NAD^+ kinase activities in *Euglena gracilis* and *Phaseolus vulgaris*

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Abstract

After an electrophoretic separation of proteins from *Euglena gracilis* and dry seeds of *Phaseolus vulgaris* in native conditions in polyacrylamide gels, gels were incubated in mixtures containing NAD^+, $Mg\text{-}ATP^{2-}$, glucose 6-phosphate, G6P dehydrogenase, and either phenazine ethosulfate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (PES/MTT) or phenazine methosulfate and nitro blue tetrazolium (PMS/NBT) as coupled redox system for NAD^+ kinase activity detection. In the presence of PES/MTT, 4 bands were revealed for *E. gracilis*, among which two corresponded to NAD^+ kinase activity, the other corresponding to a NAD^+ reductase activity due to alcohol dehydrogenase (ADH). In the presence of PMS/NBT, only the bands of NAD^+ kinase activity were revealed. With *Phaseolus vulgaris*, 3 bands of ADH were always revealed in both mixtures, and only the use of PMS/NBT allowed the detection of NAD^+ kinase as a fourth band. With both materials, NAD^+ reductase staining in gels was intensifed in the presence of GTP or ATP and even further with ADP or GDP. The results demonstrate that: *1*) the NAD^+ kinase and NAD^+ reductase are two distinct enzymes; *2*) the NAD^+ reductase corresponds to ADH.

Additional key words: alcohol dehydrogenase; electrophoresis study.

Introduction

The NAD^+ kinase (EC 2.7.1.23), which catalyzes the phosphorylation of NAD^+ into $NADP^+$, is present in prokaryotes as well as in eukaryotes (McGuiness and Butler

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Abbreviations: ADH - alcohol dehydrogenase; Bicine - *N,N,*-bis (2-hydroxyethyl) glycine; BSA - bovine serum albumin; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; G6P - glucose 6-phosphate; G6PDH - glucose 6-phosphate dehydrogenase; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT - nitro blue tetrazolium; PES - phenazine ethosulfate; PMS - phenazine methosulfate; PMSF - phenylmethylsulfonyl fluoride; PVP - polyvinylpyrrolidone; PVPP - polyvinylpolypyrrolidone.

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1985). In eukaryotes, NAD^+ kinase activity was detected in all cellular compartments, revealing the existence of different isoforms.

NAD^+ kinase activity is generally measured using a two-step colorimetric method (McGuiness and Butler 1985). During the first step, $NADP^+$ is produced by NAD^+ kinase in the presence of NAD^+ and Mg-ATP^{2-}. The second step consists in measuring the $NADP^+$ produced by the NAD^+ kinase, using an auxiliary enzyme, $NADP^+$ dependent, like G6PDH which reduces $NADP^+$ in NADPH in the presence of G6P. The NADPH finally allows the reduction of a tetrazolium salt, which is colorimetrically monitored. The NAD^+ kinase isoforms can be separated and detected in polyacrylamide gel, after an electrophoretic separation of proteins in native conditions (Apps 1975, Afanasieva *et al.* 1982, Filippovich *et al.* 1990). The detection of the NAD^+ kinase activity in gels is adapted from these colorimetric methods. It consists in an incubation of the gels in a mixture containing NAD^+, Mg-ATP^{2-}, G6P, G6PDH and a coupled redox system, PMS/NBT, which leads to the formation of coloured band(s) of reduced NBT at the level of the active NAD^+ kinase(s) in the gel. This technique was used to test the homogeneity of a pigeon-liver NAD^+ kinase preparation (Apps 1975); or to follow the NAD^+ kinase behavior during the life cycle of *Neurospora crassa* (Afanasieva *et al.* 1982) and the development of *Bombyx mori* (Filippovich *et al.* 1990).

