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Tween-20 Activates and Solubilizes the Mitochondrial Membrane-bound, Calmodulin Dependent NAD^+ Kinase of *Avena sativa* L.

M.-A. Pou de Crescenzo, S. Gallais, A. Léon, D.L. Laval-Martin
Equipe de Biochimie et de Biologie Moléculaire, UFR Sciences, Université d'Angers, 2 Boulevard Lavoisier, 49 045 Angers Cedex 01, France

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Abstract. Among different treatments assayed, a mix of a nonionic detergent (5% Tween-20) with 0.5 M NaCl was found to solubilize a large part of the calmodulin-dependent NAD^+ kinase bound to the inner mitochondrial membrane. It also stimulated its activity by increasing 7 times the maximal velocity. Activity stimulation was also observed with phosphatidylcholine, phosphatidylethanolamine and with reductants (HSO_3 and DTT). This solubilized NAD^+ kinase and the calmodulin-dependent cytosoluble isoform displayed distinct molecular masses, as well as different kinetic parameters. We propose that solubilization of membrane-bound NAD^+ kinase could occur in vivo in *Avena sativa* and could generate a soluble isoform.

Key words: Calmodulin-dependent NAD^+ kinase — K_m — Mitochondrial membranes — Solubilization — Tween-20 — V_{max}

Introduction

NAD^+ kinase (ATP:NAD^+ 2′-phosphotransferase; EC 2.7.1.23) catalyzes the synthesis of $NADP^+$ by phosphorylation of NAD^+. This enzyme is widely distributed in animals, higher plants and microorganisms (McGuinness & Butler, 1985) and controls the cellular metabolism through the intracellular concentration of $NADP^+$ (Yamamoto, 1969; Laval-Martin et al., 1990a and 1990b). Although NAD^+ kinase has neither been purified to homogeneity nor cloned, it has been studied in numerous organisms. Two isoforms of NAD^+ kinase were evidenced: one dependent on the calcium-calmodulin complex (CaCam), and the other independent (Marmé, 1985; Muto & Miyachi, 1986; Slaski, 1990). The CaCam-dependent NAD^+ kinase is involved in the stress tolerance of wheat lines to Al^{3+} (Slaski, 1989, 1990), and of tomato cells to NaCl (Delumeau et al., 2000), as well as in the dormancy breaking of lettuce seeds (Zhang, Ross & Orr, 1994a; 1994b) and *Avena sativa* (Gallais & Laval-Martin, 1999). Recently, Harding and Roberts (1998) have demonstrated that the CaCam-dependent NAD^+ kinase was implicated in tobacco-cell defense in response to an incompatible pathogen infection, through an elicitor-stimulated oxidative burst.

In *A. sativa*, three isoforms of NAD^+ kinase were previously evidenced (Gallais, Pou de Crescenzo & Laval-Martin, 2000a and 2000c): CaCam-dependent and -independent soluble isoforms of 63 and 410 kDa, respectively, and a membrane-bound CaCam-dependent NAD^+ kinase. The activity of the membrane-bound isoform was shown to increase during the *sensu stricto* germination of seeds, and to be further enhanced 7.5-fold in the growing seedlings compared to 3-fold for the soluble isoforms.

A CaCam-dependent membrane-bound NAD^+ kinase was already described in mitochondria of maize: either linked to the outer membrane (Dieter & Marmé, 1984), or to both outer and inner membranes (Sauer & Robinson, 1985). Such membrane-bound isoform was also reported to increase after dormancy breakage in *Avena* seeds (Gallais & Laval-Martin, 1999).

In this paper, the CaCam-dependent NAD^+ kinase, linked to the internal mitochondrial membrane of embryos isolated from *A. sativa* L., has been solubilized and some of its characteristics have been compared to those of the already studied cytosoluble calmodulin-dependent isoform (Gallais, Pou de Crescenzo & Laval-Martin, 2000c). The stimulation of its activity and its sensitivities to different molecules are also reported. Finally, the role of this membrane-bound NAD^+ kinase is discussed.

Materials and Methods

Plant Material

Naked seeds of oat (*Avena sativa* L. cv. Noire de Moyencourt) were germinated during 30 hr on cotton layer imbibed with deionized water

Correspondence to: M.-A. Pou de Crescenzo

in Petri dishes (10-cm diameter, 25 seeds per dish), at 30°C in absolute darkness. Embryos were excised using a scalpel blade and leaving as little endosperm as possible. They were either immediately used for fractionation or stored at −20°C.

Cell Fractionation

Cells of embryo tissues (250 to 700) were fractionated by differential centrifugation according to Gallais et al. (2000b) into: mitochondrial, cytosolic plus vacuolar, and microsomal fractions. All steps were performed in one day at 4°C to minimize damages to organelles. Freshly harvested embryos (1 g) were gently ground in a mortar with a pestle, for 10 min in 12 ml of buffer A (100 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.2% bovine serum albumin, 1 mM PMSF, 1 mM DTT, 0.5 M sucrose, 1 $\mu g \cdot ml^{-1}$ leupeptin and 1 $\mu g \cdot ml^{-1}$ pepstatin A). The crude extract was centrifuged at 500 × g for 10 min (Rotor Sorvall SS34). The supernatant ($S_{0.5}$) obtained was then centrifuged for 30 min at 20,000 × g to gather the mitochondria in a pellet, P_{20}. The resulting supernatant (S_{20}) was submitted to a 105,000 × g centrifugation for 60 min (Rotor Beckman SW41) to separate the pelletable microsomes (P_{105}) from the cytoplasmic and vacuolar contents (S_{105}). The pellets (P_{20}, P_{105}) were respectively resuspended in a glass Potter homogenizer (10 strokes) with a buffer identical to A but depleted of sucrose, then sonicated three times for 30 sec with a Branson Sonifier B 15 to disrupt the organelles membranes.

Aliquots of all mitochondrial, cytosolic plus vacuolar, and microsomal fractions were immediately used for NAD^+ kinase assays, or stored at −20°C for further assays and for protein determination. Marker enzymes for estimation of cross-contamination were: G6PDH for the cytosol (Côme, Corbineau & Lecat, 1988); cytochrome *c* oxidase for the mitochondrial membranes (Tolbert, 1974); and malate dehydrogenase for the mitochondrial matrix (Labrou & Clonis, 1997).

Mitochondrial Preparation

Concentrated mitochondria in P_{20} were washed twice in cold buffer A, then resuspended and sonicated as described above. This mitochondrial suspension, diluted to a protein concentration of 1.5 $mg \cdot ml^{-1}$, was stored in aliquots at −20°C after addition of 10% glycerol for further subfractionation and solubilization experiments.

