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STUDIES BY AFFINITY CHROMATOGRAPHY ON THE NAD(P)H AND FAD SITES OF NITRATE REDUCTASE FROM *ANKISTRODESMUS BRAUNII**

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SUMMARY

Both native NAD(P)H-nitrate reductase (E.C. 1.6.6.2.) from *Ankistrodesmus braunii* and the FAD-depleted enzyme are adsorbed on blue dextran–Sepharose at two different sites. The binding of the native enzyme involves the NAD(P)H active site, while that of the deflavoenzyme uses the FAD site. The holoenzyme can be specifically eluted by 0.5 m*M* NAD(P)H, but the elution of the deflavoenzyme was achieved only by the simultaneous addition of 1 m*M* FAD and 0.5 *M* KCl to the washing buffer.

Incubation of native or flavin-free nitrate reductases with *p*-hydroxymercuribenzoate prevents adsorption of both types of enzyme on blue dextran–Sepharose, which indicates the presence of sulphydryl groups in the sites for NAD(P)H and FAD.

Binding studies of the holoenzyme on three different kinds of NAD-Agarose indicate that the NAD(P)H-domain is acting as a crevice in which the nicotinamide ring of the nucleotide should be placed at the bottom. In addition, the structure of the binding site for FAD seems to be similar to that for NAD(P)H.

INTRODUCTION

NAD(P)H-nitrate reductase (E.C. 1.6.6.2.) from the green alga *Ankistrodesmus braunii* catalyzes the assimilatory reduction of nitrate to nitrite using reduced pyridine nucleotides as electron donors. In addition, two enzymatic activities which participate sequentially in the transfer of electrons from NAD(P)H to nitrate are present in the enzyme complex; the first is an FAD-dependent NAD(P)H-diaphorase and the sec-

* Abbreviations: FAD = flavin–adenine dinucleotide; FMN = riboflavin 5′-phosphate; NAD = nicotinamide–adenine dinucleotide, oxidized; NADH = nicotinamide–adenine dinucleotide, reduced; NADP = nicotinamide–adenine dinucleotide phosphate, oxidized; NADPH = nicotinamide–adenine dinucleotide phosphate, reduced.

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ond is the molybdoprotein terminal nitrate reductase[1]. FAD-depleted nitrate reductase lacks all the NAD(P)H-dependent activities.[2]

Thompson *et al.*[3] have proposed that blue dextran forms complexes with a wide range of proteins because it is specific for a super-secondary structure called the "dinucleotide fold". This structure involves about 120 amino acids arranged in a β-sheet core composed of five or six parallel strands which are connected by α-helical intrastrand loops located above and below the β-sheet[4,5]. The dinucleotide fold is known to form the NAD- and ATP-binding sites of several enzymes, and to be present in the structure of others. Thus, blue dextran–Sepharose columns have been used in the final purification stages of several enzymes, which were displaced from the affinity columns by addition of low concentrations of their nucleotide substrates to the elution solvents[3].

Recently, affinity chromatography on blue dextran–Sepharose was used successfully in the purification of nitrate reductase from several organisms[6]. In particular, nitrate reductase from *A. braunii* has been purified to homogeneity by a simple method which includes affinity chromatography on blue dextran–Sepharose as the main step, the enzyme being specifically eluted from the column by addition of NADH to the washing buffer[7,8]. From these results, it was suggested that NAD(P)H-nitrate reductase from *A. braunii* also contains the dinucleotide fold in its NAD(P)H-domain[9].

Affinity chromatography on FAD-Sepharose has been employed for the purification of fungal nitrate reductase[10,11], since assimilatory nitrate reductase from eukaryotic organisms is an FAD-containing enzyme[6].

In this paper, we describe a study of the interaction of *A. braunii* nitrate reductase with blue dextran–Sepharose and with three different NAD-Agaroses, in order to obtain information about the nature of the NAD(P)H-binding site of the enzyme. We have found that the FAD-free enzyme is specifically adsorbed on blue dextran–Sepharose by the flavin-binding site, which may mean that the enzyme possesses a local FAD-domain structure similar to the dinucleotide fold.

