Table IV). Concerning the K_m ratios, a value inferior to 1 indicated an increase in affinity of the prepared NAD$^+$ kinase for its substrate NAD$^+$ by effector(s) present in the considered fraction; on the contrary, a ratio superior to 1 indicated a decrease in affinity. Concerning the V_{max} ratios, a value inferior to 1 indi-

Table IV. Ratio between expected and experimental kinetic parameters, apparent K_m (app. K_m) and V_{max}.

NAD⁺ CONCENTRATION RANGES	SUPERNATANT		PELLET	
	app. K_m ratio	V_{max} ratio	app. K_m ratio	V_{max} ratio
0 to 0.05 mM NAD⁺				
CT 09	0.42	0.48	4.97	1.33
CT 17	2.17	1.03	4.28	1.88
0.067 to 0.4 mM NAD⁺				
CT 09	0.69	0.71	2.09	0.90
CT 17	1.70	1.27	0.36	0.45
0.5 to 2.5 mM NAD⁺				
CT 09	0.51	0.58	0.45	0.27
CT 17	1.20	0.94	0.86	0.61

cated an inactivation of the prepared NAD⁺ kinase by the effector(s), a ratio superior to 1 an activation.

Whatever the NAD⁺ concentration tested, S09 and S17 acted in an opposite way on the binding of the substrate NAD⁺ on the prepared NAD⁺ kinase: S09 increased it, when S17 decreased it, both by a factor of about 2 (Table IV). Moreover, while S17 did not change significantly the maximal velocity of the prepared NAD⁺ kinase activity, S09 did modulate it negatively. The effects of pellets were more complex since they depended more on the NAD⁺ concentration range tested than on the CT time. In the lowest range of NAD⁺ concentrations (0 to 0.05 mM), the binding of the substrate NAD⁺ on the prepared NAD⁺ kinase was reduced by a factor 5, while the V_{max} value was almost doubled. Conversely, in the highest range of NAD⁺ concentrations (0.5 to 2.5 mM) the binding of the NAD⁺ was enhanced by a factor about 2, while the V_{max} was depressed by about 50%.

This study demonstrated, on the one hand, in soluble fractions: (i) the presence of modulators of the binding of the NAD⁺ on the NAD⁺ kinase, positive in S09 and negative in S17; and (ii) only in S09, the presence of an inhibitor invalidating about half of the maximal NAD⁺ kinase activity (V_{max} values ranging from 0.71 to 0.48). On the other hand, in P09 as well as in P17, the presence of two kinds of effectors was shown: the first one, only detectable in the very low range of NAD⁺ concentrations, increased the V_{max}'s while decreasing the binding of the NAD⁺ by a factor about 5; the second one provoked complete reverse effects within the highest range of NAD⁺ concentrations.

Discussion

Characterization of the prepared NAD⁺ kinase

NAD⁺ kinase isolated from *Euglena gracilis* ZC mutant displays a severe unstability (Table I). The drastic loss of activity during the dialysis step was checked (by complementation experiments) not to be caused by the loss of factors of low molecular weight, but likely by a thermo-unstability of the enzyme itself. Such an unstability was already observed after dialysis of NAD⁺ kinase from rabbit liver (Chung, 1971) and pigeon liver (Apps, 1968), after 7 days at 4 °C of the NAD⁺ kinase of *Candida utilis* (Butler and Mc Guinness, 1982), and after freezing at -20 °C (Apps, 1968). In our study, after dialysis, the activity is partially restored during the last step of chromatography (Table I); this suggests that, in addition to the thermo-unstability, an inactivation by aggregation occurs during dialysis, as it was the case for the NAD⁺ kinase of pigeon liver (Apps, 1975).

