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EDITED BY

G. H. BOURNE J. F. DANIELLI

ASSISTANT EDITOR

K. W. JEON

VOLUME 92

Nuclear Genetics

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Contents

Chromosome and DNA-Mediated Gene Transfer in Cultured Mammalian Cells

A. J. R. DE JONGE AND D. BOOTSMA

DNA Methylation in Eukaryotic Cells

AHARON RAZIN AND HOWARD CEDAR

INTERNATIONAL REVIEW OF CYTOLOGY, VOL. 92

Nitrate Assimilation in Eukaryotic Cells

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I. Introduction

Nitrate is the predominant form of combined nitrogen available within our oxidative environment, and its assimilation is achieved through its biological reduction to ammonium. The subsequent utilization of ammonium to form amino

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and amido-N compounds provides the link between the plethora of pathways of organic nitrogen metabolism and the pathways of inorganic nitrogen assimilation of which there are only two: nitrate assimilation and dinitrogen (N_2) fixation. Losada and colleagues (Losada *et al.*, 1981; Guerrero *et al.*, 1981) have calculated, on the basis of relative carbon and nitrogen content of plants and the levels of CO_2 fixed by plants, that roughly 2×10^4 megatons of inorganic nitrogen are assimilated annually. Since Burns and Hardy (1975) estimated that the overall chemical and biological fixation of dinitrogen was 2×10^2 megatons, it follows that nitrate assimilation exceeds nitrogen fixation by over 100-fold. Thus, the preponderance of nitrogen acquisition by the biosphere occurs via nitrate assimilation. Its significance to agriculture is enormous.

The capability to assimilate nitrate is possessed by certain bacteria, some fungi, and virtually all algae and higher plants. It is absent from the animal kingdom. Nitrate assimilation represents a substantial energy expenditure by the cell when compared with ammonium utilization since eight reducing equivalents are consumed in the reduction of nitrate to ammonium. Consequently, cells which assimilate nitrate regulate this pathway to avoid wasteful use of reducing power when the end product, ammonium, is available. The form of regulation adopted varies in accordance with the metabolic pattern and status of the cell type but the fundamental purpose of the regulation is the same: to effect an economy of existence. For example, photosynthetic tissues assimilating nitrate show a rapid biochemical inactivation of this process when ammonium is presented. On the other hand, more rapidly proliferating fungal cells regulate through nitrate assimilation-specific gene expression.

This review addresses the biochemistry, genetics, and regulation of nitrate assimilation in eukaryotic cells; particular emphasis is placed on the genetics of nitrate assimilation with an attentive eye to emerging molecular biological studies. Nitrate assimilation both invites the investigation of molecular biologists wishing to understand the regulation of metabolic potentialities in eukaryotic cells and intices manipulation by genetic engineers seeking to enhance plant productivity through application of recombinant DNA technology. Neither group has yet met fulfillment but the opportunities for both seem most promising.

II. Biochemical Aspects of Nitrate Assimilation

As indicated, the assimilation of nitrate is achieved through the eight-electron reduction of this oxidized inorganic anion, resulting in the formation of ammonium. This transformation requires two enzymatic steps, the two-electron reduction of nitrate to nitrite followed by the six-electron reduction of nitrite to ammonium:

$$NO_3^- \xrightarrow[\text{nitrate reductase}]{2e^-} NO_2^- \xrightarrow[\text{nitrite reductase}]{6e^-} NH_4^+$$

The enzymes mediating these steps are nitrate reductase (EC 1.6.6.1-3) and nitrite reductase (EC 1.6.6.4 and 1.7.7.1), respectively.

A. General Features of Assimilatory Nitrate Reductases

The biochemical aspects of assimilatory nitrate reductases have been extensively reviewed recently (Garrett and Amy, 1978; Hewitt and Notton, 1980; Beevers and Hageman, 1980; Guerrero *et al.*, 1981; Vennesland and Guerrero, 1979; Losada *et al.*, 1981). This section will initially focus on features shared by all assimilatory nitrate reductases and subsequently detail unique characteristics of the enzyme from algal, fungal, and higher plant sources.

Assimilatory nitrate reductases are soluble, electron-transferring proteins, 200,000–300,000 in molecular weight. Electron transfer is generally regarded to be mediated by enzyme-bound heme iron, flavin adenine dinucleotide (FAD), and molybdenum cofactor. These components function as electron carriers between the physically separated pyridine nucleotide oxidation site and nitrate reduction site (Campbell and Smarrelli, 1978, 1983). The physiological activity involves the reduction of nitrate to nitrite occurring at the molybdenum site, with reducing power generated by pyridine nucleotides donated to the catalyst probably via a specific enzyme sulfhydryl group (Amy *et al.*, 1977). In addition to this physiological activity, apparent nonphysiological activities can be shown *in vitro*. The first is termed a dehydrogenase (diaphorase) activity in which nitrate reductase can mediate the pyridine nucleotide-linked reduction of one- or two-electron acceptors such as ferricyanide, cytochrome *c*, or dichlorophenolindophenol. The molybdenum moiety is not involved in these reactions. The second type of activity involves the reduction of nitrate with reducing power generated either by reduced flavins or viologen dyes. The pyridine nucleotide reduction site is not involved in this reductase activity and electrons are added probably to either heme or molybdenum cofactor.

