

Dissociation of FAD from the NAD(P)H-Nitrate Reductase Complex from *Ankistrodesmus braunii* and Role of Flavin in Catalysis

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Ankistrodesmus braunii NAD(P)H-nitrate reductase is a complex hemoflavomolybdoprotein composed by eight similar subunits. The flavin prosthetic group, identified as FAD, is essential for the NAD(P)H-dependent activities of the complex, and is located before the heme chromophore in the enzyme electron transport chain from reduced pyridine nucleotides to nitrate.

Fluorescence studies indicate that nitrate reductase can dissociate about 80% of its FAD by incubation at room temperature, the flavin dissociation being followed by a parallel decrease of NADH-nitrate reductase activity. Dissociation of FAD from the protein is easily increased by dilution or prolonged dialysis of the enzyme preparations. However, exogenous FAD specifically prevents the dissociation of enzyme-bound flavin, and protects the NAD(P)H-dependent activities. The K_m for FAD, as a protector of NADH-cytochrome *c* reductase activity, is 4 nM. In addition, dithioerythritol also prevents the flavin dissociation, and therefore the presence of free sulphhydryl groups in the FAD-domain is suggested.

FAD-depleted nitrate reductase, obtained by several methods, is unable to recover its original activity when incubated in the presence of FAD alone or with thiols.

Introduction

Assimilatory NAD(P)H-nitrate reductase (EC 1.6.6.2.) from *Ankistrodesmus braunii* is a soluble enzyme that catalyzes the two-electron reduction of nitrate to nitrite. The native enzyme, $M_r = 467\,400$, is composed by eight similar subunits, $M_r = 58\,750$, and contains four molecules of FAD, four *b*-type cytochromes and two atoms of molybdenum [1, 2].

In addition to catalyzing the normal reaction, NAD(P)H-nitrate reductase exhibits two other activities that involves only part of its electron transport chain. NAD(P)H-diaphorase activity constitutes the first moiety of the enzyme complex, and catalyzes the reduction of a variety of one- or two-electron acceptors such as ferricyanide, cytochrome *c* or 2,6-dichlorophenolindophenol. The action of the so-called terminal nitrate reductase activity, which constitutes the second moiety of the complex, results in the reduction of nitrate to nitrite by reduced flavin nucleotides or viologens [3, 4].

Nitrate reductase from *A. braunii* has previously been purified to homogeneity by a procedure that

utilizes as the main step dye-ligand chromatography on blue-Sepharose [1]. FAD had to be added to the buffers used in the purification of the enzyme in order to protect its NAD(P)H-dependent activities, and was also required to carry out the specific elution by NADH of the enzyme retained in the affinity columns [1]. Although this information suggests an important role of FAD as component of *A. braunii* nitrate reductase, a direct study on the flavin of this enzyme has not yet been realized.

In this paper we report the absorption and fluorescence emission spectra of the flavin component associated to nitrate reductase from *A. braunii*, which is essential for the reduction by NAD(P)H of the heme prosthetic group and for the NAD(P)H-dependent activities associated to the enzyme complex. On the other hand, FAD can be easily dissociated from the protein, and studies of protection against this dissociation lead to assume that free sulphhydryl groups are involved in the FAD-binding site.

Materials and Methods

Materials

Cytochrome *c*, Tris, EDTA, and FAD were purchased from Sigma (St. Louis, USA). Methyl

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viologen and dithioerythritol were from Serva (Heidelberg, West Germany). Pyridine nucleotides were from Boehringer (Mannheim, West Germany). Sephadex G-25 was from Pharmacia (Uppsala, Sweden).

Purification of nitrate reductase

Ankistrodesmus braunii nitrate reductase was purified by affinity chromatography on blue-Sepharose, according to the procedure described by De la Rosa *et al.* [1]. Purified nitrate reductase preparations were dialyzed against 5 l of 0.1 M potassium phosphate buffer, pH 7.0, containing 20 μ M FAD to saturate all flavin-binding sites of the enzyme. After 24 h, the dialyzed enzyme solutions were filtered through a Sephadex G-25 column (1 $\times$ 13 cm) equilibrated with the same buffer without FAD to remove free flavin but not enzyme-bound flavin. The resulting native nitrate reductase preparations were electrophoretically homogeneous, with a specific activity of about 50 units/mg of protein and an A_{278}/A_{414} ratio ranging from 3 to 6.

