

## Detection and characterisation of NAD(P)H-diaphorase activity in *Dictyostelium discoideum* cells (Protozoa)

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### Abstract

In *Dictyostelium discoideum* (*D. discoideum*), compounds generating nitric oxide (NO) inhibit its aggregation and differentiation without altering cyclic guanosine monophosphate (cGMP) production. They do it by preventing initiation of cyclic adenosine monophosphate (cAMP) pulses. Furthermore, these compounds stimulate adenosine diphosphate (ADP)-ribosylation of a 41 kDa cytosolic protein and regulate the glyceraldehyde-3-phosphate dehydrogenase activity. Yet, although *D. discoideum* cells produce NO at a relatively constant rate at the onset of their developmental cycle, there is still no evidence of the presence of nitric oxide synthase (NOS) enzymes. In this work, we detect the nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity in *D. discoideum* and we characterise it by specific inhibitors and physical-chemical conditions that allegedly distinguish between NOS-related and -unrelated NADPH-d activity.

### Introduction

In his paper entitled *Chemical messengers development: a hypothesis*, McMahon<sup>1</sup> postulated how simple derivatives of metabolites – including the small molecules of classical neurotransmitters and cyclic nucleotides – were employed as regulatory molecules throughout the eukaryote evolution. From this perspective, the author assumed that their role evolve: from being intracellular messengers they evolve into intercellular messengers for the relatively slow cell-cell communication between single-cell eukaryotes and for the related development of intercellular messages. This hypothesis has been supported by the detection and characterisation of molecules correlated to neurotransmitters in non-nervous tissues and systems, such as: non-neuromuscular cells and gametes,<sup>2-8</sup> animal embryos,<sup>9-15</sup> coelenterates<sup>16</sup>, plants<sup>17,18</sup> and, most of all, protozoa.<sup>19-31</sup>

The identification of neurotransmitters in

protozoa, later termed the *pre-nerve* transmitter system by Buznikov,<sup>8</sup> indicates that these signal molecules are acquired in early evolution before the appearance of the nervous system, as they are already present in, or synthesised by, protozoa. Among neurotransmitters, nitric oxide (NO) is known to be a ubiquitous biological messenger molecule that may play a role in neurotransmitter release, neural development, synaptic plasticity, and the regulation of gene expression. Furthermore, excessive production of NO is neurotoxic and is implicated in a variety of neurological disorders.<sup>32</sup> NO is a very unstable molecule synthesised by the nitric oxide synthase (NOS) enzyme that catalyses the conversion of L-arginine to citrulline and NO.<sup>33,34</sup>

The NOS enzyme can transfer electrons from co-substrate NAD(P)H to an electron acceptor substrate – a soluble tetrazolium salt – bringing about a coloured insoluble compound.<sup>35</sup> For some years this NOS ability, known as nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity, has been considered as synonymous to NOS.<sup>36,37</sup> This activity can also be produced by enzymes other than NOS.<sup>38-41</sup> Among protozoa, NOS activity and NO production have been identified and characterised in *Paramecium*,<sup>25,26,42</sup> *Leishmania donovani*,<sup>19</sup> *Physarum polycephalum*,<sup>22</sup> *Trypanosoma cruzi*,<sup>21</sup> as well as in *Dictyostelium discoideum* (*D. discoideum*). In these protozoa, NO-generating compounds: i) inhibit its aggregation and differentiation without altering cyclic guanosine monophosphate (cGMP) production<sup>43</sup> by preventing initiation of cyclic adenosine monophosphate (cAMP) pulses;<sup>44</sup> ii) stimulate adenosine diphosphate (ADP)-ribosylation of a 41 kDa cytosolic protein;<sup>45,46</sup> and iii) regulate the glyceraldehyde-3-phosphate dehydrogenase activity.<sup>47</sup> Although it has been demonstrated that cells in *D. discoideum* produce NO at a relatively constant rate at the onset of their developmental cycle,<sup>48</sup> the presence of NOS enzymes has not been reported yet. In addition, although the whole *D. discoideum* genome has been sequenced, there is no information about the presence of a coding region for NOS and only a putative NOS-interacting protein (dictyBase, gene ID: DDB\_GO270882) has been deduced. In this work, we detect NADPH-d activity in *D. discoideum* and we characterise it using specific inhibitors and physical-chemical conditions that supposedly distinguish between NOS-related and -unrelated NADPH-d activity.<sup>25,49,50</sup>

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Key words: NADPH-diaphorase activity, protozoa, nitric oxide synthase.