The study of these partial activities has provided valuable insights into the overall nature of the enzyme since the characteristic response of these activities reflect intrinsic properties of the nitrate reductase. For example, these reactions have been found to be differentially inhibited. Sulfhydryl binding agents such as *p*-hydroxymercuribenzoate inhibit both the pyridine nucleotide-linked nitrate reductase and dehydrogenase reactions (Garrett and Nason, 1969; Schrader *et al.*, 1968). Metal-binding agents such as cyanide inhibit only the reactions involving the molybdenum moiety, while having no influence over the dehydrogenase activity of nitrate reductase (Garrett and Nason, 1969; Hewitt and Notton, 1980).

Further, polyvalent monospecific antisera against nitrate reductase differentially inhibit the partial activities (Amy and Garrett, 1979; Funkhouser and Ramadoss, 1980; Smarrelli and Campbell, 1981). From these and similar studies, the general features of the typical assimilatory nitrate reductase have been revealed, as depicted in the following scheme:

$$\text{NADPH} \rightarrow [\text{FAD} \rightarrow \text{cytochrome-}b_{557} \rightarrow (\text{Mo})] \rightarrow \begin{matrix} \nearrow \text{MVH} \\ \text{NO}_3^- \\ \searrow \text{cytochrome } c,\ \text{Fe}_3(\text{CN})_6^- \end{matrix}$$

Additional information and analogy provides a more complete description of overall electron flow from the binding and oxidation of reduced pyridine nucleotide to the terminal reduction of nitrate to nitrite. Pyridine nucleotides are thought to bind to a supersecondary structure of the enzyme called the dinucleotide fold (Solomonson, 1975). Dinucleotide folds are typically composed of about 120 amino acid residues in five or six parallel strands to form a β-sheet core, the strands being connected by α-helical intrastrand loops located above and below the β-sheet (Rossman *et al.*, 1974). Following A-side oxidation of the reduced pyridine nucleotide (Guerrero *et al.*, 1977), the flavin region of the enzyme becomes reduced. Flavin adenine dinucleotide appears noncovalently bound to nitrate reductase. The FAD is easily dissociable from the enzyme from *Neurospora crassa* (Garrett and Nason, 1967), while showing very tight binding in other assimilatory nitrate reductases. Amy *et al.* (1977) described an important sulfhydryl group which apparently mediates electron transfer between NADPH and FAD. From FAD, electrons are subsequently shuttled to a *b*-type cytochrome. First identified by Garrett and Nason (1967) for the enzyme from *N. crassa*, and subsequently confirmed for other nitrate reductases (Solomonson *et al.*, 1975; Guerrero and Gutierrez, 1977; Notton *et al.*, 1977; De la Rosa *et al.*, 1981; Minagawa and Yoshimoto, 1982), it has been termed cytochrome b_{557} since it displays an α-peak at 557 nm in a reduced versus oxidized difference spectrum.

The terminal electron acceptor of the nitrate reductase protein is thought to be the molybdenum cofactor. Molybdenum cofactor was once regarded as a proteinaceous component (Nason *et al.*, 1970). However, more recent studies reveal that it is most likely a low-molecular-weight, molybdenum-binding, urothione-like molecule termed molybdopterin (Johnson *et al.*, 1980; Johnson and Rajagopalan, 1982). The actual oxidation states of molybdenum during nitrate reduction have not been established unambiguously (Jacob and Orme-Johnson, 1980).

Although many of the biochemical features of assimilatory nitrate reductases appear well established, the actual structure and role of the components involved in electron flow are only poorly understood. Elucidation of these aspects, however, involves the use of large quantities of protein, a formidable task given the low

cellular concentration and relative instability of all nitrate reductases. The low cellular concentration of this enzyme is attributed to its remarkable catalytic efficiency; its turnover number or molecular activity is 18,000 (as a point of comparison, the molecular activity of succinate dehydrogenase is 1200) (Garrett and Amy, 1978).