Such a method was previously used to compare the electrophoresis patterns of the NAD^+ kinase isoforms present in different materials of interest for our group (Jalouzot *et al.* 1994): *Euglena gracilis* Z and seeds of different plants, as well as NAD^+ kinase from chicken liver (purchased from the *Sigma Co.* and taken as reference). In that study, NAD^+ kinase appeared to display a NAD^+ reductase activity. Proteins from soluble extracts were separated in 8 - 25 % polyacrylamide gels, which were incubated in a complete mixture (NAD^+, Mg-ATP^{2-}, G6P, G6PDH, PES, MTT). In this mixture, a specific pattern of bands was obtained for each material, revealing a great polymorphism in the number of bands and in their migration rates among the studied species. Unexpectedly, the same patterns were also observed in a simplified mixture (NAD^+, Mg^{2+}, PES, MTT), indicating the reduction, by a NAD^+ reductase, of NAD^+ in NADH which is able to reduce MTT. The authors then proposed that the reaction mechanism of the NAD^+ kinase implied the formation of a NADH intermediate and suggested that both activities, NAD^+ kinase and NAD^+ reductase, were two different functions of the same protein. A further study on NAD^+ reductase showed that NAD^+ reductase is a stress marker which allowed to distinguish two differently resistant bean cultivars by their responses to NaCl and wounding stresses (Poupard *et al.* 1995).

In order to study, in the future, the effects of stresses on NAD^+ kinase activity, we had to establish more precisely the correlation between NAD^+ kinase and NAD^+ reductase. The purpose of the present work was then to study thoroughly activities of two the enzymes, NAD^+ kinase and NAD^+ reductase, focusing on the possibility that they could be carried by two different proteins co-migrating in the gel.

Materials and methods

Biological materials: *Euglena gracilis Z* was heterotrophically grown in darkness at 27 °C for 7 d in a medium containing 33 mM lactate as sole carbon source and 2 mM Al^{3+}. Plant material consists of dry seeds of common bean (*Phaseolus vulgaris* L.), genotype "Plant Introduction No. 165426".

Preparation of the soluble extracts: *E. gracilis* cells were harvested by centrifugation (500 *g*, 7.5 min at 4 °C) and the cell pellet was resuspended [(5 cm^3 g^{-1}(f.m.)] in an extraction buffer containing 100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 10 % glycerol, 2 mM PMSF, 1 mM DTT. The cell suspension was homogenized by sonication at 4 °C (sonicator *Vibracel172434, Bioblock Scientific*, Illkirch, France) for 4 periods of 30 s (60 % of maximal power) and 3 periods of 15 s (100 % of maximal power), separated by 60 s rest periods for cooling. After centrifugation (46 000 *g*, 1 h, 4 °C) of the cell homogenate, the supernatant, which corresponds to the soluble extract, was used for electrophoresis. Dry seeds of *Phaseolus vulgaris* were ground in a mortar in the presence of liquid nitrogen. Extraction buffer (100 mM Tris-HCl pH 7.5, 2.4 mM EDTA, 10 % glycerol, 0.2 % Triton X100, 1 % PVPP, 10 mM 2-mercaptoethanol, 1 mM DTT and 2 mM PMSF) was added to the powder [(5 cm^3 g^{-1} (powder)]. After 2 h of maceration at 4 °C, the crude extract was centrifuged for 30 min at 25 000 *g* and the supernatant (*i.e.* soluble extract) used for electrophoresis.

The protein concentrations in soluble extracts were estimated according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Proteins electrophoresis: Gel electrophoresis were carried out in native conditions using a *Mini-protean II* apparatus (*Biorad*, Hercules, USA). Proteins of the soluble extracts were separated by an electrophoresis either within 10 % polyacrylamide gels in 175 mM Tris-HCl (pH 8.5) for *E. gracilis*, or within 4 - 15 % or 10 - 15 % gradient of polyacrylamide gels in 112 mM Tris acetate (pH 6.4) for *P. vulgaris*, 0.05 % TEMED and 0.04 % ammonium persulfate were added for polymerization. The electrophoresis separations were performed at 20 mA within 25 mM Tris-192 mM glycine (pH 8.3) as a running buffer.

Activities in gels: After electrophoresis, NAD^+ kinase, NAD^+ reductase and ADH activities were detected by immersion of the gel in a 50 mM Tris HCl, pH 7.5, 4.5 mM $MgCl_2$, 2 mM $CaCl_2$ buffer supplemented with: 2.5 mM NAD^+, 2.5 mM ATP (or GTP), 1 mM PES and 0.42 mM MTT, for NAD^+ reductase activity (referred as simplifed mixture); 2.5 mM NAD^+, 2.5 mM ATP (or GTP), 5 mM G6P, 2 U cm^{-3} baker yeast G6PDH, 1 mM PES (or 0.16 mM PMS) and 0.42 mM MTT (or 0.37 mM NBT), for NAD^+ kinase activity (referred as complete mixture); and 2.5 mM NAD^+, 2.5 mM ethanol (or butanol-1, or methanol), for ADH activity. After 30 min or up to 12 h, reactions were stopped by transferring the gels in a solution containing 10 % acetic acid and 5 % glycerol.