Subfractionation of Mitochondrial Preparations

To separate the submitochondrial fragments, a freshly thawed mitochondrial suspension was layered on the top of a discontinuous sucrose gradient containing five 2-ml layers of 50, 40, 30, 20 and 10% sucrose (Sauer & Robinson, 1985). After a 105,000 × g centrifugation for 60 min at 4°C, each gradient layer was removed, centrifuged again and the pellets were resuspended in 2 ml of buffer A. NAD^+ kinase activities were immediately assayed. Cytochrome *c* oxidase and malate dehydrogenase activities were measured later as marker enzymes for inner membranes and matrix, respectively (Sauer & Robinson, 1985). Protein determination was performed as described below.

Solubilization Assays of Membrane-Bound NAD^+ Kinase

A freshly thawed mitochondrial suspension, diluted to a protein concentration of 1 $mg \cdot ml^{-1}$, was gently incubated during 30 min at 30°C in the presence of (in mM): 1 hydroxylamine specifically cutting thioester linkages to fatty acids (Magee & Courtneidge, 1985); 1 cholate or 1 deoxycholate as anionic detergents; 1 CHAPS as zwitterionic detergent; 1 DTT or 1 HSO_3 as reductants; 1 H_2O_2 or 1 tetrathionate as oxidants and 1% Triton X-100 or 1% Tween-20 as nonionic detergents. After a 105,000 × g centrifugation for 60 min at 4°C, NAD^+ kinase activities were assayed in the supernatant and resuspended pellet, and referred to as solubilized and not-solubilized enzyme, respectively.

Chromatographies

The NAD^+ kinase activity, freshly solubilized from P_{20} by 5% Tween-20:0.5 M NaCl, (1 ml corresponding to 0.145 mg fresh weight extract) was directly applied on an analytical Sephadex G200 column (1.5 × 50 cm, Biorad) and eluted at a flow rate of 0.15 $ml \cdot min^{-1}$ with buffer B (in mM: 100 Tris-HCl, pH 7.5, 50 NaCl, 1 EGTA, 1 EDTA, 1 DTT and 1 $\mu g \cdot ml^{-1}$ leupeptin). The column calibration was performed with: dextran blue (2,000 kDa), β-galactosidase (595 kDa), catalase (232 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), cytochrome *c* (12.4 kDa). The S_{105} (1 ml corresponding to 0.145 mg fresh weight extract) was comparatively chromatographed under the same conditions.

NAD^+ kinase activity was immediately measured in the presence of either 1 mM Ca^{2+} and 400 U ml^{-1} calmodulin, or 5 mM EGTA as described below. Aliquots of each fraction were frozen in liquid nitrogen and stored at −20°C for subsequent protein determinations.

NAD^+ Kinase Assay

The NAD^+ kinase activity was assayed according to Gallais and Laval-Martin (1999), in a final volume of 250 μl of medium containing in mM: 100 Bicine pH 8, 5 ATP, 5 NAD^+, 7 $MgCl_2$ and 6 nicotinamide. The reaction, initiated by the addition of the sample, was either immediately stopped by heating for 3 min in a boiling water bath in order to measure the endogenous $NADP^+$, or performed for 60 min at 40°C and then stopped as described. The reaction medium was supplemented with either: 1 mM $CaCl_2$ (Ca^{2+}) plus 400 U ml^{-1} of calmodulin from bovine brain to determine the total NAD^+ kinase activity (CaCam-dependent and independent); or 5 mM EGTA to measure the CaCam-independent NAD^+ kinase activity.

For $NADP^+$ measurements, the microassay used was modified from Goto (1984) and Pou de Crescenzo et al. (1997). In a microplate well, 50 μl of the reaction mixture was mixed with 200 μl of a medium containing (in mM) 100 Bicine-NaOH pH 8, 5 G6P, 1 PES, 0.42 MTT and 1% PVP. The assay was initiated by the addition of 0.25 U G6PDH (E.C. 1.1.1.49, from baker's yeast, Sigma G4134). The reduction velocity of MTT was proportional to the amount of $NADP^+$, and was followed at 570 nm using an EL 340 microplate reader (CERES UV900 Hdi, Bio-Tek Instruments Inc.). The results were quantified by comparing the velocities with those obtained in the presence of known amounts of $NADP^+$ ranging from 0 to 100 pmol. The values of the samples at time 0, corresponding to the endogenous $NADP^+$ contents of the initial extracts, were subtracted from the results of the samples at time 1 hr of incubation. The NAD^+ kinase activity was expressed as total activity (nmol or pmol of $NADP^+$ formed hr^{-1}).

The determination of the CaCam-independent activity and NAD^+ kinase total activity was performed supplementing the kinase reaction medium with either 5 mM EGTA, or 1 mM $CaCl_2$ plus 400 U ml^{-1} calmodulin (Bovine Brain; Sigma P2277), respectively. The CaCam-dependent activity corresponded to the difference between the total activity (measured with exogenous CaCam) and the CaCam-independent activity.

For the kinetics studies, solubilized NAD^+ kinase (obtained with buffer B supplemented with 5% Tween-20 and 0.5 M NaCl) was de-

Table 1. Activities of CaCam-dependent NAD^+ kinase and of marker enzymes in subcellular fractions from embryos of *Avena sativa* L. seeds

	NAD^+ kinase isoforms		Marker enzymes		
	Dependent	Independent	G6PDH-6PGDH	Cyt. *c* oxidase	MDH
	nmole · hr^{-1} · 100 $embryos^{-1}$		%	%	%
Cytosol + vacuoles	847 ± 14.8	398 ± 2.1	100 ± 0	0 ± 5.8	0 ± 30
Mitochondria	209 ± 1.4	27 ± 1.1	0 ± 0	90 ± 0.0	86 ± 25
Membranes	71 ± 1.4	14.6 ± 1.4	0 ± 0	90 ± 0.0	6 ± 1.2
Matrix	21 ± 1.3	11.7 ± 0.6	0 ± 0	0 ± 5.1	80 ± 6.0
Microsomes	21 ± 2.2	6 ± 2.4	0 ± 0	10 ± 5.1	7 ± 6.5

For each fraction, marker enzymes have been represented as percentage of the total enzyme activity measured in cytosol + vacuoles, mitochondria and microsomes. Means of 4 experiments expressed in percentage ± SD. For the NAD^+ kinase experiments, data expressed in nmol · hr^{-1} · 100 $seeds^{-1}$ ± SD, are means of 3 experiments.

salted on Sephadex G25 column equilibrated with buffer B containing only 5% Tween-20. Membrane-bound enzyme was also used after resuspension of P_{20} with buffer B.