MATERIALS AND METHODS

Materials

Tris, EDTA, FAD, *p*-hydroxymercuribenzoate, reactive blue dextran 2–Sepharose CL-6B and β-nicotinamide adenine dinucleotide–Agaroses (N-1008, N-6130 and N-9505) were purchased from Sigma (St. Louis, MO, U.S.A.). NADH and NADPH were obtained from Boehringer (Mannheim, G.F.R.), methyl viologen and dithioerythritol from Serva (Heidelberg, G.F.R.), alumina for disrupting cells from Alcoa (Arkansas, U.S.A.) and streptomycin sulphate was from Cía. Española de Penicilina (Spain).

Preparation of native and flavin-depleted nitrate reductases

Ankistrodesmus braunii strain 202-7c from Göttingen University's culture collection was grown as previously described[12]. The cells were harvested by low speed centrifugation and ground at 0°C with alumina in a mortar. The broken material was extracted with 10 m*M* potassium phosphate buffer, pH 7.0, containing 0.15 m*M* dithioerythritol, 0.10 m*M* EDTA and 20 μM FAD (buffer A) for native nitrate

reductase, and the same buffer without FAD (buffer B) for the flavin-free enzyme; 7 ml buffer per g, wet weight, of cells were added. The homogenate was centrifuged at 27,000 *g* for 15 min, and the supernatant was used as the crude extract.

A solution of 0.1 *M* streptomycin sulphate, pH 7.0, was added dropwise to the crude extract (1 ml per 10 ml extract). After 10 min at 0°C with continuous stirring, the suspension was centrifuged at 27,000 g for 15 min, and the resulting supernatant was used for affinity chromatography.

Affinity chromatography

Blue dextran–Sepharose and NAD-Agaroses were packed in columns of 65 × 7.5 mm and 40 × 5 mm, respectively. Before use, the column beds were washed with ten volumes of 2 *M* KCl and then equilibrated with twenty volumes of the starting buffer. A constant flow-rate of 12 ml/h was used for the blue dextran–Sepharose column, and 6 ml/h for the NAD-Agarose ones.

Enzyme activity

NADH- and reduced methyl viologen-nitrate reductase activities were determined colorimetrically by measuring the nitrite formation. The reaction mixture contained (in a final volume of 1 ml) 0.1 *M* Tris–HCl buffer, pH 7.5, 10 m*M* potassium nitrate, a donor of electrons and an adequate amount of enzyme. The donors were present at the following concentrations: NADH, 0.3 m*M*; or sodium dithionite, 4.6 m*M*, plus methyl viologen, 0.15 m*M*. The reaction was started by addition of the enzyme and stopped after 5 min by rapid oxidation of the electron donor system in a Vortex mixer.

Analytical methods

Protein was estimated by the method of Bailey[13], with bovine serum albumin as standard. The absorbance of standards at 279 nm was measured to determine their concentration ($\varepsilon^{1\%}_{279\,nm}$ = 0.067 g^{-1} ml cm^{-1}). Nitrite was estimated as described by Snell and Snell[14].

RESULTS AND DISCUSSION

Behaviour of native nitrate reductase and flavin-free enzyme on blue dextran–Sepharose

A phosphate buffer of low ionic strength and pH 7.0, supplemented with 0.15 m*M* dithioerythritol, 0.10 m*M* EDTA and 20 μM FAD (buffer A), was used for optimum adsorption of native nitrate reductase on blue dextran–Sepharose[9]. The enzyme retained in the column was eluted by addition of NADH to the eluting buffer (Fig. 1A). NADPH was also able to promote the elution of nitrate reductase from the affinity column, although it was less efficient than NADH. FAD-free nitrate reductase in buffer B (without FAD) was also adsorbed on blue dextran–Sepharose, but in this case NADH alone (Fig. 1B) or in the presence of FAD (Fig. 1C) was unable to elute the enzyme. In all cases, 3 *M* KCl promoted the elution of both holoenzyme and deflavoenzyme.

It can be deduced, therefore, that native nitrate reductase from *A. braunii* is adsorbed on blue dextran–Sepharose via its NAD(P)H-domain. Because the Michaelis constant (K_M) of the enzyme for NADH and NADPH are 13 and 23 μM respectively[1], it is logical that NADH has a higher efficiency than NADPH as eluent.