Enzyme assays

Assays of the enzymatic activity were performed in 1.0 ml reaction volumes containing 0.1 M Tris-HCl buffer, pH 7.5; 0.3 mM NADH; acceptor of electrons; and an appropriate amount of enzyme. Acceptors were present at the following concentrations: potassium nitrate, 10 mM; or cytochrome *c*, 60 μ M. Reactions were carried out in cells of 1 cm light path at 30 °C. Kinetics were followed using a Pye Unicam SP8-100 spectrophotometer, with the reference cuvette containing all components except the enzyme. Absorbance changes were followed at 340 nm for NADH-nitrate reductase activity, and at 550 nm for NADH-cytochrome *c* reductase activity. Extinction coefficients used were: $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for NADH at 340 nm; and $1.96 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for reduced minus oxidized cytochrome *c* at 550 nm.

NADH-nitrate reductase activity was also determined colorimetrically by measuring the nitrite formation after 5 min of incubation at 30 °C of the reaction mixture.

One unit of activity is the amount of enzyme which catalyzes the oxidation of 1 μ mol of NADH per min under the assay conditions.

Analytical determinations

Protein was determined by the method of Bailey [5], with bovine serum albumin as standard. Absorbance of the standards at 279 nm was measured to determine their concentration ($\epsilon_{279 \text{ nm}}^{1\%} = 6.7$). Nitrite was estimated as described by Snell and Snell [6].

Spectroscopic measurements

Absorption spectra were recorded *versus* appropriate solvent blanks with an Aminco DW-2a spectrophotometer. Band width was 1.5 nm. Fluorescence spectra were recorded with an Aminco-Bowman SPF spectrophotofluorometer equipped with a Varian-Aerograph F-100 recorder. For determinations of flavin fluorescence intensity, the excitation and emission wavelengths were 450 nm and 535 nm, respectively. Flavin-containing solutions were protected from light at all times prior fluorometer measurement. All spectroscopic determinations were performed at room temperature utilizing silica cuvettes of 1 cm path length.

Dialysis

Dialysis were carried out at 4 °C with membranes of 1 cm in diameter (Visking, Union Carbide, Chicago, USA), which were previously boiled in 1% NaHCO_3 /0.01% EDTA for 30 min, and then washed thoroughly with distilled water.

Results

Spectrophotometric studies on nitrate reductase and enzyme-bound flavin.

The absorption spectrum of the flavin component of *A. braunii* nitrate reductase is shown in Fig. 1 *upper*, and presents absorption maxima at 266, 374, and 448 nm, with a shoulder at 480 nm. This spectrum resulted by subtracting that obtained with an exhaustively dialyzed nitrate reductase preparation (Fig. 1 *lower*, dashed line) from the absorption spectrum of the native enzyme (Fig. 1 *lower*, solid line). These data indicate that the flavin component of nitrate reductase seems to be non-covalently bound to the enzyme and it is easily dissociated.

NADH was able to reduce the heme chromophore of native nitrate reductase [1], but not that of the flavin-depleted enzyme (Fig. 2, *Curve B*), which can be fully reduced by dithionite (Fig. 2, *Curve C*).

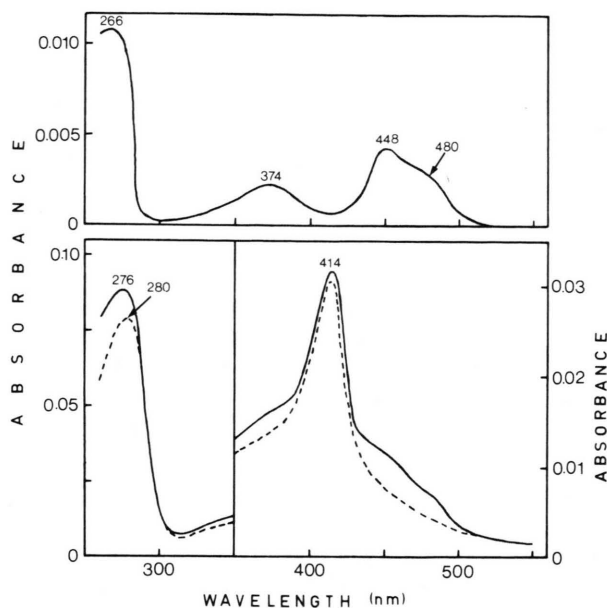


Fig. 1. Absorption spectrum of the flavin component of *A. braunii* nitrate reductase. Lower, the solid line shows the absorption spectrum of nitrate reductase in 0.1 M potassium phosphate buffer, pH 7.0, as isolated in the native form (0.3 mg/ml), recorded against the buffer without enzyme; the dashed line represents the spectrum of the same enzyme preparation after 24 h dialysis against 5 l of the same phosphate buffer, and using the dialysis buffer as reference. Upper, it shows the absorption spectrum obtained by subtracting the latter spectrum from the former one in the lower picture.