NAD^+ kinase assays (modifed from Goto 1984): NAD^+ kinase activity was measured by a $NADP^+$ production in a final volume of 0.25 cm^3 containing a slice of polyacrylamide gel (1.25 cm × 0.25 cm), 50 mM Tris-HCl (pH 7.5), 2.5 mM NAD^+, 2.5 mM ATP, 4 mM $MgCl_2$, and 6 mM nicotinamide. Reactions were either immediately stopped by heating for 5 min in a boiling water bath (t_0 sample) or performed, at 30 °C, for either 30 min (for *E. gracilis*) or for 4 h (for *P. vulgaris*), and then stopped as described (t_{30} or t_4 sample, respectively). The $NADP^+$ generated was measured by a recycling procedure using an auxiliary enzyme, the glucose-6-phosphate dehydrogenase, G6PDH, which, in the presence of excess of G6P, initiates a redox cascade (PES, MTT) starting with the reduction of the $NADP^+$ (endogenous $NADP^+$ in t_0, endogenous $NADP^+$ plus the $NADP^+$ formed during the incubation in t_{30} or t_4). For each assay, 50 mm^3 of reaction medium were added to 0.2 cm^3 of the following mixture: 0.1 M Bicine (pH 8), 5 mM G6P, 2.5 mg of soluble PVP 40000, 0.5 mM PES, 1 mM MTT, then and extemporaneously, 2.5 U of G6PDH. The rate of reduction of MTT, proportional to the amount of $NADP^+$, was monitored by following the absorbance at 570 nm and directly computed by a microplate reader *CERES UV900 HDi* (*Bio-Tek Instruments* Inc., Winooski, USA).

Alcohol dehydrogenase assays: ADH from bakers yeast (purchased from the *Sigma Co.*) activity was determined by colorimetrically monitoring the conversion of NAD^+ to NADH in 250 mm^3 of the following reaction mix: 50 mM Tris-HCl pH 7, 2.5 mM NAD^+, 2.5 mg of soluble PVP 40 000, 0.5 mM PES, 1 mM MTT, with or without 1 M ethanol and 2.5 mM GTP, GDP or ADP. The reaction was started by adding 2×10^{-3} U of ADH, and the rate of reduction of MTT, directly correlated to the NADH level, was monitored at 570 nm, using a microplate reader *CERES UV900.*

Results and discussion

Detection of NAD^+ kinase and NAD^+ reductase activities: In order to separate NAD^+ kinase from NAD^+ reductase by native electrophoresis, gels of longer sizes than those used for the previous study (Jalouzot *et al.* 1994) were prepared (6 cm instead of 3.5 cm) and different conditions were tested: pH of the gels (8.3 in Tris-HCl or 6.4 in Tris-acetate) and polyacrylamide concentration (10 % homogenous, 4 - 15 % or 10 - 15 % gradients). After electrophoresis, the gels were cut vertically in several lanes which were incubated in either simplifed or complete mixtures (Jalouzot *et al.* 1994).

After separation of soluble proteins of *E. gracilis* in a 10 % homogenous polyacrylamide gel in Tris-HCl, pH 8.3, two bands were detected in the simplifed mixture (Fig. 1*A*, lane 1) and four bands in the complete mixture (Fig. 1*B*), instead of the unique band detected previously in both mixtures (Jalouzot *et al.* 1994). Among the four bands, the two upper ones corresponded to those detected in the simplifed mixture and therefore resulted from NAD^+ reductase activity. Only the two lower bands could then correspond to NAD^+ kinase activity. In order to verify this hypothesis, the production of $NADP^+$ was monitored by spectrophotometry. Soluble

proteins from *E. gracilis* were separated as above and the gel was cut, perpendicularly to the direction of migration, in 25 slices. Each slice was incubated for 0 or 30 min in the presence of NAD^+ and $Mg\text{-}ATP^{2-}$; then, after protein denaturation at 100 °C for 15 min, the $NADP^+$ produced was measured (using G6PDH as the auxiliary enzyme) by the kinetics of MTT reduction. Two overlapping peaks of $NADP^+$ production were obtained, which were located at the level of the two lower bands revealed in the gel (Fig. 1*B*). Therefore, soluble proteins of *E. gracilis* contain two isoforms of NAD^+ reductase, as well as two isoforms of NAD^+ kinase (isoform 1 and isoform 2 according to their decreasing Rf).