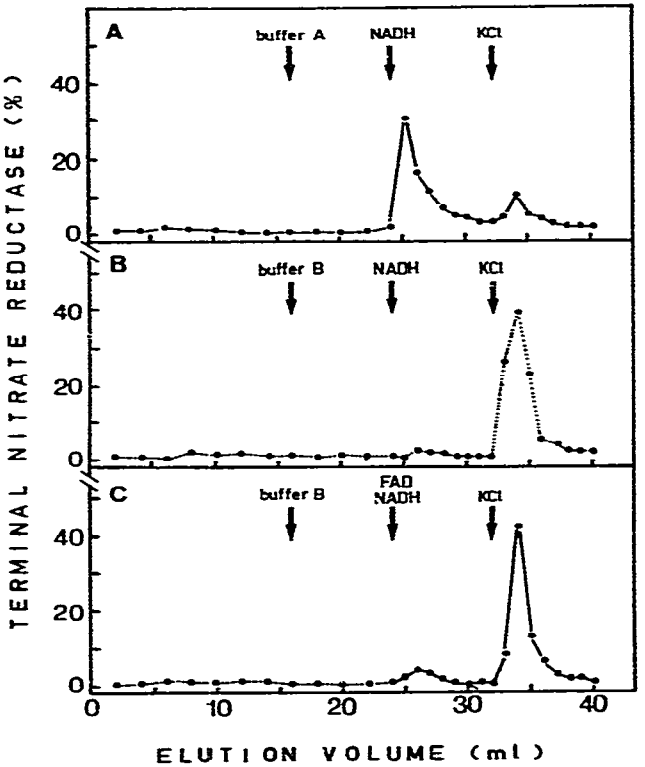

Fig. 1. Elution profiles of nitrate reductase adsorbed on blue dextran–Sepharose in the absence or presence of FAD. Three nitrate reductase preparations were obtained, as described in Materials and methods, from 2 g of cells. The native enzyme was in buffer A (———), while the flavin-depleted nitrate reductase was prepared with buffer B (– – –). The enzyme solutions were separately applied to a blue dextran-Sepharose column previously equilibrated with the corresponding buffer. After adsorption, the column bed was washed with the same buffer, which was supplemented as indicated with 0.5 m*M* NADH, 1 m*M* FAD or 3 *M* KCl. 1-ml Fractions were collected, and reduced methyl viologen-nitrate reductase activity was measured by adding 0.1-ml aliquots of each fraction to the reagents of the standard assay. 100% activity corresponds to the sum of activities of the pool of fractions collected in each case.

On the other hand, the adsorption of FAD-free nitrate reductase on blue dextran–Sepharose seems proceed through its FAD-binding site, probably due to the higher affinity of the enzyme for FAD (K_M = 4 n*M*)[2] than that for NADH.

Both types of interaction of nitrate reductase with blue dextran–Sepharose possess ionic character, because the elution from the affinity column can be carried out in both cases by increasing the ionic strength of the buffer. Moreover, the results shown in Fig. 2 indicate that FAD-depleted nitrate reductase is more strongly bound to blue dextran–Sepharose than the holoprotein, since a lower ionic strength was necessary to elute the latter.

Similar results have recently been reported for NADH-cytochrome b_5 reductase, which is an FAD-requiring enzyme[15]. Holoenzyme is adsorbed on blue dextran–Sepharose at low ionic strength and is eluted by NADH, while the flavin-free enzyme is more strongly bound and is eluted by the simultaneous addition of FAD and NADH