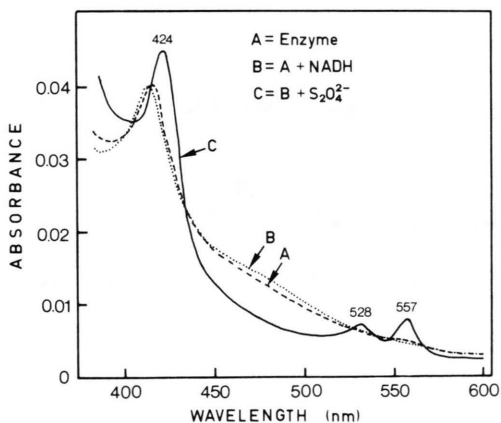


Fig. 2. Reducibility of the heme prosthetic group of flavin-depleted nitrate reductase. 1 ml of a nitrate reductase solution containing 40 μ g of protein, was exhaustively dialyzed against 5 l of 0.1 M potassium phosphate buffer, pH 7.0. Afterwards, the absorption spectrum of the enzyme was recorded against the dialysis buffer after the following additions: A, nothing; B, a little amount of NADH; and C, a few crystals of sodium dithionite.

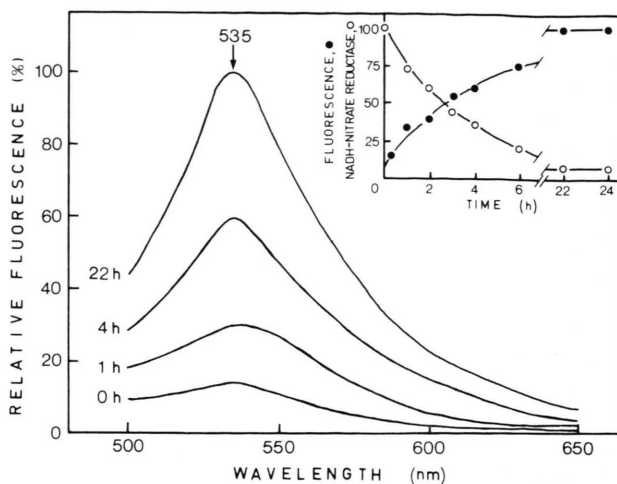


Fig. 3. Flavin fluorescence of nitrate reductase and role of flavin in the catalytic activity. 2.5 ml of a solution containing 60 μ g of native nitrate reductase in 50 mM potassium phosphate buffer, pH 7.0, were incubated at room temperature in the dark. The flavin fluorescence emission spectrum (excitation at 450 nm) was registered at the indicated times. On the other hand, NADH-nitrate reductase activity was also measured by adding 10 μ l aliquots of the enzyme solution to the reagents of the standard assay. Inset, time courses of the flavin fluorescence emission at 535 nm and the enzyme activity.

On the other hand, addition of FAD prior to NADH did not result in the reduction of the deflavoenzyme nor did it restore the NAD(P)H-dependent activities (not shown).

Fluorometric studies on flavin from nitrate reductase

The fluorescence emission spectrum of native nitrate reductase when excited at 450 nm, presented significant changes upon incubation of the enzyme solution at room temperature in the dark. At the beginning, fluorescence was practically zero, but it was increasing as a function of time with an emission maximum at 535 nm, typical of flavin nucleotides (Fig. 3). In addition, the NADH-nitrate reductase (Fig. 3, inset) and NADH-cytochrome *c* reductase (not shown) activities were on the decrease parallel to the increase of fluorescence. These results indicate that flavin bound to nitrate reductase, but not free flavin, is essential for the NADH-dependent activities. On the other hand, it is interesting to emphasize that the amount of flavin spontaneously dissociated from nitrate reductase represented about 75–80% of the total content in FAD of the enzyme,

since if the protein was denaturalized by treatment with trichloroacetic acid [7], the fluorescence at 535 nm increased about 20–25% (not shown).