All concentrations of the ATPMg complex were corrected according to Brooks and Storey (1992). To calculate the kinetic parameters from the saturation curves for the substrates, the Hill equation was used for sigmoidal curve fitting,

$$v = \frac{V_{max}\,[substrate]^{n_H}}{S_{0.5}{}^{n_H} + [substrate]^{n_H}} \quad (1)$$

where V_{max} is the maximum velocity of catalysis, $S_{0.5}$ the half-saturating concentration of the substrate, and n_H the Hill coefficient (Cornish-Bowden, 1979). For hyperbolic saturation curves, the Michaelis-Menten equation was used (n_H of the Hill equation = 1; $S_{0.5} = K_m$). Data were also analyzed according to two other models (Cornish-Bowden, 1979): the substrate inhibition model (Eq. 2) and the random ternary complex Bi-Bi mechanism model (Eq. 3):

$$v = \frac{V_{max}\,[substrate]}{K_m + [substrate] + [substrate]^2/K_{si}} \quad (2)$$

where K_{si} is the inhibition constant for the substrate;

$$v = \frac{V_{max}\,[A][B]}{K'_a K_b + K_b[A] + K_a[B] + [A][B]} \quad (3)$$

where [*A*] and [*B*] are the two substrates concentrations, K'_a the dissociation constant for *A* from the free enzyme, K_a and K_b the Michaelis constants (K_m's) for *A* and *B*, respectively, calculated in the presence of saturating concentrations of the other substrate (Price & Stevens, 1989).

Protein Assay

The protein contents were determined according to Lowry et al. (1951), using a Biorad protein DC assay kit and bovine serum albumin as standard.

Abbreviations

Alcohol dehydrogenase, ADH; adenosine triphosphate, ATP; calcium-calmodulin complex, CaCam; dithiothreitol, DTT; ethylenediamine tetraacetic acid, EDTA; ethylene glycol-bis(aminoethyl ether)-N,N,N′,N′-tetraacetic acid, EGTA; glucose-6-phosphate dehydrogenase, G6PDH; nicotinamide adenine dinucleotides, NAD^+; nicotinamide adenine dinucleotide phosphate, $NADP^+$; malate dehydrogenase, MDH; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; oxidative pentose phosphate pathway, OPP; $P_{0.5}$, P_{20}, P_{105}, pellets of 500 × g, 20,000 × g, and 105,000 × g centrifugations, respectively; phenazine ethosulfate, PES; phenylmethylsulfonyl fluoride, PMSF; polyvinylpyrrolidone, PVP; $S_{0.5}$, S_{20}, S_{105}, supernatants of 500 × g, 20,000 × g, and 105,000 × g centrifugations, respectively.

Results

Subcellular Distribution

Previous studies of NAD^+ kinase during the breaking of dormancy of *A. sativa* seeds (Gallais & Laval-Martin, 1999; Gallais et al., 2000a) demonstrated an increase in activity due to a membrane-bound CaCam-dependent isoform (pelletable at 28,000 × g for 90 min at 4°C).

To identify the precise intracellular location of this isoform, a subcellular fractionation of the embryo tissues was performed. Assays of subcellular fraction markers (Table 1) revealed that almost all the cytochrome *c* oxidase and malate dehydrogenase (MDH) activities were in P_{20}, and all G6PDH-6PGDH activities were detected in the S_{105} fraction. This confirmed the reliability of the fractionation procedure (Rickwood, Ford & Steensgaard, 1994), with P_{20} corresponding to mitochondria (membranes plus matrix), and S_{105} to cytosol plus vacuolar content. After disruption of mitochondria and a 105,000 × g centrifugation, the pelletable cytochrome *c* oxidase activity was exclusively associated with the membranes (P_{105}) and separated from the soluble MDH activity of the matrix. The NAD^+ kinase activity assays were performed on each fraction (Table 1). The activity was mostly soluble (82%), and more than 60% was CaCam-dependent. The remaining activity (18%), located in mitochondria, was totally CaCam-dependent and mainly linked to membranes. It is noticeable that this last activity was greatly diminished by disruption and centrifugation.

Table 2. Submitochondrial localization of the CaCam-dependent NAD^+ kinase activity

	MDH %	Cyt. *c* oxidase %	Protein mg	NAD^+ kinase	
				Activity (%)	CaCam dependency (%)
Mitochondria homogenate	100	100	3.8	100	85
Sucrose fractions					
0%	34	0	0.9	5	21
10%	41	0	0.2	1	0
20%	4	0	0.3	1	0
30%	8	0	0.6	21	92
40%	12	100	1.5	61	89
50%	1	0	0.3	11	82

Enzyme activities are represented as percentage of the total activity summed from all fractions. Experiment was carried out three times with reproducible data. MDH is malate dehydrogenase.

Submitochondrial Localization

The different layers from the sucrose gradient were assayed for cytochrome *c* oxidase and MDH (Table 2). SDS-PAGE was moreover made to detect the 30-kDa porin, specific of the outer membrane (*data not shown*) (Douce, 1985). In agreement with Sauer and Robinson (1985), the CaCam-dependent NAD^+ kinase was co-located with cytochrome *c* oxidase in the 40% sucrose layer containing the inner membranes (Table 2). Very little NAD^+ kinase was found in the 0–10% sucrose layer containing mitochondrial matrix, and none in the 10–20% sucrose layer corresponding to the outer membranes (Sauer & Robinson, 1985; Dieter & Marmé, 1984), and displaying 30-kDa porin (*data not shown*).

Solubilization Effect of Different Molecules

The solubilization of the CaCam-dependent NAD^+ kinase from P_{20} membranes was tested in the presence of (in mM) 1 hydroxylamine, 1 deoxycholate, 1 cholate, 1 CHAPS, 1% Triton-X100, or 1% Tween-20. The activities of the solubilized and not-solubilized enzyme were measured after incubation followed by a 105,000 × g centrifugation. The P_{20} aliquots displayed an initial activity of 243 ± 33 pmol $NADP^+$ hr^{-1}, 82% CaCam-dependent. All treatments solubilized 40 to 80% of the enzyme (Table 3), but only CHAPS and Tween-20 maintained a slight CaCam dependency (17 and 30%, respectively), a dependency, which was completely lost with the other molecules. On the contrary, except with deoxycholate which totally suppressed the dependency, the not-solubilized activity remained 30 to 95% dependent of the CaCam in the pellet fractions, the CHAPS and Tween-20 being the most efficient. Tween-20 remarkably stimulated the membrane-bound CaCam-dependent NAD^+ kinase (5 times) as compared to the initial activity of P_{20}.

The Tween-20 was then selected, and the solubilization was performed with different concentrations of this detergent, from 0 to 5% (Fig. 1). The results clearly document a progressive solubilization by increasing concentrations. One percent was the optimal concentration but a great amount of enzyme remained in the pellet. Moreover, a maximal CaCam activation of the not-solubilized enzyme was obtained with 1% Tween-20. Such activation was confirmed by directly incubating the mitochondrial suspension, P_{20}, with increasing Tween-20 concentrations (Fig. 2); the CaCam-dependent NAD^+ kinase was stimulated 7-fold by 1 to 5% Tween-20.

The activation of the not-solubilized NAD^+ kinase (Fig. 1) could result from protein aggregates. Since 1% Tween-20 corresponds to 8 mM concentration, high above the 0.049 mM critical micelle concentration, NaCl was then used to dissociate such aggregates (Von Jagow & Schägger, 1994). For this experiment, mitochondrial membranes were incubated in the presence of 4 fixed Tween-20 concentrations and varying NaCl concentrations. After centrifugation, the activities of the solubilized and not-solubilized enzymes were measured (Fig. 3). The results confirmed the preceeding ones (Fig. 1), and clearly illustrated that the enzyme solubilization was most efficient for the highest Tween-20 concentration (5%) supplemented by 0.5 M NaCl.