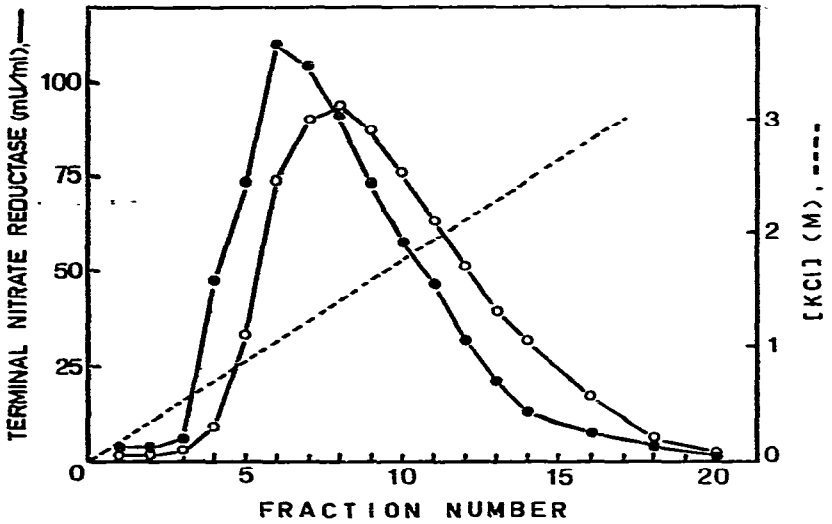

Fig. 2. Elution with a linear gradient of KCl of native nitrate reductase and FAD-free enzyme from a blue dextran–Sepharose column. A nitrate reductase preparation obtained from 2 g of cells with buffer A was applied to a blue dextran–Sepharose column. After a brief washing with buffer A, the elution was carried out with a linear gradient of 0–3 *M* KCl in the same buffer. 1-ml Fractions were collected, and reduced methyl viologen-nitrate reductase activity (●) was determined by adding 0.1 ml of each fraction to the reagents of the standard assay. A similar experiment was repeated under identical conditions, but using buffer B throughout the procedure. In this case, the terminal nitrate reductase activity is represented by open circles (○).

to the eluting buffer. Separately, the cofactors have no effect. It has been also reported that Cibacron blue and blue dextran–Sepharose bind to the FMN-binding site of flavocytochrome b_2, and the authors[16] suggested that the enzyme possesses a local flavin-binding structure similar to the dinucleotide fold.

Elution by FAD of flavin-depleted nitrate reductase adsorbed on blue dextran–Sepharose

When a preparation of nitrate reductase free from FAD was adsorbed on a blue dextran–Sepharose column, the enzyme could be eluted by the simultaneous addition of 1 m*M* FAD and 0.5 *M* KCl to the washing buffer (Fig. 3). Separately, FAD or KCl was unable to elute the enzyme. These results are compatible with the idea that flavin-depleted nitrate reductase is specifically retained by blue dextran–Sepharose through its flavin-domain, forming a tight complex with the dye ligand. Apparently, it is necessary to weaken the bond between deflavoenzyme and blue dextran–Sepharose by increasing the ionic strength so that FAD can elute the apoenzyme. It is of interest that FAD by itself did not elute flavin-free cytochrome b_5 reductase from a blue dextran–Sepharose column[15], while an FMN gradient displaced the FMN-depleted flavocytochrome b_2 (ref. 16).

Effect of NADH and p-hydroxymercuribenzoate on adsorption of nitrate reductase on blue dextran–Sepharose

When native nitrate reductase was incubated with 1 m*M* NADH for 10 min at

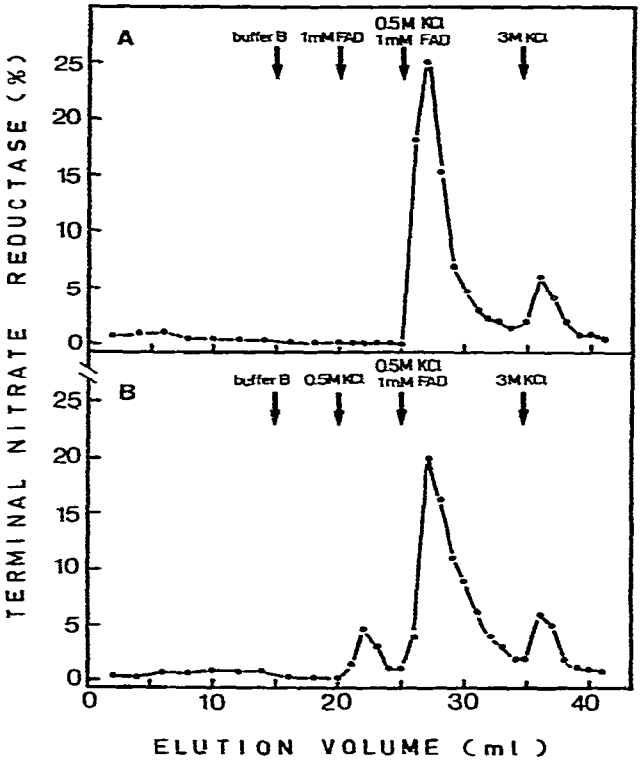

Fig. 3. Elution profile with FAD plus KCl of flavin-depleted nitrate reductase adsorbed on blue dextran–Sepharose. Two nitrate reductase preparations, each from 2 g of cells, were obtained with buffer B as described in Materials and methods. After adsorption on blue dextran–Sepharose, the column bed was washed with buffer B, and then 1 m*M* FAD, 0.5 *M* KCl or 3 *M* KCl was added to the washing buffer as indicated by arrows. 1-ml Fractions were collected and the reduced methyl viologen-nitrate reductase activity was measured by adding 0.1 ml of each fraction to the reagents of the standard assay. 100% activity corresponds to the sum of activities of pooled fractions collected in each case.