Conflict of interests: the authors declare no conflict of interests.

Received for publication: 2 March 2012.

Accepted for publication: 26 July 2012.

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European Journal of Histochemistry 2012; 56:e47  
doi:10.4081/ejh.2012.e47

## Materials and Methods

### Cell reproduction and development

The *amoebae* used in this work were axenically induced by inoculating the fruiting bodies in Falcon flasks (BD Biosciences, San Jose, CA, USA) containing AX-2 axenic medium and supplemented with tetracycline, as described in Amaroli *et al.*<sup>51</sup> The fruiting bodies were induced by transferring some drops of the AX-2 culture onto a *Enterobacter aerogenes* monolayer growing on a nutrient agar-N plate.<sup>24</sup> The plates were incubated in a moist chamber for three days at 25°C<sup>52</sup> to allow the cells to exhaust the supply of bacteria (*i.e.* to reach starving conditions) and migrate and aggregate. When the fruiting bodies had developed, the plates were kept at 4°C.

### Experimental samples

The experimental samples used in this study were taken as follows:

- the total sample (T-sample) was obtained by centrifuging a 30 mL sample of an AX-2 culture of logarithmically growing single-cell *amoebae* at 130 g for 10 min.
- the total extract sample (TE-sample) was obtained by transferring the T-samples to -80°C for 10 min. After protease inhibitors [2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 µg/mL leupeptine] were added, the samples were homogenised in 0.3% Triton X-100 and centrifuged at 1000 g for 30 min.
- the membrane sample (M-sample) (pellet) and the cytoplasm sample (C-sample) (supernatant) were obtained by centrifug-

ing six T-samples frozen overnight at  $-20^{\circ}\text{C}$  and resuspended in 1000  $\mu\text{L}$  of 50 mM tris (hydroxymethyl)aminomethane (Tris-HCl), pH 8.0 at 1000 g for 20 min.

- the membrane extract sample (ME-sample) – after protease inhibitors (2 mM PMSF, and 5  $\mu\text{g}/\text{mL}$  leupeptine) were added – was obtained by homogenising the M-samples in 0.3% Triton X-100 and then by centrifuging at 1000 g for 30 min.

### Non-denaturing electrophoresis

To characterise electrophoretically NADPH-d activity, the total protein content of TE-samples was evaluated using the BioRad Assay Kit (Hercules, CA, USA), within factory specifications. A 30 mg total protein extract was layered on 8% polyacrylamide gel. The molecules were electrophoretically separated for 2 h at 80 V. Afterwards, the gels were removed and agitated for 15 min at  $25^{\circ}\text{C}$  in 50 mM Tris-HCl, pH 7.4. A staining bath containing 0.35% Triton X-100 in 50 mM Tris-HCl, pH 7.4, 0.25 mM nitroblue tetrazolium (NBT), and 0.25 mM  $\beta$ -NADPH or  $\beta$ -NADH, was added to cover the gel for 1 h at  $25^{\circ}\text{C}$ . Enzyme activity was blocked in the gels by replacing the reaction mixture with a solution of 10% methanol and 7.5% acetic acid in distilled water.<sup>25,53</sup> We used ImageJ 1.33 software (NIH, Bethesda, MD, USA) to evaluate the optical density (OD) and the apparent molecular mass of the experimental samples bands.

### Immunoblot analysis

In order to detect the presence of proteins immunocitochemically NOS-related, the total protein content of TE-samples was evaluated using the BioRad Assay Kit (Bio-Rad, Hercules, CA, USA), within factory specifications. A 30  $\mu\text{g}$  total protein extract was run in 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with a blocking buffer containing Tris-buffered saline (pH 7.4) and 0.1% Tween-20 with 3% non-fat milk powder for 45 min at  $25^{\circ}\text{C}$ . The blot was incubated with a blocking buffer containing the anti-NOS universal (Sigma, N217), or brain (Sigma, N7155), or endothelial (Sigma, N3893) antibody produced in rabbit, diluted 1:500, overnight at  $4^{\circ}\text{C}$ . After washing, the blot was incubated with an anti-rabbit immunoglobulin secondary antibody conjugated with alkaline phosphatase, diluted 1:500 for 3 h at  $25^{\circ}\text{C}$ . After rinsing thoroughly, detection was performed directly on the membrane using the one-step NBT/5-Bromo-4-chloro-3-indolyl phosphate (BCIP) kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).<sup>25</sup> A 20  $\mu\text{g}$  total protein of mouse tissue extracts was used as a positive control for the NOS antibodies.