Activating Effects of Tween-20 and Lipids

To confirm the double effect of Tween-20, activation and solubilization, P_{20} aliquots were resuspended with either buffer alone (control), 5% Tween-20:0.5 M NaCl, 5% Triton X-100, 5% Triton X-100 plus 0.5 M NaCl or 20 mM CHAPS. After a 105,000 × g centrifugation, the NAD^+ kinase activities of pellet and supernatant fractions were then assayed in the presence or absence of 5% Tween-20 (Fig. 4). If Tween-20 provoked only a solubilization of the enzyme, its addition would not have any effect on supernatants resulting from Triton X-100- or CHAPS treatments. In fact, activities of all fractions were at least 2-fold enhanced by Tween-20, except that of the Tween-20:NaCl supernatant already containing this detergent. This supernatant presented the highest amount of solubilized activity; nevertheless, the remaining activity of the corresponding pellet was still 20-fold amplified by the Tween-20, as it was previously demonstrated in Fig. 1. These data clearly demonstrate the double action of the Tween-20, namely solubilizing and activating the membrane-bound NAD^+ kinase.

Table 3. Solubilization of membrane-bound NAD^+ kinase by different molecules

Condition	Concentration	Solubilized enzyme			Not solubilized enzyme		
		+CaCam	+EGTA	%[a]	+CaCam	+EGTA	%[a]
Hydroxylamine	1 mM	111 ± 14	115 ± 5	0	169 ± 7	120 ± 9	30
Cholate	1 mM	146 ± 10	147 ± 20	0	79 ± 16	43 ± 10	45
Deoxycholate	1 mM	237 ± 11	240 ± 26	0	63 ± 3	61 ± 12	0
CHAPS	1 mM	163 ± 20	136 ± 7	17	140 ± 10	33 ± 3	76
Triton X-100	1%	183 ± 17	189 ± 18	0	182 ± 25	91 ± 11	50
Tween 20	1%	290 ± 17	203 ± 17	30	1177 ± 18	61 ± 29	95

Solubilized and not-solubilized activities were assayed in the presence of either EGTA (to measure the CaCam-independent NAD^+ kinase activity) or Ca^{2+} and calmodulin (to determine the total NAD^+ kinase activity: CaCam-dependent and independent).

[a] The CaCam dependency was expressed as percentage corresponding to 100 × (total activity − CaCam-independent activity)/total activity.

Each assay was performed with 250 μg of protein from a mitochondrial homogenate, P_{20}, characterized by a total NAD^+ kinase activity of: 243 ± 33 pmol of $NADP^+$ formed per hr in the presence of CaCam and 44 ± 6 pmol of $NADP^+$ formed per hr in the presence of EGTA. Means of 3 experiments expressed in pmol of $NADP^+ \times hr^{-1}$ ± SD.

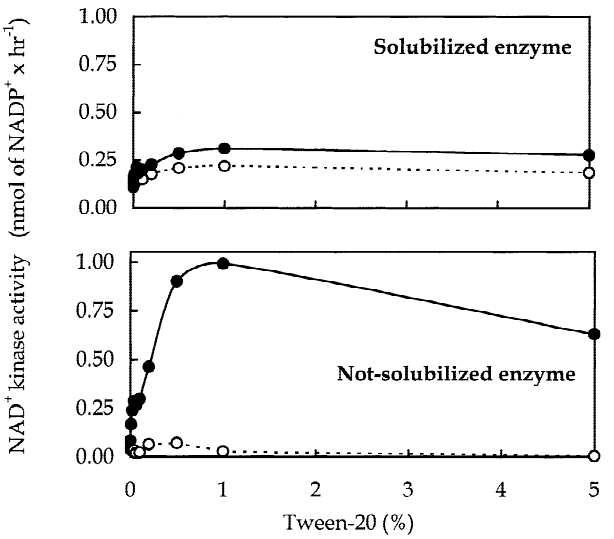

Fig. 1. Solubilization of mitochondrial NAD^+ kinase activity by Tween-20. A mitochondrial suspension was incubated with different Tween-20 concentrations (0 to 5%), and the solubilized and not-solubilized activities were measured in the presence of 5 mM EGTA, ○), or of 1 mM $CaCl_2$ plus 400 U ml^{-1} calmodulin (CaCam, ●). The CaCam dependency was deduced and expressed in nmol of $NADP^+$ hr^{-1}. Experiment was reproduced three times with similar results.

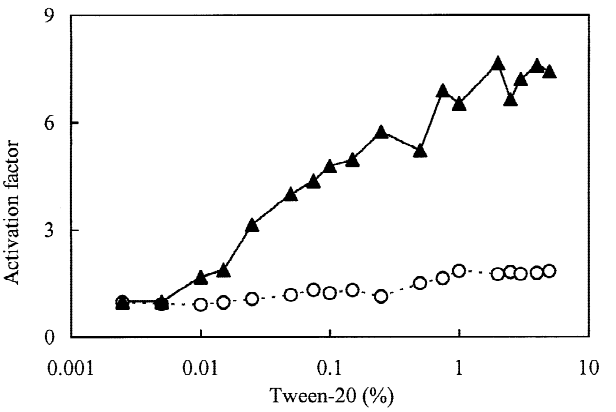

Fig. 2. Activating effect of Tween-20 on mitochondrial NAD^+ kinase activity. A mitochondrial suspension was incubated with different Tween-20 concentrations. The activation factors were deduced from measurements performed in the presence of either EGTA (○) or $CaCl_2$ plus calmodulin, ▲. Experiment was reproduced three times with similar results.

Since several membrane-bound enzymes are lipid-dependent (Johansson, Sommarin & Larsson, 1994; Orr & Newton, 1992; Chang et al., 1998; Bogdanov & Dowhan, 1998), and/or sometimes detergent-dependent (Palmgren et al., 1990), the effects of oleic and linoleic acids, phosphatidylethanolamine (PE), phosphatidylcholine (PC) and lysophosphatidylcholine (lyso-PC), were studied on the NAD^+ kinase activity of mitochondria, P_{20}. Phospholipase A_2, known to hydrolyse PC in lyso-PC, as well as PEG 600 characterized by structural analogies with the Tween-20, were also tested. The effects of these molecules on the enzyme solubilized by Tween-20 were also reported (Table 4). As a matter of fact, some lipids activated the membrane-bound enzyme: slightly in case of PE and more importantly in case of PC. For a ten times lower concentration, the PC was slightly more efficient than the Tween-20. On the contrary, PC and PE inhibited partly the solubilized enzyme. Lyso-PC and phospholipase A_2 presented similar effects: they diminished by about 30% the membrane-bound activity and by 50% the activity of solubilized enzyme. The strongest inhibition of both activities could be noticed with the unsaturated oleic and linoleic acids. Finally, although fatty acids and PEG 600 where structurally related to Tween-20, they inhibited both the membrane-bound and the solubilized enzyme.