Effect of dilution of enzyme on NADH-cytochrome c reductase activity

NADH-diaphorase activity of the nitrate reductase complex, measured as reduction of cytochrome *c* by NADH, was selected for this study of enzymatic activity as a function of the enzyme dilution since it was the most sensitive assay available at low concentrations of enzyme.

Nitrate reductase underwent a loss in its ability to reduce cytochrome *c* with NADH when the enzyme was incubated in dilute solution at 30 °C. This inactivation followed a first order kinetics, with a half time of 11 min, after dilution of an enzyme preparation (0.7 units/ml) with 100 volumes of buffer. The results of Fig. 4 show that NADH-cytochrome *c* reductase activity depends on the concentration of the enzyme solutions during the preincubation, although the degree of inactivation becomes constant at dilution factor higher than 26. The remaining activity was about 20–25% of the control, which is in good agreement with the relative amount of flavin undissociated from the protein (see Fig. 3).

Protection by FAD against inactivation of NADH-cytochrome c reductase activity by dilution.

Table I shows that FAD completely protects NADH-cytochrome *c* reductase activity against its

inactivation upon incubation at 30 °C of a dilute nitrate reductase solution. Only partial protection was observed when FMN or NADH was included in the enzymatic preincubation mixture. However, no significant effect of FAD on NADH-cytochrome *c* reductase activity was detected when a concentrate nitrate reductase solution was used.

Fig. 5 shows the results obtained using different concentrations of FAD to protect NADH-cytochrome *c* reductase activity against inactivation upon dilution. A Lineweaver-Burk plot of the increment in activity as a function of the concentration of added FAD yielded a K_m value for FAD of 4 nM.

Table I. Protection by FAD against inactivation of NADH-cytochrome *c* reductase in dilute enzyme solutions.

Preincubation system	NADH-cytochrome <i>c</i> reductase $\Delta A_{550}/\text{min}$
E_c	0.128
$E_c + \text{FAD}$	0.124
E_d	0.030
$E_d + \text{FAD}$	0.177
$E_d + \text{FMN}$	0.075
$E_d + \text{NADH}$	0.052

10 μl samples of 11 nM native nitrate reductase were used in these experiments, either directly (E_c) or after 80-fold dilution with 100 mM Tris-HCl buffer, pH 7.5 (E_d). Where indicated, the nucleotides were added at 30 μM final concentration. After 30 min of incubation at 30 °C, NADH-cytochrome *c* reductase activity was measured by supplementing each preincubation mixture with the reagents of the standard assay and adjusting the final volume, in all cases, until 1 ml. Reaction rates were determined by following absorbance changes at 550 nm.

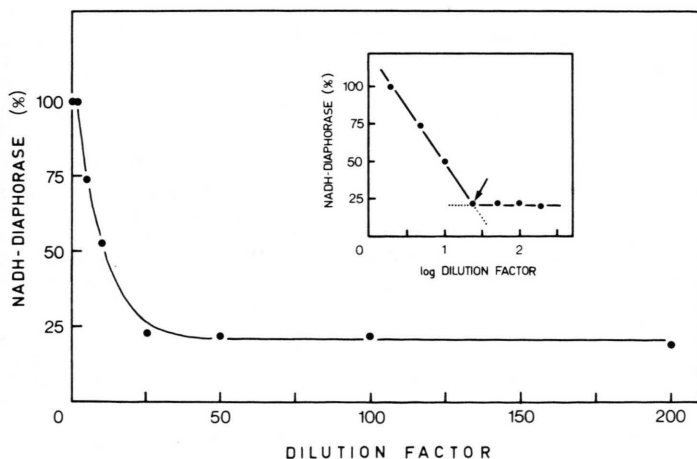


Fig. 4. Inactivation of NADH-cytochrome *c* reductase depending on the concentration of the nitrate reductase solution. 5 μl samples of 9 nM native nitrate reductase were diluted, at the indicated proportions, with 100 mM Tris-HCl buffer, pH 7.5, and incubated at 30 °C for 30 min. Then, NADH-cytochrome *c* reductase activity was measured in each case by adding the reagents of the standard assay to the dilute enzyme solutions, adjusting the final volume until 1 ml, and following the absorbance change at 550 nm.