### Histochemical evaluation of the nicotinamide adenine dinucleotide phosphate-diaphorase activity

Cells were fixed in 0.1 M phosphate buffer (PB) (pH 7.4) by the presence of 2% paraformaldehyde (PFA). The fixed cells were transferred onto the slide and after air-drying they were incubated for 15 min at  $37^{\circ}\text{C}$  in 50 mM Tris-HCl (pH 7.4), and then for 45 min at  $37^{\circ}\text{C}$  in 1 mM  $\beta$ -NADPH or  $\beta$ -NADH co-substrate, 0.8 mM NBT and 1% Triton X-100 in 50 mM Tris HCl (pH 7.4). The controls were performed omitting the co-substrates.<sup>25</sup>

### Standard reaction for spectrophotometric evaluation of the nicotinamide adenine dinucleotide phosphate-diaphorase activity

The evaluation of NADPH-d activity was performed as suggested by Kuonen *et al.*,<sup>53</sup> appropriately modified. Aliquots of TE-sample were placed in cuvettes containing 1.0 mL of reaction mixture (50 mM Tris-HCl, pH 7.5, containing 0.1% vol/vol Triton X-100, 0.020 mM  $\beta$ -NADH and 0.100 mM NBT) at  $25^{\circ}\text{C}$ . Once the extract was added to the cuvette, the increase in absorbance at 525 nm was monitored by a Nikon 930 spectrophotometer and found to be linear for about 10 min.

### Spectrophotometric characterisation of the nicotinamide adenine dinucleotide phosphate-diaphorase activity

To spectrophotometrically characterize NADPH-d activity, the standard reaction was carried out under the following modified conditions. To evaluate the spectral properties: the standard reaction for NADPH-d activity and the standard reaction mixture – with the omission of Triton X-100 or sample and  $\beta$ -NADH or sample and Triton X-100 – were analysed to the wavelength range 440 to 800 nm. To evaluate the co-substrate affinity:  $\beta$ -NADPH was substituted for  $\beta$ -NADH. To evaluate the influence of the Triton X-100 concentration: the standard reaction was carried out in the presence of 0.1%, 0.3%, 1.0%, 2.0%, 2.5%, 5.0% or 10.0% Triton X-100. To evaluate the influence of pH: the standard reaction was carried out at pH 3.0, 5.0, 7.4, 8.0 or 10.0. To evaluate the influence of fixative solutions: T-samples were pre-incubated for 2 h, at  $2^{\circ}\text{C}$ , with a solution of 50 mM Tris-HCl (pH 7.4) and 1%, 2%, 4%, 10% PFA, 2% glutaraldehyde or 90% methanol/3.7% phormaline. Afterwards, the samples were rinsed in 50 mM Tris-HCl (pH 7.4) and the TE-sample processed for the standard NADPH-d reaction. To evaluate the influence of inhibitors: the standard spectrophotometric

evaluation was carried out in the presence of one of the following compounds:

- 0.1 mM *N*-nitro-L-arginine-methylester (L-NAME) – an analogous compound of arginine – inhibiting NO synthesis;<sup>49</sup>
- 60 mM pyruvate, a competitive inhibitor of mitochondrial respiratory enzymes;<sup>50</sup>
- 0.5 mM warfarin, an inhibitor of NADPH-quinone oxidoreductase activity;<sup>54</sup>
- 0.1 mM dichlorophenolindophenol (DPIP), an electron acceptor and bNOS competitor;<sup>55</sup>
- 5 mM sodium azide, a competitive inhibitor of mitochondrial respiratory enzymes;
- 1 mM 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), a sulphhydryl inhibitor.<sup>50</sup>

### Cellular localisation

In order to evaluate the localisation of NADPH-d activity, the total protein content of TE-sample, ME-sample and C-sample, was quantified by using the BioRad Assay Kit (Bio-Rad, Hercules, CA, USA), within factory specifications. NADPH-d activity itself was measured by the standard reaction for spectrophotometric evaluation of NADPH-d activity.