Assuming that NAD⁺ kinase behaved as a typical globular protein on Sephacryl S-300 column, our procedure for isolation of NAD⁺ kinase reveals five forms of NAD⁺ kinase of comparable specific activity, respectively characterized by MW of: 40, 90, 170, 350, and more than 500 kDa for the last form. This suggests that NAD⁺ kinase preparation from *Euglena gracilis* ZC mutant might consist of distinct molecular forms of enzyme, or of one polymeric native form of 350 kDa composed of 40 kDa subunits (the others detected forms, 170 and 90 kDa, corresponding to dissociated forms of the native enzyme). Structural studies on NAD⁺ kinase from rabbit skeletal muscle (Telepneva and Insarova, 1976), pigeon heart

(Bulygina and Telepneva, 1982), and *Saccharomyces cerevisiae* (Tseng *et al.*, 1979; Iwahashi *et al.*, 1989), revealed the presence of multiple active forms in the weight range 31–370 kDa. In contrast, there is a strong consensus that a subunit of MW 30–35 kDa is a component of the pigeon (Apps, 1975) and yeast NAD⁺ kinase (Tseng *et al.*, 1979). In line of these reports, the present study suggests that the quaternary structure of *Euglena gracilis* NAD⁺ kinase could be a polymer of 40 kDa subunits.

Only the three molecular forms ranging from 90 to 250 kDa (Fig. 2A), and displaying identical behavior to the assayed effectors (Fig. 2B), were pooled together and referred as prepared enzyme. The kinetics studies indicate two distinct apparent K_m *values for NAD⁺*, as it was the case for the pigeon liver NAD⁺ kinase (Apps, 1975). The existence of two (or more) distinct molecular forms is then suggested; an alternative explanation is that NAD⁺ kinase could present two classes of NAD⁺ binding sites (characterized by different affinities) which arise from the association of monomers to form the polymeric enzyme. Such an association-dissociation of the multi-subunit complex could depend on calcium. An other possibility is that identical subunits in the dissociated form could interact negatively, in an homotropic manner, in the presence of NAD⁺. Such an homotropic effect has already been hypothesized to explain the interaction of NAD⁺ and NADP⁺ with either NAD⁺ kinase from pigeon-liver, or with glutamate dehydrogenase (Apps, 1975).

Recently, two distinct genes of NAD⁺ kinase, nadF et nadG, were described in *Salmonella typhimurium* (Cheng and Roth, 1994). However, these various hypothesis remained, because it was proposed that nadF and nadG encode: (i) either for two independent kinases; (ii) or for a kinase and a transcriptional regulator of this kinase; or for dissimilar subunits of a single kinase.

Effects of Ca^{2+}, Ca^{2+}-CaM, or EGTA on the prepared NAD⁺ kinase activity

In each of the two ranges of NAD⁺ concentrations, the study of the effects of Ca^{2+}, Ca^{2+}-CaM, and EGTA on the enzyme properties of the prepared NAD⁺ kinase might allow the detection of a possible heterogeneity between the NAD⁺ binding sites. As a matter of fact, although it has already been shown that NAD⁺ kinase could be activated by Ca^{2+}, Ca^{2+}-CaM (Muto and Miyachi, 1986) and inhibited by EGTA (Slaski, 1989), no report has ever defined the direct modulation of the kinetic parameters of the NAD⁺ kinase by these commonly used effectors. This paper describes how these effectors do modify the apparent K_m and V_{max} values of the prepared enzyme, Fig. 3 and Table II. The EGTA eliminates a competitive inhibitor in the lowest range of NAD⁺ concentrations, and an noncompetitive inhibitor in the highest range. These results reinforce the hypothesis of heterogeneous sites for NAD⁺ binding. The fact that the prepared NAD⁺ kinase, still partially purified (Fig. 2B), displays a maximal velocity only in the presence of EGTA, suggests that either the Ca^{2+}, or another divalent ion (Fe^{2+}, or Mg^{2+}...), linked to the NAD⁺ binding sites of the enzyme, acts as an inhibitor. If the EGTA would chelate the endogenous Ca^{2+}, the assays in the presence of Ca^{2+} excess might display reinforcements of: (i) the competitive inhibition in the lowest range of NAD⁺ concentrations; and (ii) the noncompetitive inhibition in the highest range. Since it is not the case, the divalent ion trapped by EGTA is not Ca^{2+}, but probably part of the Mg^{2+} present in excess in the reaction medium, either under the Mg-ATP^{-2} form or under a free form.