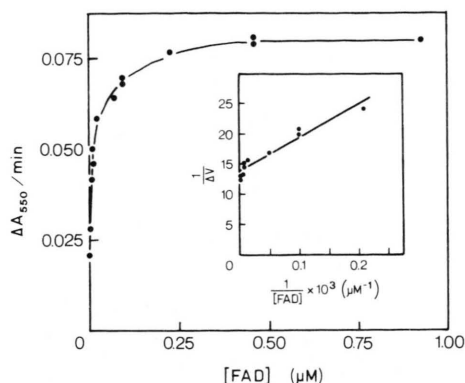


Fig. 5. Dependence of NADH-cytochrome *c* reductase activity of diluted enzyme on concentration of added FAD. 10 μ l samples of 9 nM native nitrate reductase were diluted 1:100 with Tris-HCl buffer, pH 7.5. FAD was added at the indicated concentrations, and the resulting solutions were incubated at 30 °C during 30 min. Then, NADH-cytochrome *c* reductase activity was measured by adding the reagents of the standard assay to the enzyme solutions, and adjusting the final volume until 1 ml. Activity is expressed on basis of the changes in absorbance at 550 nm per min. Inset, a Lineweaver-Burk plot of the obtained data.

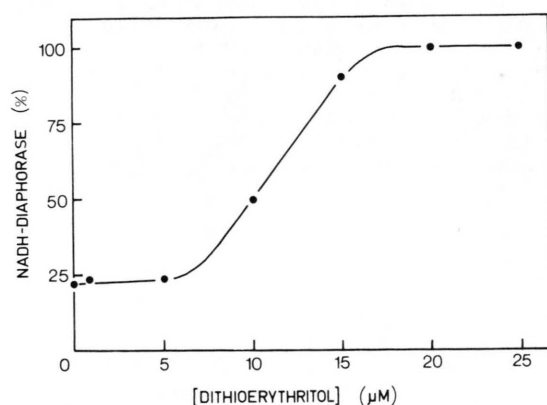


Fig. 6. Protection by dithioerythritol against inactivation of NADH-cytochrome *c* reductase of diluted enzyme. 10 μ l samples of 8 nM native nitrate reductase were diluted 1:100 with Tris-HCl buffer, pH 7.5. Dithioerythritol was added at the indicated concentrations, and the resulting solutions were incubated at 30 °C during 30 min. NADH-cytochrome *c* reductase activity was measured, after incubation, by adding the reagents of the standard assay to the enzyme solutions, and adjusting the final volume until 1 ml. Reaction rate was determined by following absorbance change at 550 nm.

Table II. Flavin fluorescence emission of nitrate reductase in the absence or presence of dithioerythritol.

Time [h]	Relative fluorescence [%]	
	– DTE	+DTE
0.25	39	33
1	48	39
2	50	40
5	64	45
24	100	64

A 2 ml sample containing 8 μ g of native nitrate reductase in 50 mM potassium phosphate buffer, pH 7.0, was incubated at room temperature in the dark, in the absence or presence of 1 mM dithioerythritol (DTE). In both cases, the fluorescence intensity at 535 nm (excitation at 450 nm) was registered at the indicated times.

Protection by dithioerythritol against inactivation of NADH-cytochrome c reductase activity by dilution.

The presence of dithioerythritol in a dilute solution of nitrate reductase (4 mU/ml of NADH-cytochrome *c* reductase activity), protected efficiently the diaphorase activity of the enzyme complex against its inactivation by incubation at 30 °C. This protection was maximal at 20 μ M of dithioerythritol or higher (Fig. 6).

Table II shows that the fluorescence emission at 535 nm (excitation at 450 nm) of a nitrate reductase solution incubated at room temperature in the dark, is increasing as a function of time. However, when the enzyme solution was previously supplemented with 1 mM dithioerythritol, the intensity of the flavin fluorescence was minor.

It may be concluded therefore that dithioerythritol protects NADH-cytochrome *c* reductase activity of nitrate reductase by preventing, or at least delaying, the dissociation of FAD from the protein.

Discussion

Nitrate reductase from *Ankistrodesmus braunii* requires the presence of FAD in the buffers in order to protect the associated NAD(P)H-dependent activities, either in crude extracts or in purified preparations [1]. By contrast, nitrate reductase from *Chlorella fusca* or leaves of higher plants did not exhibit the requirement of added FAD for catalytic activity of the diaphorase moiety, except when the enzyme preparations were subjected to gel filtration [8, 9]. In the case of *Chlorella vulgaris* nitrate reduc-

tase, the flavin prosthetic group remains bound to the enzyme even after a thorough purification procedure [10, 11].