### Statistical analysis

The experiments were carried out at least three times. The statistical analysis was performed using a two-way ANOVA test followed by the Student-Newman-Keuls multi-comparison test to discriminate statistically significant results (significance level:  $P < 0.05$ ).

## Results

### Detection of the nicotinamide adenine dinucleotide phosphate-diaphorase activity by non-denaturing electrophoresis

The electrophoretic analysis carried out in the presence of the co-substrate  $\beta$ -NADPH (Figure 1 A) or  $\beta$ -NADH (Figure 1 B) showed the presence of the same band of apparent molecular mass of about 175 kDa. The OD measures obtained with ImageJ 1.33j software (NIH, Bethesda, MD, USA) revealed that the intensity of the diaphorase reaction depends on the co-substrate used. This is less evident in  $\beta$ -NADPH than in  $\beta$ -NADH.

### Detection of nitric oxide synthase-related molecules by immunoblot analysis

In the immunoblot analysis, the anti-NOS antibodies did not recognise immunoreactive band in the samples of *D. discoideum* and a

band in mouse brain homogenate (these data are not shown here).

### Histochemistry detection of the nicotinamide adenine dinucleotide phosphate-diaphorase activity

The histochemistry analysis showed that diaphorase staining in the cytoplasm is more intense in the presence of  $\beta$ -NADH (Figure 2 B) than in the presence of  $\beta$ -NADPH (Figure 2 C).

### Spectrophotometric detection of the nicotinamide adenine dinucleotide phosphate-diaphorase activity

The spectrophotometric analysis carried out on T-sample showed that diaphorase activity is higher in the presence of co-substrate  $\beta$ -NADH (Figure 3 B) than in the presence of  $\beta$ -NADPH (Figure 3 A).

### Characterisation of the nicotinamide adenine dinucleotide phosphate-diaphorase activity by spectrophotometric analysis

The spectral analysis of NAD(P)H-d reaction showed a maximal absorbance value at 525 nm (Figure 4, corresponding symbol: circle). NAD(P)H-d activity had a wide range of tolerance to non-ionic detergent Triton X-100. When the reaction was carried out in the presence of 0.1% to 2% Triton X-100, no significant variations were observed (Figures 5, A, B, C, D, and E). On the contrary, 5% Triton X-100 partially inhibited the activity (Figure 5 F) and 10% Triton X-100 made it very low (Figure 5 G).

The increasing pH values exposure affected the intensity of NAD(P)H-d reaction in a pH-

dependent manner (Figure 6). The activity was highest at pH 8.0 to 10.0 (Figures 6 D and E) and lowest at pH 3.0 (Figure 6 A).

The pre-incubation of T-samples in solutions with fixatives also influenced the diaphorase activity (Figure 7). T-samples pre-incubated with 1% to 4% PFA showed some 25% decrease in the activity (Figures 7 B, C, and D), while T-samples pre-incubated with 10% PFA approximately decreased by 38% (Figure 7 E). Finally, exposition to methanol/formalin (90%/37%) or 2% glutaraldehyde decreased NADPH-d activity by about 38% (Figure 7 F) and 73%, respectively (Figure 7 G).

The sensitivity of the T-samples to inhibitors of enzymes having NADPH-d activity is showed in Figure 8. In comparison with the control activity obtained with the standard NADPH-d reaction (Figure 8 A), this activity was unaffected by exposure to 0.1 mM L-NAME, 5.0 mM sodium azide, 0.5 mM warfarin (Figures 8 B, C, and D). The activity decreases significantly after exposure to 60.0 mM pyruvate (Figure 8 E), 0.1 mM DPIP (Figure 8 F), and 1.0 mM DTNB (Figure 8 G) (the power of inhibition was  $DPIP > DTNB = \text{pyruvate}$ ). Finally, as showed in Figure 9, TE-sample and C-sample had the same NAD(P)H-d activity.