The Ca^{2+}-CaM complex is known to be an activator increasing the maximal velocity of the NAD⁺ kinase when assayed in the presence of NAD⁺ concentrations equal to or greater than 2.5 mM (Muto and Miyachi, 1986; Slaski, 1989). The data presented in Table II confirm, in the highest range of NAD⁺ concentrations, such an increase of V_{max}. The apparent K_m value, seldom studied, is yet diminished. In the lowest range of NAD⁺ concentrations, opposite effects are observed: the V_{max} value is reduced and the apparent K_m value amplified, suggesting that the inhibition by the Ca^{2+}-CaM complex is not strictly noncompetitive towards the NAD⁺.

It is interesting to notice that: (i) the two substrates of the NAD⁺ kinase, ATP and NAD⁺, comprise a common ADP motive; (ii) NAD⁺ kinase is able to form NADP⁺ from other phosphate donors than ATP, like GTP, ITP, CTP, UTP [Mc Guinness and Butler, 1985); (iii) the 2'-hydroxyl group is unimportant in the binding of NAD⁺ and NADH to

the NAD^+ kinase (Apps, 1971); and finally (iv) $NAD(P)H$, or $NADP^+$, have been reported as competitive inhibitors of the NAD^+ kinase (Oka and Field, 1968; Apps, 1975). It is then possible to propose that NAD^+ binding sites could recognize the two adjacent phosphates of either NAD^+, or ATP. In such a case, in the lowest range of NAD^+ concentrations (0–0.12 mM) and in the presence of 2.5 mM of ATP and 7 mM Mg^{2+}, most of the NAD^+ binding sites would be occupied by $Mg-ATP^{2-}$, and an EGTA treatment would liberate some of these from the competitive inhibition exerted by $Mg-ATP^{2-}$. Moreover, the $Ca-ATP^{2-}$ being a donor of phosphate less efficient than the $Mg-ATP^{2-}$ (Apps, 1970), the observed non competitive inhibition exerted by Ca^{2+} could be explained by $Ca-ATP^{2-}$ occupying the $Mg-ATP^{2-}$ sites.

In the highest range of NAD^+ concentrations (0.15–2.5 mM), NAD^+ and $Mg-ATP^{2-}$ concentrations being quasi-equimolar, the NAD^+ sites are probably mostly occupied by their own substrate, and only occasionally by $Mg-ATP^{2-}$. Since in these conditions, the chelating effect exerted by EGTA on $Mg-ATP^{2-}$ suppresses an uncompetitive effect, perhaps the protein could adopt a new conformation with coalescence of the NAD^+ and $Mg-ATP^{2-}$ binding sites. In the presence of Ca^{2+}, and considering both the dissociation constants of the ligands $Mg-ATP^{2-}$ and $Ca-ATP^{2-}$ (Thompson-Aman et al., 1992), and the new conformation of the enzyme, the exposed NAD^+ sites could become more easily accessible to the weak concentration of $Ca-ATP^{2-}$ complex, which would then exert a competitive inhibition. The Ca^{2+}-CaM could provoke a change in the enzyme structure invalidating some of the NAD^+ binding sites, and therefore could act as a competitive inhibitor. This kind of inhibition has already been described for the phosphorylase kinase of mammalian muscle (Babu et al., 1985).

The physiological situations might be far more complex than the ones hypothesized, since different NAD^+ kinase forms exist, which have been voluntarily eliminated from this study: (i) a form of NAD^+ kinase activable by Ca^{2+} (Fig. 2B); and (ii) the membrane-bound forms (Table I), characterized by different kinetic behaviors (Laval-Martin et al., 1990a). It would be of interest to undertake comparable studies on each of these isoforms.