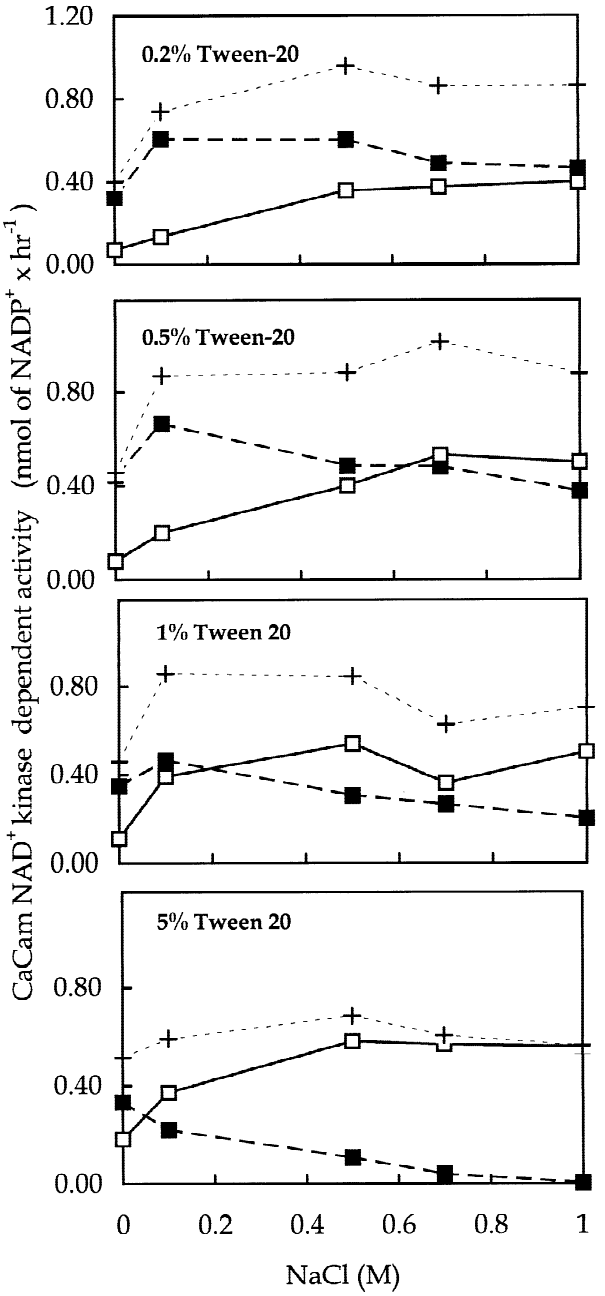

Fig. 3. Solubilizing effect of Tween-20-NaCl mixes. After incubation with a fixed Tween-20 concentration and varying NaCl concentrations, a centrifugation at 20,000 × g for 30 min at 4°C was applied on mitochondrial membranes. Solubilized (□), not-solubilized (■) and total activities (+) of CaCam dependent NAD^+ kinase were represented. Experiment was reproduced three times with similar results.

Effects of Reducers and Oxidants on the NAD^+ Kinase Activity from the Mitochondrial Preparation

Tween-20 used in our experiments contains low concentrations of peroxides known to disrupt membranes (Hjelmeland, 1990). Moreover, an important role of sulfydryl group(s) has been extensively reported for the activity of the soluble NAD^+ kinase (McGuinness & Butler, 1984). Effects of DTT and HSO_3 were then analysed on mitochondrial NAD^+ kinase and compared to those of H_2O_2 and tetrathionate (Fig. 5). An activation by reducers was clearly observed (7- and 11-fold with 1 mM DTT and 1 mM HSO_3, respectively). Surprisingly, these reducers were also able to partially solubilize the enzyme activity: 40% for DTT, 70% for HSO_3. In contrast, H_2O_2 and tetrathionate inhibited the membrane-bound activity, a slight release of the NAD^+ kinase being, however, observed with tetrathionate.

Effect of Tween-20 Solubilization on the Kinetic Parameters of the Mitochondrial Membrane-bound NAD^+ Kinase

Kinetic and chromatographic studies were undertaken, in order to determine (i) how the extraction of the membrane-bound protein by Tween-20 modified its enzymatic parameters, and (ii) if the NAD^+ kinase solubilized from mitochondrial membranes (P_{20}) was distinct from the calmodulin-dependent isoform of the cytosoluble fraction (S_{105}).

Apparent Kinetic Parameters

The mitochondrial NAD^+ kinase activity was measured before and after solubilization (plus Sephadex G-25 desalting) with varying concentrations of ATPMg and NAD^+. Saturation curves displayed typical inhibitions for high ATPMg concentrations (Fig. 6). By comparison, high concentrations of NAD^+ were not inhibitory (*data not shown*). The substrate inhibition model (Eq. *2*) provided the best fit of the data, and the apparent kinetic parameters were deduced from these equations. Similar $K_{i\ ATPMg}$ values of 11.1–12.3 mM were determined for both membrane-bound and solubilized enzymes, suggesting the same inhibitory mechanism. The inhibition by high concentrations of ATPMg could not be avoided by increasing the concentration of NAD^+, contrary to the case of the cytosoluble NAD^+ kinase (Gallais et al., 2000c).

At low concentrations of ATPMg, according to Cornish-Bowden (1979), the inhibition could be neglected for the analyses of the enzyme mechanisms. Linear double-reciprocal plots for fixed NAD^+ or ATPMg, at varying ATPMg or NAD^+ concentrations, respectively, were represented for both enzymes. In the low concentration range, similar multiple intersecting lines were obtained for each of the substrates, indicating a sequential mechanism. Depending on the second substrate concentration, apparent K_m values decreased and apparent V_{max} values increased, and a common intersection point above the abscissa was observed. The binding of the first sub-