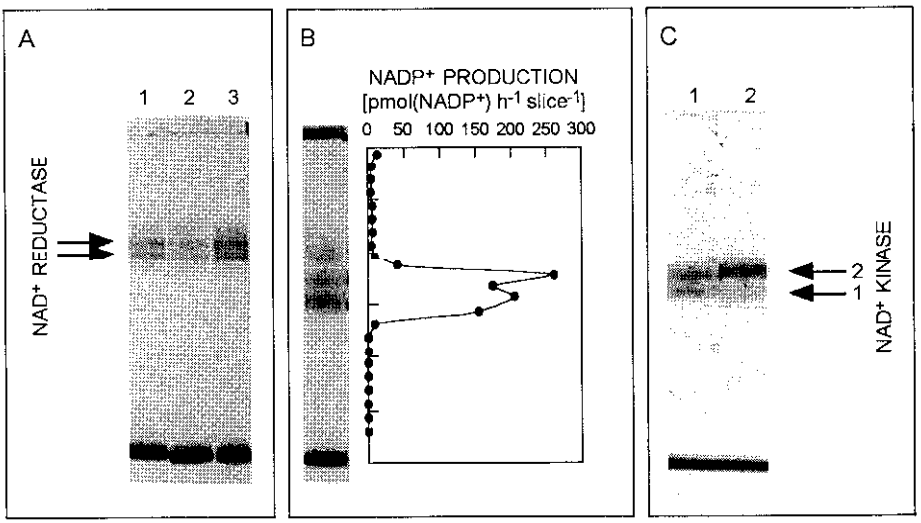

Fig. 1. NAD^+ kinase and NAD^+ reductase activities of *E. gracilis* in gel. Proteins of soluble extract (1 mg on the total width of the gel) were separated by a 10 % polyacrylamide gel electrophoresis in 175 mM Tris-HCl (pH 8.5) under native conditions. After electrophoresis, the gel was vertically cut in several lanes, which were incubated: *A* - simplifed mixture, NAD^+, PES, MTT - lane 1; supplemented with ATP - lane 2; or GTP - lane 3. *B* - complete mixture, NAD^+, ATP, G6P, G6PDH, PES, MTT. The remaining gel was cut perpendicularly to the direction of migration in slices (1.25 cm × 0.25 cm), assayed for $NADP^+$ production and graphed. *C* - complete mixture in which PES, MTT was replaced by PMS, NBT and in which phosphate donor was either ATP - lane 1; or GTP - lane 2.

Three bands were always observed in both mixtures with soluble extract of *P. vulgaris* seeds, in agreement with previous data (Jalouzot *et al.* 1994), whatever the electrophoresis condition used. Among the different conditions tested, Fig. 2 presents the results obtained after an electrophoresis in a 4 - 15 % polyacrylamide gradient gel in Tris-acetate at pH 6.4 and incubation in simplifed mixture (Fig. 2*A*, lane 1) or in complete mixture (Fig. 2*B*). But, when $NADP^+$ production was measured in gel slices as above for *E. gracilis*, none of the three coloured bands did correspond to a protein able to phosphorylate NAD^+ to form $NADP^+$ (Fig. 2*B*). On the contrary and unexpectedly, a $NADP^+$ production was found in a part of the gel,

facing a non-coloured region. The NAD^+ kinase activity was therefore under the limit of detection in the gel, probably because of the very low quantity of $NADP^+$ formed by the *P. vulgaris* NAD^+ kinase: for similar quantities of total protein extract loaded on the gels, $NADP^+$ production for *P. vulgaris* was nearly 20 times lower than that for *E. gracilis*.

However, when MTT was replaced by NBT, also used as final acceptor for NAD^+ kinase activity detection in gels (Apps 1975, Afanasieva *et al.* 1982, Filippovich *et al.* 1990), a large coloured zone was observed (Fig. 2*C*, lane 1), which corresponded to the NAD^+ kinase activity (Fig. 2*B*), in addition to the three bands corresponding to the NAD^+ reductase activities (detectable with MTT). For unknown reasons, the use of NBT with gels corresponding to *E. gracilis* (Fig. 1*C*, lane 1) only permitted the revelation of the two isoforms of NAD^+ kinase, the two