Modulation of the prepared NAD^+ kinase activity by soluble and membrane-bound effectors present at different circadian times

The kinetic parameters of the prepared NAD^+ kinase, from asynchronous *Euglena gracilis* cells, and of the NAD^+ kinase(s) present at CT's 09 and 17, under soluble (S) and pelletable (P) forms, in synchronously dividing *Euglena gracilis* in a LD: 12,12 regimen, are distinct in three ranges of NAD^+ concentrations (Table III). In the range 0.5–2.5 mM NAD^+: between S09 and S17, the decrease of the V_{max} value was stronger than for the apparent K_m values; on the contrary, the apparent K_m values increase between P09 and P17, while V_{max} values do not vary significantly. These results corroborate previous findings (Laval-Martin et al., 1990a; Laval-Martin et al., 1990b) that indicated a circadian rhythm of NAD^+ kinase activity, in the presence of saturating NAD^+ concentration (2.5 mM), with peak and trough eight circadian hours apart.

The search for effectors – soluble and/or membrane-bound, and susceptible to variations in a circadian manner – that may modulate the binding of the substrate NAD^+ on the NAD^+ kinase (apparent K_m) and/or the maximum velocity of the enzyme activity (V_{max}), was then undertaken. For that end, the effects of S09, P09, S17 and P17 fractions were analyzed on the prepared enzyme, using mixing experiments. Whatever be the NAD^+ concentration, a strong modulating effect of S09 is observed on the prepared NAD^+ kinase, with a diminution of the maximal velocity associated to an increase in the binding of the substrate NAD^+ by the same factor (about 2). This result is consistent with previous findings (Laval-Martin et al., 1990b) that described a maximal affinity for NAD^+ at time when the enzyme activity was minimal (Laval-Martin et al., 1990a). In contrast, the binding of the substrate NAD^+ on the enzyme becomes minimal in the presence of S17, without any variation of the maximal velocity (Table II and Laval-Martin et al., 1990a). In other respects, P09 and P17 provoke similar effects on the prepared NAD^+ kinase, that are specific of the NAD^+ concentration ranges: in the highest range of NAD^+ concentrations, an effector inactivates 50% of the enzyme activity, but improves the affinity of the remaining active enzyme by a factor 2; conversely,

in the lowest range of NAD^+ concentrations, the addition of P09 or P17 doubles the enzyme activity, while it reduces the affinity by a factor 4. These results suggest the presence, in P fractions, of two kinds of effectors associated to membranes and acting in opposite ways on the prepared enzyme, according to the NAD^+ concentration range. Each of these effectors could be alternatively and rhythmically (eight circadian hours apart), partially released from the pelletable into the soluble fractions, and modulate the NAD^+ kinase activity. Thus, the hypothesis proposed by Laval-Martin *et al.* (1990a), that the circadian could be due to variations in the cellular concentration of effector(s) seems to be confirmed.

In the lowest range of NAD^+ concentrations, when P09 or P17 was mixed with the prepared NAD^+ kinase, because the kinetics parameters increased, a conformational change probably occurred due to an effector E1 present in P fractions. A second effector, E2, would coexist in P fractions, because a complete reversed effect is observed in the highest range of NAD^+ concentrations.

It had earlier been proposed that endogenous Ca^{2+} and the Ca^{2+}-CaM could be good candidates for the understanding of the circadian oscillations in NAD^+ kinase activity (Goto *et al.*, 1988). It is then interesting to consider the identity of the E1 and E2 effectors. Because a parallel could be established between the effect of S09 and that of the Ca^{2+}-calmodulin complex already described, this complex might correspond to the endogenous effector E2. Similarly, the effect of the E1 effector present in S17 could be correlated to that of Ca^{2+}. Thus the Ca^{2+} and the Ca^{2+}-calmodulin complex would be membrane-bound in P09 and P17, and alternatively, rhythmically, and partially released (eight circadian hours apart) into S09 and S17. The protein nature of E2, supposed to be the Ca^{2+}-calmodulin complex released in S09, has been verified by treatments of S09 by either trypsin or heat (100 °C) before the mixing with the prepared NAD^+ kinase. These treatments destroyed all in S09 (or part in P09) of the E2 effector. In an attempt to understand better the regulation mechanisms of the NAD^+ kinase, it would be interesting to try to annihilate the effect of S09 by EGTA, as well as to perform the purification of the E1 and E2 effectors in order to assure their identity. As a matter of fact, there is a good possibility of other modulators besides Ca^{2+} (and Ca^{2+}-CaM) being able to regulate the NAD^+ kinase.

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