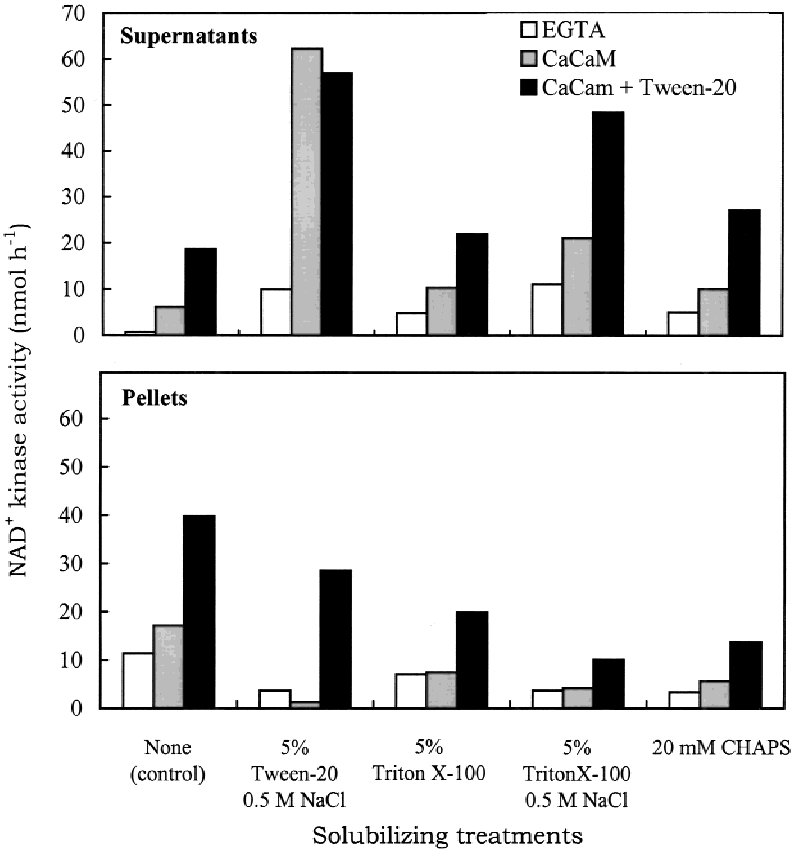

Fig. 4. Tween-20 effects on NAD^+ kinase activities extracted by Triton X-100, Triton X-100 plus 0.5 M NaCl or CHAPS. Mitochondrial membranes were incubated with Tween-20 (5%) plus 0.5 M NaCl, Triton X-100 (5%), Triton X-100 (5%) plus 0.5 M NaCl or CHAPS (20 mM). The activities of pellet and supernatant of a 105,000 × g centrifugation were measured in presence of EGTA, or $CaCl_2$ plus calmodulin, with or without 5% Tween-20.

Table 4. Effects of lipids on NAD^+ kinase from either mitochondria or Tween 20-solubilized fraction

	Mitochondria	Solubilized fraction
Control	100 ± 8	100 ± 13
Oleic acid (0.05%)	0 ± 0	24 ± 3
Linoleic acid (0.05%)	34 ± 10	11 ± 1
Phosphatidylcholine (0.05%)	255 ± 7	76 ± 3
Phosphatidylethanolamine (0.05%)	144 ± 16	70 ± 7
Lysophosphatidylcholine (0.05%)	80 ± 7	61 ± 4
Phospholipase A2 (3U)	70 ± 4	47 ± 6
Tween 20 (0.5%)	212 ± 13	67 ± 8
PEG 600 (0.5%)	68 ± 10	84 ± 8

Values are the means of 4 replicates ± SD.

strate (ATPMg or NAD^+) consistently increased the enzyme affinity for the second substrate. Michaelis constants were determined from secondary plots, namely apparent V_{max} values versus fixed substrate concentrations. Similar apparent K_m ATPMg of 0.5 and 0.4 mM were calculated for the not-solubilized and the solubilized enzyme, respectively. The not-solubilized NAD^+ kinase was characterized by an apparent $K_{m\ NAD^+}$ of 1.3 mM, while a value of 1 mM was found for the solubilized enzyme.

A restriction might be introduced for the apparent $K_{m\ ATPMg}$ provided by the plots derived from the primary NAD^+ saturation curves, because the value observed was a combination of the $K_{m\ ATPMg}$ and the $K_{i\ ATPMg}$ previously evidenced (Fig. 4). Nevertheless, according to the random ternary complex Bi-Bi model (Eq. 3) using the low concentrations of substrates, the apparent dissociation constants from the free enzyme K'_{ATPMg} and K'_{NAD^+} could be calculated equal to 0.8 and 5.5 mM, respectively, for the not-solubilized enzyme, against 0.7 and 7.7 mM, respectively, for the solubilized one. By comparison with the K_m values, the higher values of K'_{ATPMg} and K'_{NAD^+} indicate a synergy of the two substrates for the ternary complex formation (Cornish-Bowden, 1979; Price & Stevens, 1989). In conclusion, a low concentration of ATPMg favours the NAD^+ binding, and *vice versa*, while the high concentrations of ATPMg diminished the enzyme activity. If the affinity of the mitochondrial NAD^+ kinase toward its substrates was very similar before and after solubilization, it greatly differed from that of the cytosoluble calmodulin-dependent isoform (31 ± 2 μM for the $K_{m\ ATPMg}$, and 232 ± 32 μM for the $K_{m\ NAD^+}$) (Gallais et al., 2000c).

The K_m and K'_m values of the not-solubilized and the solubilized enzyme were not different enough to explain the stimulation by Tween-20. The stimulation occured

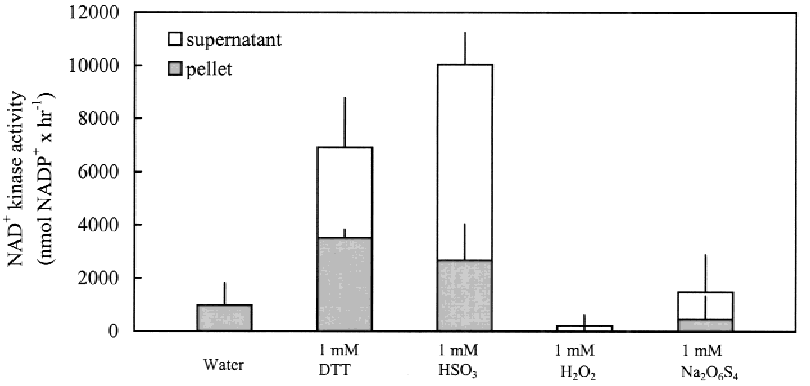

Fig. 5. Effect of reducers and oxidants on membrane-bound NAD^+ kinase activity. Mitochondrial membranes were incubated with 1 mM DTT, 1 mM HSO_3, 1 mM H_2O_2, or 1 mM tetrathionate. Activities of pellets and supernatants of a 105,000 × g centrifugation were measured. Means of three experiments expressed in nmol of $NADP^+$ hr^{-1} ± SD.