## Discussion

The presence of NAD(P)H-d activity in *D. discoideum* cells has been identified first in this study. NAD(P)H-d activity shows different levels of co-substrate affinity, higher for  $\beta$ -NADH than for  $\beta$ -NADPH, as is demonstrated by non-denaturing electrophoresis, histochemical and spectrophotometrical analyses. This

enzyme activity is almost completely localised in the cytoplasmic fraction, which is in line with what Kuonen *et al.*<sup>53</sup> observed in the rat brain for NOS-related NADPH-d reaction.

The spectral characterisation of the NAD(P)H-d reaction using the co-substrate  $\beta$ -NADH reveals a maximal absorbance value at 525 nm, which is similar to what observed in the case of NADPH-d activity in rat brain<sup>53</sup> and opossum tissues,<sup>55</sup> and with commercially-available water-insoluble formazan.<sup>53,55</sup> The

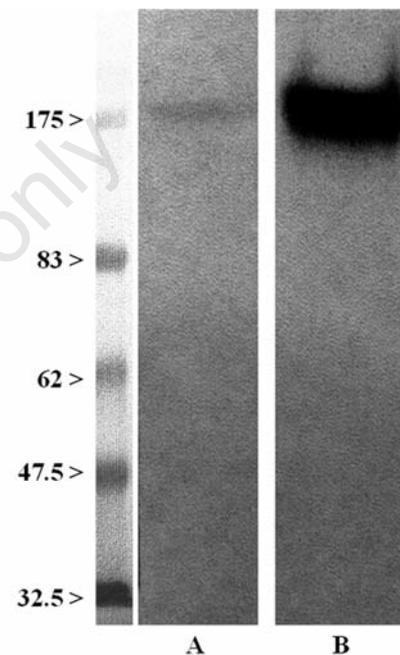


Figure 1. Pattern of NAD(P)H-d activity in *D. discoideum* by non-denaturing electrophoresis. The co-substrate used were  $\beta$ -NADPH (A) and  $\beta$ -NADH (B).

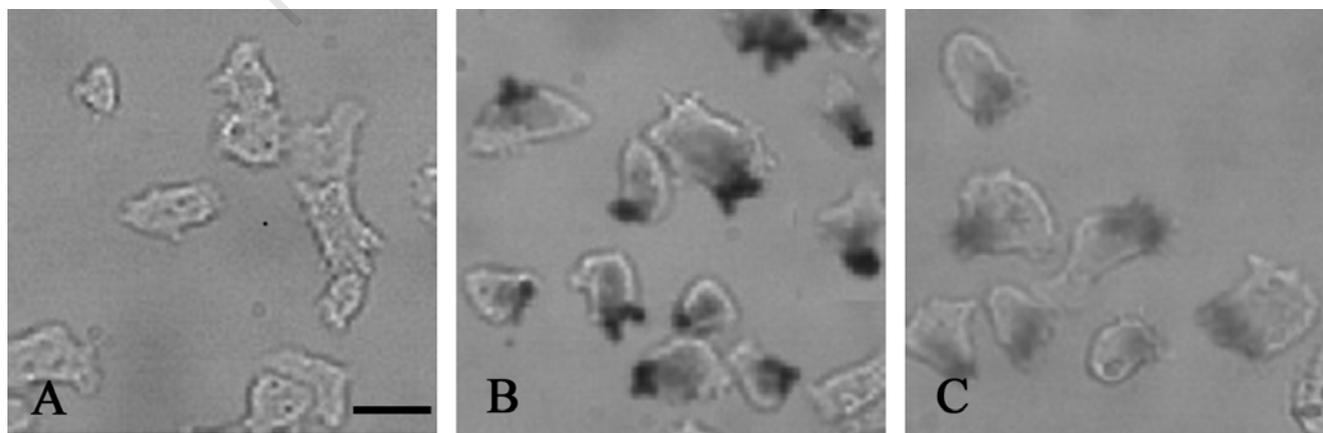


Figure 2. Histochemistry detection of NAD(P)H-d activity. The reaction was carried out in the presence of co-substrates  $\beta$ -NADH (B) and  $\beta$ -NADPH (C) and in the absence of the co-substrate (A). Scale bars=10  $\mu$ m.