0°C, before application to a blue dextran–Sepharose column, the enzyme was not retained by the affinity column (not shown). This may be explained by the fact that reduced pyridine nucleotide binds to the enzyme through its active site for NAD(P)H and, therefore, the complex NADH–holoenzyme cannot be adsorbed on blue dextran–Sepharose. Similar results were obtained when FAD-free nitrate reductase was incubated with 1 m*M* NADH before chromatography. This behaviour can be understood if the structure of the flavin-domain in nitrate reductase is similar to that of the NAD(P)H, *i.e.*, both sites fit the dinucleotide fold, and NADH is also able to bind to the enzyme through the flavin-binding site. Support for this idea is provided by the fact that FAD may be substituted by NADH in the protection of the NADH-diaphorase activity of nitrate reductase against inactivation in dilute solutions[2].

When nitrate reductase in buffer A without dithioerythritol was incubated at 0°C for 10 min with 5 μM *p*-hydroxymercuribenzoate, a typical sulphydryl groups reagent, before application of the enzyme solution to a blue dextran–Sepharose column, only 37% of nitrate reductase was adsorbed on the column (Table I). Like-

wise, if FAD-depleted enzyme was first incubated in buffer B under the same conditions, only 40% of the apoenzyme applied to the column was retained (Table I). These results confirm the similarity of the two dinucleotide-binding sites of nitrate reductase, and indicate that the presence of –SH groups is essential for the binding of NAD(P)H and FAD to the enzyme.

TABLE I

EFFECT OF *p*-HYDROXYMERCURIBENZOATE ON THE ADSORPTION OF NITRATE REDUCTASE ON BLUE DEXTRAN–SEPHAROSE

Native and flavin-depleted nitrate reductases were prepared from 1 g, wet weight, of cells, as described in Materials and methods, except that the phosphate buffers did not contain dithioerythritol. The enzyme preparations were incubated alone or with 5 μM *p*-hydroxymercuribenzoate (*p*HMB) at 0°C for 10 min. They were separately applied to a blue dextran–Sepharose column, and the amount of enzyme retained in the column was determined after elution with 3 *M* KCl.

Preincubation system	*Nitrate reductase adsorbed (%)*	
	Native	*Flavin-depleted*
Enzyme	95	93
Enzyme + *p*HMB	37	40

It has previously been suggested that free sulphydryl groups of nitrate reductase from eukaryotic organisms participate in the binding of NAD(P)H to the enzyme[17,18]. Similar results have been reported for the enzyme from *A. braunii*[1,7]. In addition, an active participation of –SH groups in the electron flow from NAD(P)H to FAD has been reported for nitrate reductase from *Neurospora crassa*[19].

From the above data it can be deduced that sulphydryl groups are also required for the binding of FAD. It is obvious that multiple interactions between flavin and protein are involved in the binding. Thus, Choi and McCormick[20] suggest that the interaction of flavin with hen egg white riboflavin-binding protein is mainly due to hydrophobic interaction of the flavin ring system with non-polar groups of the protein, and hydrogen bonding of the hydroxyl groups of the flavin. Yubisui and Takeshita[21] have reported that tryptophan and tyrosine seem to participate in the binding of FAD to NADH-cytochrome b_5 reductase. In all cases, the isoalloxazine ring seems to be placed in the crevice of the flavin-domain of flavoproteins[15,20,22,23].

Adsorption of nitrate reductase on three different NAD-Agaroses

Three different NAD-Agaroses were used to study the geometrical orientation of reduced pyridine nucleotides when bound to nitrate reductase. The Agaroses contained β-nicotinamide adenine dinucleotide covalently bound through: (1) C-8 of adenine, with a carbon chain of six carbons between the NAD and the Agarose matrix (N-1008); (2) ribose hydroxyls, with a carbon chain of six carbons between the NAD and the Agarose matrix (N-6130) and (3) N^6 of adenine, with a spacer of eight carbons (N-9505). The code numbers for the NAD-Agarose are those of Sigma.