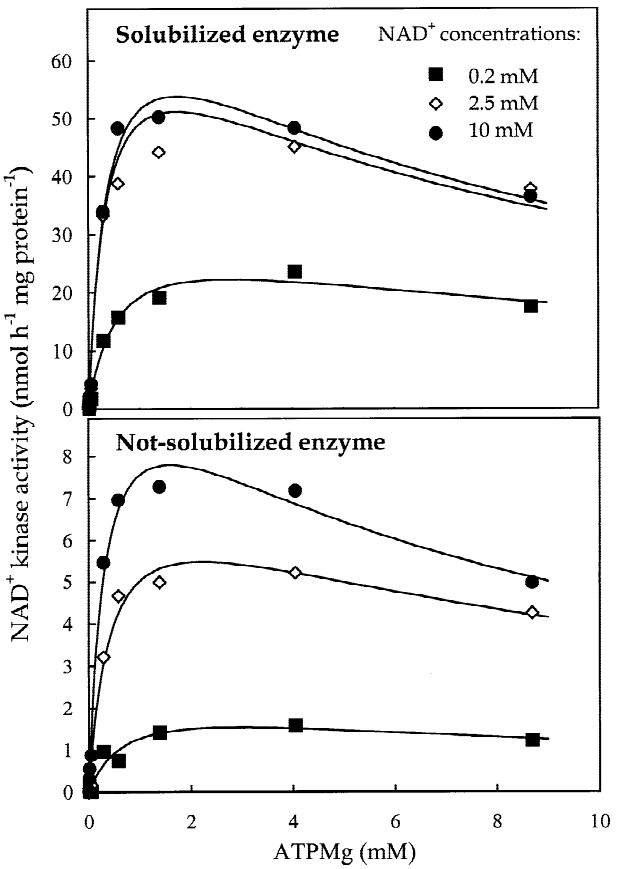

Fig. 6. Saturation curves for NAD^+ as the variable substrate and at various fixed ATPMg concentrations of not-solubilized and solubilized NAD^+ kinase. Values were plotted according to substrate inhibition model (*see* Materials and Methods, Eq. 1).

mainly through an enhancement of the maximal velocities: 9.2 nmol · hr^{-1} · mg $protein^{-1}$ for the membrane-bound NAD^+ kinase, against 63 nmol · hr^{-1} · mg $protein^{-1}$ for the Tween-20 solubilized one.

Chromatographic Analysis

The analytical Sephadex G-200 pattern of proteins solubilized from mitochondrial membranes (P_{20}) by 5% Tween-20:0.5 M NaCl was compared to that of the cytosoluble proteins (S_{105}) obtained after a 105,000 × g, 1 hr, 4°C centrifugation. The cytosoluble fraction displayed two peaks of NAD^+ kinase activity (Fig. 7*A*): the first one being a 410-kDa protein mostly CaCam-independent (about 90%), while the second (63 kDa) corresponds to the calmodulin-dependent isoform. The analysis of the membrane solubilized proteins revealed a unique peak of total NAD^+ kinase activity, 80% CaCam-dependent, but different from the cytosoluble CaCam-dependent isoform, since it was eluted in the void volume (Fig. 7*B*), suggesting a molecular weight greater than 410 kDa. Distinct retardation factors (Rfs) could be also observed by native-PAGE for the two CaCam-dependent isoforms (*results not shown*).

Discussion

NAD^+ kinase from *Avena sativa* has been previously observed in soluble and membrane fractions (Gallais et al., 2000a). The membrane-bound, CaCam-dependent isoform has been reported to increase after dormancy breakage in *Avena* seeds (Gallais & Laval-Martin, 1999), and to be greatly activated during the further growth (Gallais et al., 2000a).

There was some evidence that other plant cells contained membrane-bound NAD^+ kinase, which was under the control of Ca^{2+} and calmodulin (Jarrett et al., 1982; Dieter & Marmé, 1984; Sauer & Robinson, 1985; Muto & Miyachi, 1986; Stephan & Laval-Martin, 2000). Nevertheless, the solubilization of this protein had never been attempted. This paper presents for the first time a solubilization procedure and some characteristics of the CaCam-dependent NAD^+ kinase linked to the inner mitochondrial membrane of *A. sativa.*

To our knowledge, only two reports described a membrane-bound NAD^+ kinase in corn mitochondria after density gradient centrifugation: Dieter and Marmé (1984) characterized a CaCam-dependent activity in the outer membranes from coleoptiles of dark grown seedlings, whereas Sauer and Robinson (1985) associated the

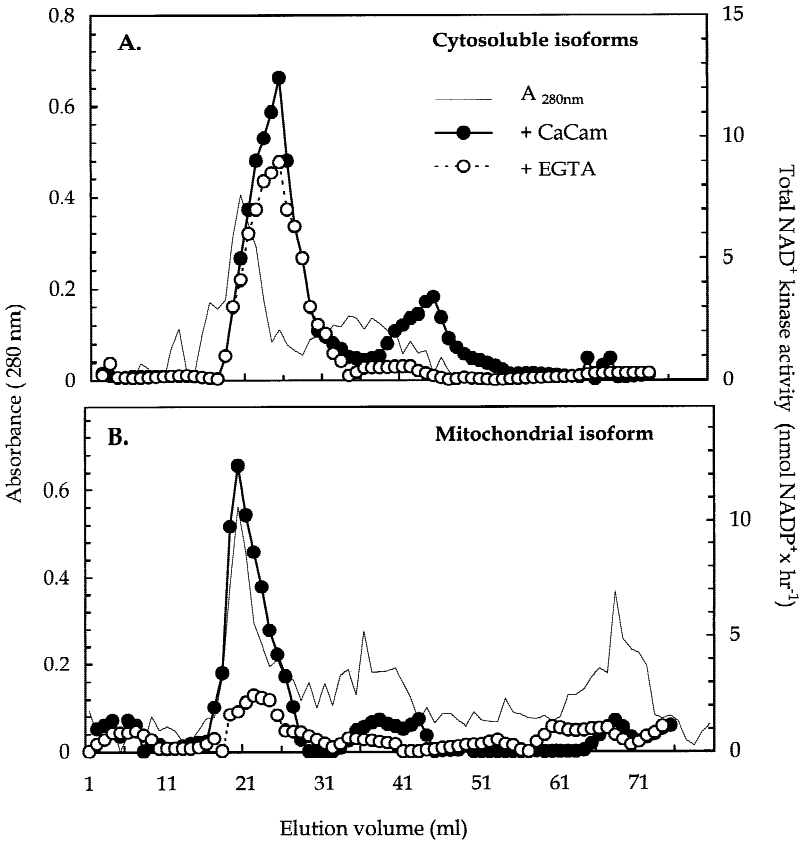

Fig. 7. Chromatographic profiles of cytosoluble and of solubilized NAD^+ kinases from Sephadex G-200 column. Cytosoluble proteins (105,000 × g, 1 hr, 4°C, supernatant S_{105}) were supplemented with Tween-20:NaCl before chromatography (*A*). The same protein quantity, solubilized by Tween-20:NaCl from a mitochondria preparation, was loaded on the same column (*B*). Enzyme activities were assayed in the presence of either EGTA (○) or $CaCl_2$ plus calmodulin (●) and expressed in nmol of $NADP^+$ hr^{-1}. Experiment was reproduced three times with similar results.

mitochondrial NAD^+ kinase from maize roots to both inner and outer membrane fractions. Our results, colocalizing the NAD^+ kinase and cytochrome *c* oxidase activities and, therefore, indicating the presence of CaCam-dependent enzyme in the inner mitochondrial membranes, seem more in agreement with the conclusion of Sauer and Robinson. The localization of such CaCam-dependent NAD^+ kinase might be physiologically important to produce $NADP^+$ within the mitochondrial matrix since (i), many metabolic processes are coupled to the turnover of the $NADP^+$ in the matrix of plant mitochondria (Møller & Rasmusson, 1998); (ii), calmodulin has been located between the inner and the outer mitochondrial membranes of oat seedlings (Biro et al., 1984); (iii), mitochondria contain significant amounts of Ca^{2+}, the transport of which being associated with the inner membrane (Akerman & Moore, 1984).