*Dictyostelium*'s NAD(P)H-d reveals a different sensitivity to cell fixative, pH and Triton X-100 concentrations. Some fixatives, such as PFA and glutaraldehyde, are known to inhibit NADPH-d activity.<sup>56</sup> However, as shown in the *Helix pomatia* nervous system, only the NOS-unrelated NADPH-d reaction is completely suppressed, while NOS-related NADPH-d activity is not particularly sensitive.<sup>57</sup> In addition, a solution of methanol/formalin in the nervous system of *Periplaneta americana* do not alter the NOS-related NADPH-d activity. In our study NAD(P)H-d activity was inhibited by exposure to either PFA (1% to 10%) or methanol/formalin, but not in a dose-dependent manner and not drastically.

Exposure to different pH shows the sensitivity of the *Dictyostelium* NAD(P)H-d to acid pH and a higher activity in pH 8.0 to 10.0. This result is in line with the observations made by Kuonen *et al.*<sup>53</sup> on the characterisation of NADPH-d activity in the rat brain, but differ from Spessert and Claassen's results.<sup>49</sup> These authors found high sensitivity to the NOS-related NADPH-d in the rat olfactory bulb at basic pH.

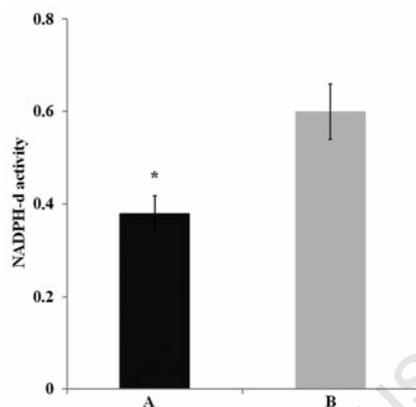
As demonstrated by Fang *et al.*,<sup>55</sup> Triton X-100 is able to improve NADPH-d reaction, probably by catalysing the activity of diaphorase, by keeping staining nitroblue diformazan in solution thus suppressing the straining of non-neuronal structure, and by increasing the permeability of the cell membrane. However, highly concentrated Triton X-100 is known to be a denaturing protein. In *Dictyostelium* cells, NAD(P)H-d activity is not sensitive to 0.1% to 2.5% Triton X-100. Yet, it is inhibited by 5% Triton X-100 and is almost completely suppressed after exposure to 10% Triton X-100. This result conforms to the finding that NOS-related NADPH-d activity is not particularly sensitive to low concentration of Triton X-100, while NOS-unrelated NADPH-d activity is inhibited by approximately 2% Triton X-100.<sup>49,53,55</sup>

Exposure to specific inhibitors of enzymes showing diaphorase activity further supports the hypothesis that the *Dictyostelium* NAD(P)H-d could be NOS-related. After incubation with DPIP – a NOS competitor –<sup>49</sup> the diaphorase activity is inhibited by about 50% compared to the control samples. A similar result has been found in *Paramecium primaurelia*, where NOS-related NADPH-d activity was inhibited by exposure to DPIP by about 50%.<sup>25</sup>

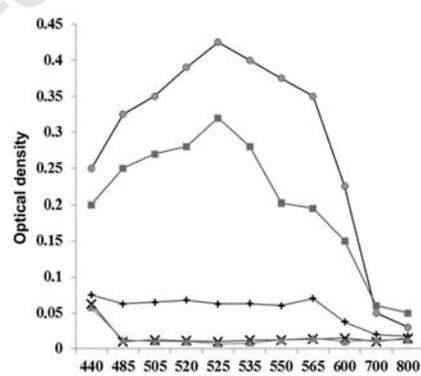
On the other hand, exposure to L-NAME – an analogous compound of arginine and inhibitor of NO synthesis – is unaffected by the diaphorase activity. The 20% and 25% decrease in the activity caused by exposure to pyruvate and DTNB, respectively, also suggests the presence of some NOS-unrelated

NAD(P)H-d activity. The diaphorase reaction appears to be unaffected by exposure to sodium azide and warfarin. The findings of the non-denaturing electrophoretic analysis show the presence of one protein, which shows diaphorase activity, of apparent molecular mass of about 175 kDa that oxidizes co-sub-