In this paper, the absorption spectrum of FAD of nitrate reductase from *A. braunii*, which is dissociated from the protein by prolonged dialysis, is reported. The flavin component bound to the enzyme is essential for the electron transfer from reduced pyridine nucleotides to the heme chromophore; and exogenous FAD can restore neither full reduction by NAD(P)H of the heme in flavin-depleted nitrate reductase nor the NAD(P)H-dependent activities. By contrast, addition of FAD to purified nitrate reductase from fungi has been reported to stimulate significantly the reduction of the cytochrome by pyridine nucleotides [12, 13].

This variability seems to reflect a difference in the strength of binding of flavin to the enzyme, which is not very striking since significant differences have been also reported with respect to the molecular weight, quantitative analysis of prosthetic groups, and subunit composition of nitrate reductase from several organisms [2].

Fluorescence emission of protein-bound flavin nucleotides is usually infrequent, and so FAD fluorescence is quenched when bound to *A. braunii* nitrate reductase. However, there are some exceptions such as lipoyl dehydrogenase [14] and the flavoprotein ETF (electron transferring protein) from *Peptostreptococcus elsdenii* [15], whose flavin is fluorescent. On the other hand, the results of Fig. 3 show an increase of flavin fluorescence emission when nitrate reductase is incubated at room temperature, as well as a parallel decrease of NADH-nitrate reductase activity. These data are in agreement with those reported in other flavoproteins such as sulfite reductase [16], formate dehydrogenase [17], flavocytochrome b_2 [18], NADH-cytochrome b_5 reductase [19], lipoamide dehydrogenase [20], xanthine oxidase and xanthine dehydrogenase [21].

Dissociation of FAD from nitrate reductase, measured as loss in NADH-cytochrome c reductase activity, depends on the concentration of the enzyme solution during the incubation. A residual catalytic activity of about 20–25% is present even in high dilute solutions, which indicates that the enzyme retains some of its flavin, as proved by fluorometric studies. Added FAD completely protects NADH-cytochrome c reductase activity against inactivation by incubation of dilute enzyme solutions. Signifi-

cant protection however has been also observed by FMN and NADH, which suggests that these nucleotides may interact with nitrate reductase in the same FAD-binding site or near it. This is compatible with the idea that FAD serves as the entry port for electrons, from reduced pyridine nucleotides, into the nitrate reductase molecule.

The K_m for FAD as a protector of NADH-cytochrome c reductase activity was 4 nM, indicative of a high affinity of the enzyme for FAD. It is interesting to note here that the K_m value for the reduced flavin, FADH₂, as an electron donor for the terminal nitrate reductase activity of the complex, is very much higher: 300 μ M [4]. According to Faeder *et al.* [16], the K_m value for FMN-stimulation of NADPH-cytochrome c reductase activity of sulfite reductase was a good measurement of the dissociation constant for FMN from the enzyme. Thus, it is possible to think that the dissociation constant for FAD from *A. braunii* nitrate reductase may be similar to 4 nM.

In general, flavoproteins can dissociate its flavin, and then are inactive in catalyzing their respective reactions. The loss in activity is reversed in a few minutes by addition of flavin [16–18]. By contrast, FAD-depleted nitrate reductase is unable to recover its original activity upon incubation with exogenous FAD alone or in the presence of thiols. It can be argued that some modifications occur in the FAD-domain of the enzyme upon dissociation of its flavin which makes impossible the reassociation of FAD with the protein. Similar situation has been observed in flavin-free lipoamide dehydrogenase, which only recovers 30–40% of its original activity by incubation for several hours in the presence of FAD and dithioerythritol. Upon addition of FAD alone, 6% of the original activity is recovered [20].

It is important to remark the highly protective effect of dithioerythritol on the dissociation of FAD from nitrate reductase. At the moment, we have not a clear explanation for this effect, although the existence of sulphydryl groups in the FAD-domain (or at least near it) can be suggested. According with these observations, Márquez *et al.* have demonstrated that the FAD-domain of *A. braunii* nitrate reductase, which seems to be very similar to the dinucleotide fold for NAD(P)H, contains sulphydryl groups required for the binding of FAD (unpublished results). In this context, a close association of FAD with sulphydryl groups has been reported for fungal nitrate reductase [22].

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