The results shown in Fig. 4 indicate that nitrate reductase is adsorbed on NAD-Agarose only when pyridine nucleotide is attached through adenine (N-1008 and N-9505). When NAD is bound through ribose hydroxyls to Agarose (N-6130), no adsorption of nitrate reductase is observed (Fig. 4, centre). It seems that the

NAD(P)H-binding site is acting as a crevice in the bottom of which the nicotinamide ring of the pyridine nucleotide would be placed. Therefore, when pyridine nucleotide is bound to Agarose through adenine, the other end of the molecule, *i.e.*, nicotinamide, may enter to the bottom of the NAD(P)H-domain. When the NAD-Agarose bond is formed through ribose hydroxyls, the molecule of pyridine nucleotide lies across the crevice and cannot bind to the enzyme.

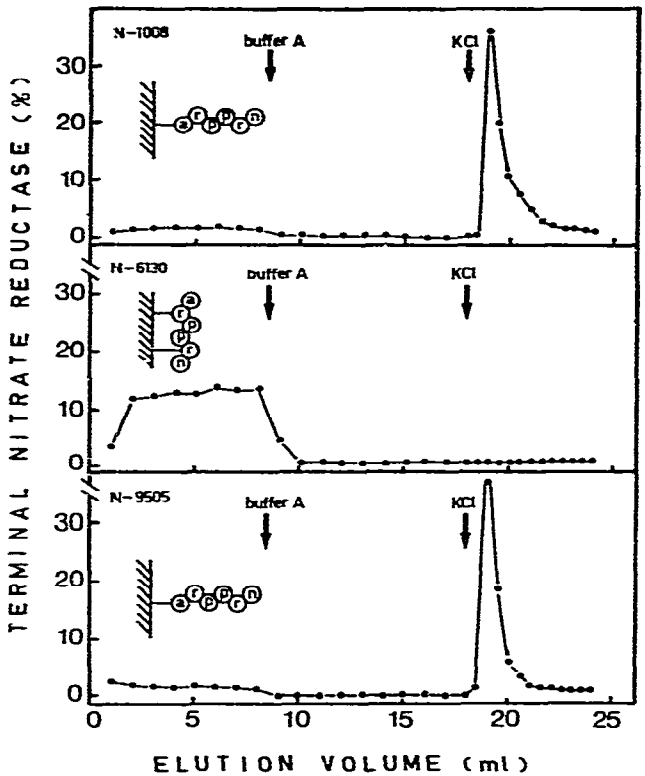

Fig. 4. Elution profile of native nitrate reductase adsorbed on three different NAD-Agaroses. Three nitrate reductase preparations, each from 1 g of cells, were obtained with buffer A as described in Materials and methods. Each enzyme solution was applied to the NAD-Agarose columns, which differed in the bonding between pyridine nucleotide and Agarose (see text). The columns were then washed with buffer A and eluted with 2 *M* KCl in the same buffer. 0.5-ml Fractions were collected, and the reduced methyl viologen-nitrate reductase activity was measured by adding 0.1 ml of each fraction to the reagents of the standard assay. 100% activity corresponds to the sum of activities of the pooled fractions collected in each case. The type of NAD-Agarose used is indicated by its code number. Note that N-1008 and N-9505 differ by the spacer (6 and 8 carbons, respectively). a = Adenine; r = D-ribose; P = phosphate and n = nicotinamide.

In conclusion, when NAD(P)H and FAD are accommodated in their respective domains in nitrate reductase, nicotinamide and isoalloxazine would be placed at the bottom of the crevices. Then, rotation of the molecule would allow the nicotinamide to take up a position where it makes contact with the isoalloxazine ring of flavin, and the electron transfer takes place between both nucleotides.

Schulz *et al.*[23] have elucidated the three-dimensional structure of the flavoenzyme glutathione reductase, which binds two dinucleotides: FAD and NADPH. They

observed that the NADPH- and FAD-domains are very similar and resemble other nucleotide-binding proteins with respect to chain fold and binding mode. Thus, the same general dinucleotide-domain pattern has been observed in the structurally known nucleotide-binding proteins[5,24]. This evolutionary relationship between the two domains indicates that a primordial gene duplication accompanied or followed by gene splicing is likely[23].

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