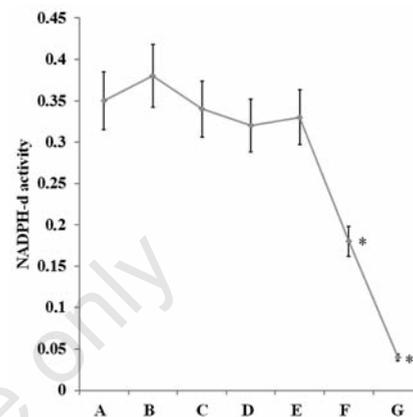
strate  $\beta$ -NADH with higher affinity than  $\beta$ -NADPH. In previous papers we have shown that *Enterobacter aerogenes* can exhibit two NADPH-diaphorase activities with an apparent molecular mass of 124 kDa and 47 kDa.<sup>25</sup> For this reason, NADPH-d activity shown for *D. discoideum* is not a consequence of bacterial pol-



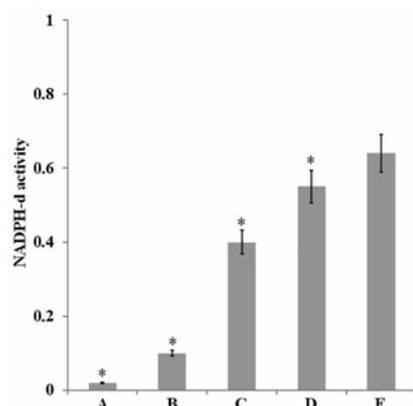
**Figure 3.** Spectrophotometric detection of NAD(P)H-d activity. The reaction was carried out in the presence of the  $\beta$ -NADPH (A) and  $\beta$ -NADH (B) co-substrates. NAD(P)H-d activity was expressed as  $\Delta$ OD/min/total proteins. The symbol \* indicates a significant difference ( $P < 0.05$ ) with regard to the reaction processed with the  $\beta$ -NADH (B) co-substrate.



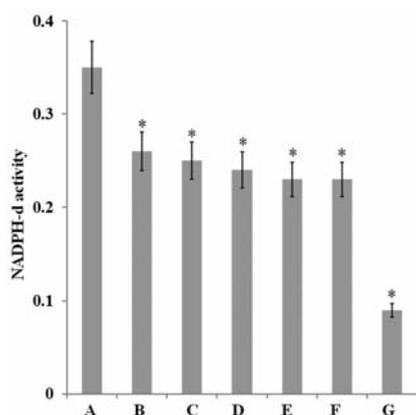
**Figure 4.** Spectral analysis of NAD(P)H-d reaction and standard reaction mixture. Symbols are used as follows. Circle: standard reaction ( $\beta$ -NADH, Triton X-100, NBT and sample); square: reaction mixture ( $\beta$ -NADH, NBT, sample); plus: reaction mixture ( $\beta$ -NADH, Triton X-100, NBT); X: reaction mixture ( $\beta$ -NADH, NBT); triangle: reaction mixture (Triton-X-100, NBT).



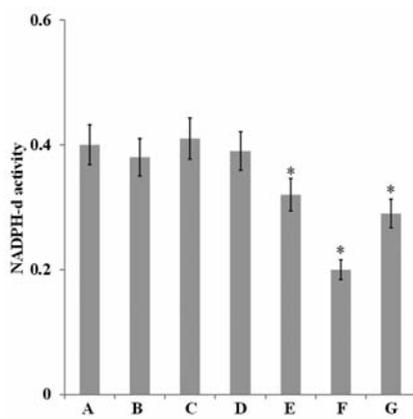
**Figure 5.** Spectrophotometric detection of the effect of Triton X-100 on NAD(P)H-d activity. The standard reaction was carried out in the presence of the following Triton X-100 concentrations: 0.1% (A), 0.3% (B), 1% (C), 2% (D), 2.5% (E), 5% (F), 10% (G). The NAD(P)H-d activity was expressed as  $\Delta$ OD/min/total proteins. The symbol \* indicates the significant difference ( $P < 0.05$ ) with regard to the reaction processed in the presence of 0.1% (A) Triton X-100.