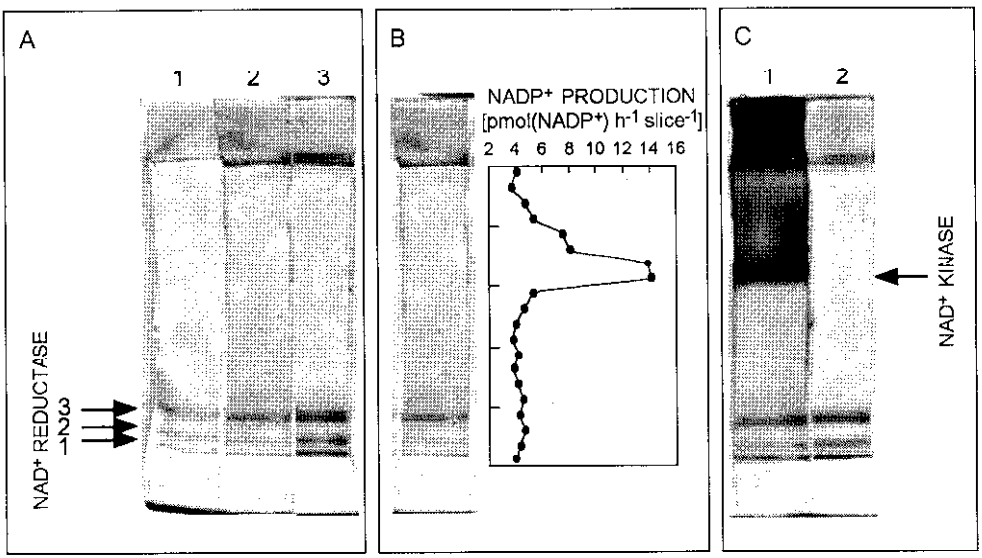

Fig. 2. NAD^+ kinase and NAD^+ reductase activities of *P. vulgaris* in gel. Proteins of soluble extract (1 mg on the total width of the gel) were separated by a 4 - 15 % polyacrylamide gel electrophoresis in 112 mM Tris-acetate (pH 6.4) under native conditions. After electrophoresis, the gel was vertically cut in several lanes, which were incubated in different mixtures for 12 h. *A* - simplifed mixture, NAD^+, PES, MTT - lane 1; supplemented with ATP - lane 2; or GTP - lane 3. *B* - complete mixture, NAD^+, ATP, G6P, G6PDH, PES, MTT. The remaining gel was cut perpendicularly to the direction of migration in slices (1.25 cm × 0.25 cm), assayed for $NADP^+$ production and graphed. *C* - complete mixture in which PES, MTT was replaced by PMS, NBT and in which phosphate donor was either ATP - lane 1; or GTP - lane 2.

NAD^+ reductase bands being no longer detectable. Therefore, NBT seemed to be necessary for the revelation of the NAD^+ kinase activity of *P. vulgaris* (Fig. 2*B* and Fig. 2*C*, lane 1) and MTT for the NAD^+ reductase activity of *E. gracilis* (Fig. 1*B* and Fig. 1*C*, lane 1).

The two isoforms of NAD^+ kinase of *E. gracilis* (Fig. 1*C*) could be detected either with ATP (lane 1) or GTP (lane 2) as a phosphoryl donor (the isoform 2 being more

active with GTP than with ATP). In contrast the NAD^+ kinase activity of *P. vulgaris* (Fig. 2*C*) was highly specific with respect to ATP (lane 1), and not detectable if GTP replaced ATP (lane 2).

Because these results showed that NAD^+ kinase and NAD^+ reductase activities were carried by distinct proteins in both materials studied, the identification of the NAD^+ reductase was undertaken.

Identification of the NAD^+ reductase: The tetrazolium salts have been extensively used to localize dehydrogenase activities after protein separation on polyacrylamide gels. Nevertheless, misleading stainings could be observed which can be due to: *1*) either direct tetrazolium reduction, by reduced thiol-group of proteins (like BSA) and thus unrelated to any enzymatic activity (Venugopal and Adiga 1980); *2*) or indirect tetrazolium reduction by the NADH resulting from the activity of intrinsic dehydrogenases, like ADH, able to use as substrate residual ethanol present in