If, using highly purified enzyme from different sources, considerable interest has been focused on properties of the soluble CaCam-dependent or -independent NAD^+ kinase (McGuinness & Butler, 1984), the membrane-bound NAD^+ kinase has never been solubilized, and its kinetic properties are unknown. A hydroxylamine treatment was previously used to identify thioester linkages of palmitate, oleate or stearate to the side chain of cystein residues of membrane proteins from chicken embryo fibroblasts (Magee & Courtneidge, 1985). Using hydroxylamine on the NAD^+ kinase, the loss of CaCam dependency of the small amount of the solubilized enzyme suggests the involvement of such thioester group(s) necessary for the linkage to membrane and for the CaCam dependency. The importance of such thioester group(s) is confirmed by the solubilization and enhancement of the membrane-bound NAD^+ kinase activity with DTT and HSO_3 (Fig. 5). The CaCam dependency of the solubilized enzyme is also lost in the presence of anionic molecules such as cholate, deoxycholate, oleic and linoleic acids, as it was reported for the cytosoluble NAD^+ kinase of pea (Jarrett, 1986).

Among all assayed solubilizing treatments, the nonionic detergent Tween-20 (5%), associated with high ionic strength (0.5 M NaCl) permitted both a solubilization and a strong activation of the NAD^+ kinase. The most efficient solubilizing action of Tween-20 occurred for concentrations above its critical micelle concentration (CMC of 0.06 mM, i.e., 0.007%). According to Von Jagow & Schägger (1994), the Tween-20 used alone is able to solubilize peripheral proteins from membranes without breaking the protein interactions. In addition to Tween-20, the NaCl requirement for the solubilization suggests that: either the NAD^+ kinase could be associated with other integral proteins, or the enzyme could be itself an integral protein able to form insoluble aggregates.

Tween-20, used with or without NaCl, activates not only the solubilized enzyme but also the remaining not-solubilized enzyme. It was already established that detergents could increase numerous enzyme activities. For example, over limited concentrations, the laurylmaltoside and zwitterionic detergents reactivate the denatured rhodanese (Tandon & Horowitz, 1987). Detergents of the Brij series and Lubrol WX stimulate the H^+-ATPase activity solubilized from plant membrane vesicles and spinach plasma membrane (Palmgren et al., 1990; Johansson et al., 1994); this enzyme is also solubilized and activated by lysophosphatidylcholine (Palmgren et al., 1990). Finally, activation of different enzymes is possible by acidic phospholipids, such as phosphatidylserine (Orr & Newton, 1992), phosphatidylcholine (Chang et al., 1998), and phosphatidylethanolamine acting as molecular chaperone (Bogdanov & Dowhan, 1998). More interestingly, some of these acidic phospholipids or detergents stimulate numerous Ca- or CaCam-dependent enzymes, such as the Ca^{2+}-dependent protein kinase which presents a calmodulin-like domain in carrot membrane cells, in oat plasma membranes and in *Arabidopsis* (Farmer & Choi, 1999). We could then hypothesize a putative phosphatidylcholine binding site close to the catalytic site of the mitochondrial CaCam-dependent NAD^+ kinase. Membrane vicinity and stimulation by phospholipids and Ca^{2+} would be consistent with a role of NAD^+ kinase in signal transduction pathways involving $NADP^+$ (Harding & Roberts, 1998), a hypothesis which was also suggested for the Ca^{2+}-dependent protein kinase (Farmer & Choi, 1994).

Activation by Tween-20 of the membrane-bound NAD^+ kinase could be explained by reinforcing action either on enzyme affinities towards one (or two) of its substrates (K_m and K'_m), or on maximal velocity. The kinetic studies of the membrane-bound and of the solubilized enzymes evidenced that the Tween-20 enhanced the maximal velocity, which suggests the unmasking of additional catalytic sites. Another hypothesis would be that this detergent promotes interaction of the NAD^+ kinase with its activator, the calmodulin.

The partial solubilizing effect of Tween 20 could perhaps mimick the disrupting effects on membranes of lipid peroxidation in response to oxidative-burst, which is correlated with an increased activity of CaCam-dependent NAD^+ kinase in tobacco cells (Harding & Roberts, 1998). As a matter of fact, the membrane-bound NAD^+ kinase was activated by reducers (DTT and HSO_3) (Fig. 5). The extensively reported evidence for the important role of sulfhydryl group(s) in the catalytic site of soluble NAD^+ kinase (McGuinness & Butler, 1984), could then be extrapolated to the membrane-bound form. Another explanation would be the stabilization by the added reducers of the linkage between NAD^+ kinase and calmodulin, as it was suggested above. In the presence of DTT and HSO_3, the membrane-bound NAD^+ kinase was labile (40% and 70% of the total enzyme solubilized, respectively; Fig. 5), and the activities were remarkably stimulated both in the remaining pellet and soluble fraction. The active ferredoxin-$NADP^+$ reductase from the thylakoid membranes becomes also labile in the stroma in response to oxido-reduction changes by methyl viologen-treatment of the plants (Palatnik, Valle & Carillo, 1997); this regulation maintains the NADPH/$NADP^+$ homeostasis in stressed plastids. In addition, environmental or biotic stresses disrupt the redox balance of cells particularly through thiol/disulfide exchange reactions via the glutathione pool and H_2O_2 production (Foyer et al., 1997). Increases in CaCam-dependent NAD^+ kinase activities have already been correlated to metabolic perturbations in response to NaCl stress (Delumeau et al., 2000), to drought stress (Stephan & Laval-Martin, 2000), or to enhanced *Avena* seeds germination (Gallais et al., 2000a). We could therefore propose that, in the stressed mitochondria, the CaCam-dependent NAD^+ kinase, able to shift from the inner membrane to soluble fractions (and *vice versa*) in response to redox modifications, could regulate the pools of NAD^+ and $NADP^+$. It seemed then interesting to compare the solubilized NAD^+ kinase with a previously characterized CaCam-dependent cytosoluble isoform.

If the affinities of the mitochondrial NAD^+ kinase toward its substrates were very similar before and after solubilization (K'_{ATPMg} of 0.7–0.8 mM and K'_{NAD^+} 5.5–7.7 mM), they greatly differed from those of the cytosoluble calmodulin-dependent isoform (31 ± 2 μM for the $K_{m\ ATPMg}$, and 232 ± 32 μM for the $K_{m\ NAD^+}$) (Gallais et al., 2000c). In addition, these two proteins display different molecular masses (above 410 kDa for the solubilized mitochondrial enzyme, and 63 kDa for the cytosoluble one). These two isoforms therefore correspond to distinct proteins. Further studies might be undertaken to demonstrate the existence of the hypothesized cytosoluble isoform and its possible analogies with the solubilized CaCam-dependent and membrane-bound enzyme.

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