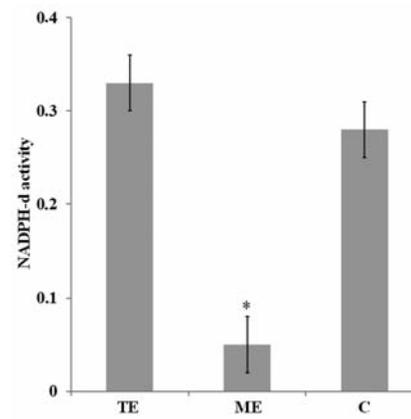
**Figure 6.** Spectrophotometric detection of the effect of pH on NAD(P)H-d activity. The standard reaction was carried out at the following pH values: 3.0 (A), 5.0 (B), 7.4 (C), 8.0 (D), 10.0 (E). NAD(P)H-d activity was expressed as  $\Delta$ OD/min/total proteins. The symbol \* indicates the significant difference ( $P < 0.05$ ) with regard to the preceding pH value.



**Figure 7.** Spectrophotometric detection of the effect of cell fixative on NAD(P)H-d activity. T-samples were pre-incubated with fixative and subsequently the standard reaction was performed. Control (A), 1% paraformaldehyde (PFA) (B), 2% PFA (C), 4% PFA (D), 10% PFA (E), 90% methanol/3.7% phormaline (F), 2% glutaraldehyde (G). NAD(P)H-d activity was expressed as OD/min/total proteins. The symbol \* indicates the significant difference ( $P < 0.05$ ) with regard to the control (A).



**Figure 8.** Spectrophotometric detection of the effect inhibitors on the NAD(P)H-d activity. The standard reaction was carried out in the presence of the following inhibitors: 0.1 mM L-NAME (B), 5.0 mM sodium azide (C), 0.5 mM warfarin (D), 60 mM pyruvate (E), 0.1 mM DPIP (F), 1 mM DTNB (G). The standard reaction carried out in the absence of inhibitors is considered as control (A). The NAD(P)H-d activity was expressed as  $\Delta OD/min/total\ proteins$ . The symbol \* indicates the significant difference ( $P < 0.05$ ) with regard to the control (A).



**Figure 9.** Spectrophotometric detection of the NAD(P)H-d localisation in *D. discoideum*. Total sample extract (TE), membrane-sample extract (ME), cytoplasm-sample (C). The NAD(P)H-d activity was expressed as  $\Delta OD/min/total\ proteins$ . The symbol \* indicates the significant difference ( $P < 0.05$ ) with regard to the total sample extract (TE).

lution by *E. aerogenes*. Lastly, the anti-NOS antibodies do not recognize NOS-related molecules in immunoblot analysis. In conclusion, it is important to point out that diaphorase activity shows how difficult it is to establish an experimental condition that clearly distinguish between NOS-related and -unrelated NADPH-d activity. The sensitivity of NOS-related NADPH-d to physical-chemical parameters changes between organisms and is tissue-specific.<sup>40,50,53,57-59</sup> With regard to the NAD(P)H-d activity described in *D. discoideum*, however, there are numerous results which suggest its correlation to the presence of NOS enzyme, not least to the inhibition by DPIP. Insensitivity to the L-NAME inhibitor does not change this hypothesis for it is known that the reduction of the NBT substrate occurs without the conversion of L-arginine to citrulline and NO, according to the hypothesis that NO synthesis does not take place in fixed tissues.<sup>25,55</sup> The effect observed after exposure to pyruvate or DTNB could indicate the presence of NAD(P)H-d activity due to NOS-unrelated enzymes, such as lactate dehydrogenase, the enzyme described in *Dictyostelium*.<sup>60</sup> However, the DTNB inhibition can be the consequence of its effect on the NOS after exposure to sulfhydryl inhibitors of the *Mesocricetus auratus*'s NOS, as described by Wehby and Frank (1999).<sup>50</sup> The non-denaturing electrophoresis analysis clearly shows that the *D. discoideum*'s NAD(P)H-d

is due to one protein of apparent molecular mass of 175 kDa. This is within the range of molecular masses indicated in literature for the NOS-related NADPH-d<sup>53</sup> and is not representative of the molecular mass of the majority of proteins showing NADPH-d activity. Our results, like those indicating NO production in *D. discoideum* cells,<sup>48</sup> provide further evidence of the presence of the NOS enzyme in *Dictyostelium*, an enzyme that does not show epitopes recognizable by the usual anti-NOS antibody.

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