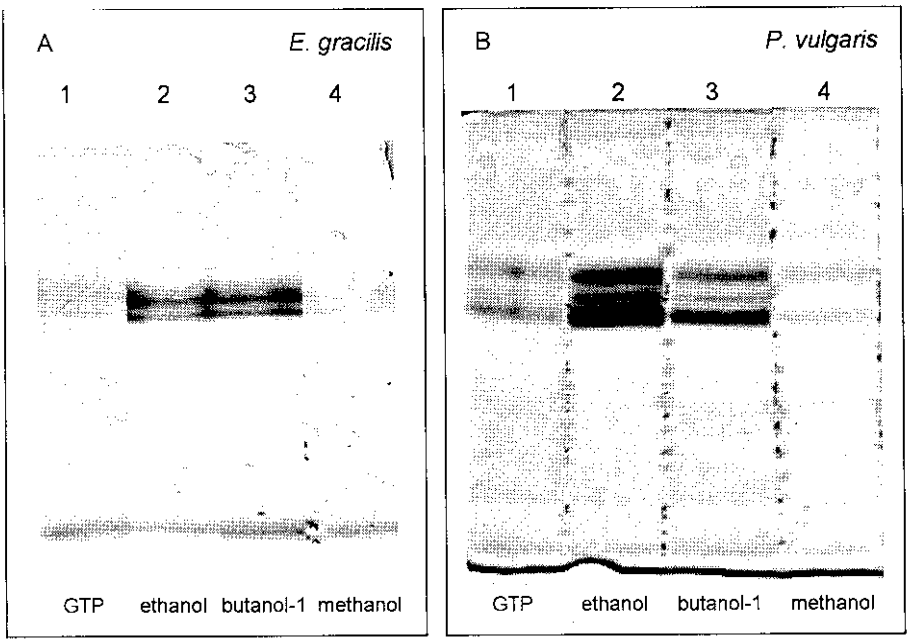

Fig. 3. ADH activity of *E. gracilis* and of *P. vulgaris* in gel. For *E. gracilis*, *A* proteins of soluble extract were separated as in Fig. 1; and for *P. vulgaris*, *B* - within a 10 - 15 % gradient of polyacrylamide gel instead of a 4 - 15 % gradient in order for the coloured bands to be located in the middle of the gel (Fig. 1). After electrophoresis, gels were cut vertically in several lanes, which were incubated, for 30 min, in simplifed mixture without alcohol, but supplemented with GTP (this compound being able to intensify NAD^+ reductase bands, Jalouzot *et al.* 1994) - lanes 1; ethanol - lanes 2; butanol-1 - lanes 3; or methanol - lanes 4.

reagents (Shaw and Koen 1967, Beutler 1967). In our study, since the misleading staining occurred only in the presence of NAD^+, we tested the possibility that the NAD^+ reductase(s) could be an ADH by adding different alcohols in the simplifed

mixture. With both materials, NAD^+ reductase bands (Fig. 3) appeared more rapidly and were more intense with ethanol (lanes 2) and butanol-1 (lanes 3), than without alcohol (lanes 1), or with methanol (lanes 4) - a compound known as being non oxidizable by plant ADH (Côme and Corbineau 1989). The three ADH bands of *P. vulgaris* may result from the expression of two *adh* genes, as it was generally shown in plant diploid species (Gottlieb 1982). In plants, which are homozygous for both genes, the simultaneous expression of the two genes provides an ADH electrophoresis pattern consisting of three isozymes: two different homodimers and a heterodimer (Schwartz and Endo 1966). In the case of the polyploid *E. gracilis*, usually presenting two ADH bands, a third one is occasionally observed (result not shown).

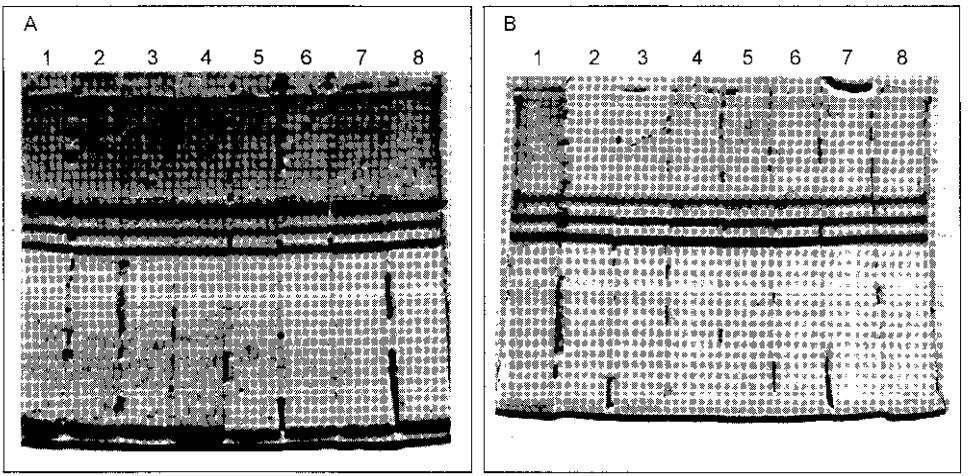

Fig. 4. Effects of some nucleotides on ADH activities of *P. vulgaris* in gel. Proteins of soluble extract were separated as in Fig. 3*B* (except that the quantities loaded on the total width of the gel were: 2. 5 mg for A, and 0. 5 mg for B). After electrophoresis, gels were cut vertically in several lanes, which were incubated, for 2 h, in simplifed mixture (*A*) or in simplifed mixture supplemented with ethanol (*B*). Lanes 1, assay without any added nucleotide; lanes 2, with GTP; lanes 3, with GDP; lanes 4, with GMP; lanes 5, with Pi; lanes 6, with ATP; lanes 7, with ADP; lanes 8, with AMP.

An interesting observation, already shown (Jalouzot *et al.* 1994), still remains unexplained for both *E. gracilis* (Fig. 1*A*) and *P. vulgaris* (Fig. 2*A*): the ADH bands, detected in the simplifed mixture (lanes 1, Figs. 1*A* and 2*A*), *i.e.* in Tris-buffer and NAD^+, were intensifed in the presence of ATP (lanes 2. Figs. 1*A* and 2*A*) and even more in the presence of GTP (lanes 3, Figs. 1*A* and 2*A*). This activation was the principal argument, previously and erroneously proposed, that NAD^+ kinase could function as an NAD^+ reductase.

Since what we had previously described as an NAD^+ reductase activity (Poupard *et al.* 1995) is here identifed as ADH activity, we needed to test the effects of some nucleotides on the ADH revelation in mixtures containing or not ethanol (Fig. 4). As expected, the bands were intensifed in simplifed mixture supplemented with GTP

(Fig. 4*A*, lane 2), however they were also intensifed with GDP (lane 3) and even more with ADP (lane 7). In contrast, in the presence of ethanol, the addition of any nucleotide tested did not modify the intensities of the bands (Fig. 4*B*). In the same manner, the purchased ADH, colorimetrically assayed, showed an increase in activity (factor 1.2 - 2) in the presence of 2.5 mM of GTP, GDP and ADP, only when the activities were measured without ethanol (Table 1).

Table 1. ADH (purchased from *Sigma Co.*) colorimetrically assayed in Tris-buffer with or without ethanol, supplemented with 2.5 mM of GTP, GDP or ADP (specific activities are expressed in nmol of NAD^+ reduced mg^{-1}(protein) s^{-1}). Values presented are the mean ± SE ($n = 4$).

	NAD^+	NAD^+ GTP	NAD^+ GDP	NAD^+ ADP
without ethanol	76.9 ± 4.5	95.2 ± 2.0	131.9 ± 3.5	152.8 ± 3.0
with ethanol	650.3 ± 6.0	640.8 ± 12.9	631.4 ± 6.9	649.8 ± 4.5

Conclusion: The detection of NAD^+ kinase activity in gels can allow to distinguish and characterize the different isoforms without any prior step of purification, while NAD^+ kinase assays performed by spectrophotometry on soluble samples (crude or partially purified extracts), only allow to measure the global activity of all present isoforms. Thus, the present work shows that *E. gracilis* displays two isoforms of NAD^+ kinase, which differ by their specificity to either ATP or GTP, and *P. vulgaris* presents an unique ATP dependent form. However, the detection of NAD^+ kinase activity in gels depends on the coupled redox system used: the NAD^+ kinase activity of *E. gracilis* can be detected either with PES/MTT or with PMS/NBT, when the NAD^+ kinase activity of *P. vulgaris* needed PMS/NBT.

Because misleading bands due to ADH activity could appear in gels, the study of the NAD^+ kinase activity becomes difficult with ADH rich materials like the here studied seeds and Al^{3+} stressed *E. gracilis* (no ADH activity was detected in gels when *E. gracilis* cells were grown without Al^{3+}) - in these cases, the staining results must be carefully analyzed and $NADP^+$ production must be checked for, in order to confirm a NAD^+ kinase activity.

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