B. Fungal Nitrate Reductases

The best characterized fungal nitrate reductases are those derived from *Neurospora crassa* (Garrett and Nason, 1969; Pan and Nason, 1978; Horner, 1983), *Penicillium chrysogenum* (Renosto *et al.*, 1981), *Rhodotorula glutinis* (Guerrero and Gutierrez, 1977), and *Aspergillus nidulans* (Minagawa and Yoshimoto, 1982). Table I summarizes several of the important physical properties of these enzymes. With the exception of the enzyme from *A. nidulans* (see also Downey and Steiner, 1979), all appear to be homodimers, with molecular weights in excess of 200,000. All are specific for NADPH as pyridine nucleotide electron donor, display pH optima of approximately 7.5, and possess an easily dissociable FAD. All apparently homogeneous enzyme preparations except that from *A. nidulans* display specific activities greater than 100 units/mg protein (1 unit being 1 μmol of nitrate reduced per minute). The best preparations of these fungal nitrate reductases were obtained using affinity chromatography (FAD-

TABLE I
Fungal Nitrate Reductases and Higher Plant NADH-Nitrate Reductases

Source	Native molecular weight ($\times 10^3$)	Subunit molecular weight ($\times 10^3$)	Reference
Fungi			
N. crassa	230	115	Garrett and Nason (1969); Pan and Nason (1978)
N. crassa	290	145	Horner (1983)
P. chrysogenum	200	100	Renosto *et al.* (1981)
R. glutinis	230	118	Guerrero and Gutierrez (1977)
A. nidulans	180	59 + 38	Minagawa and Yoshimoto (1982)
A. nidulans	—	90	Tomsett (1983, personal communication)
Higher plants			
Squash	230	115	Redinbaugh and Campbell (1983)
Barley	220	110	Kuo *et al.* (1982)
Tobacco	220	110	Mendel and Müller (1980)
Spinach	190	120 (major)	Notton and Hewitt (1979)

Sepharose or Blue Dextran-Sepharose) in combination with conventional techniques.

C. Algal Nitrate Reductases

The best characterized algal nitrate reductases are those from *Chlorella vulgaris* (Solomonson *et al.*, 1974, 1975; Giri and Ramadoss, 1979; Howard and Solomonson, 1982) and *Ankistrodesmus braunii* (De la Rosa, 1981). However, the literature reveals discrepancies regarding the subunit composition of the enzyme from *Chlorella*. Initial reports (Solomonson *et al.*, 1975) described the *Chlorella vulgaris* nitrate reductase as a trimer with a native molecular weight of 356,000. Later studies (Giri and Ramadoss, 1979) concluded the enzyme to be a trimer of molecular weight 280,000. The most recent data (Howard and Solomonson, 1982) conclude that the *Chlorella* nitrate reductase is a homotetramer with a molecular weight of 360,000. The nitrate reductase from *Ankistrodesmus braunii* has been described as an octamer with a molecular weight of 370,000. In other significant features, pH optima, pyridine nucleotide specificity, prosthetic group involvement, and molecular activity, these two representative green algal nitrate reductases are *not* significantly different from each other or from fungal nitrate reductases. FAD is not easily dissociable from either algal enzyme, in contrast to the situation found in fungal nitrate reductases.

D. Higher Plant Nitrate Reductases

Immunological data suggest a degree of similarity between algal, fungal, and plant enzymes (Smarrelli and Campbell, 1981), but structural and catalytic diversity of the enzyme exists even within a single plant. However, the basis of this diversity is obscured by the difficulty in obtaining a protein unmodified by the purification procedures. Thus, discerning intrinsic differences is problematical. Well characterized enzyme has been obtained from corn, barley, wheat, spinach, squash, and tobacco (Campbell and Smarrelli, 1978; Redinbaugh and Campbell, 1983; Kuo *et al.*, 1982; Sherrard and Dalling, 1979; Notton *et al.*, 1977; Mendel and Müller, 1980). There is not only a diversity of structure between the well characterized leaf nitrate reductases, but also different enzymes in the same tissue (e.g., soybean leaves) and tissue-specific enzymes. Typically these enzymes have been distinguished with some reliability by their pyridine nucleotide specificity (e.g., corn leaves and roots). The majority of leaf nitrate reductases are highly specific for NADH. The physical properties of representatives of this group are also shown in Table I. This group of higher plant enzymes can thus be characterized as homodimeric with subunit molecular weights of approximately 115,000. All leaves thus far examined apparently contain this type of nitrate reductase, except those from *Erythrina senegalensis* (Stewart and Orebamjo,

1979). This tropical legume contains an enzyme which can accept electrons from either NADH or NADPH.

The bispecific NAD(P)H–nitrate reductase has been described in three general cases. First, it can accompany the typical NADH–nitrate reductase in the same tissue. This pattern was found in soybean leaves and cotyledons (Jolly *et al.*, 1976; Orihuel-Iranzo and Campbell, 1980). In rice seedlings, the two enzymes respond to different induction conditions (Shen *et al.*, 1976). Despite their pyridine nucleotide specificity differences, the presence of two nitrate reductases in one tissue has not yet been adequately explained. The second case of the bispecific nitrate reductase was reported for corn roots and scutella (Redinbaugh and Campbell, 1981; Campbell, 1978). Since corn leaves contain only the typical NADH–nitrate reductase, there appears to be tissue-specific control over synthesis of the bispecific enzyme. The third case is the synthesis of the bispecific enzyme in response to mutation of the NADH–nitrate reductase (Dailey *et al.*, 1982a). Mutant barley seedlings which lack NADH–nitrate reductase activity (*nar*-1a) have been found to contain a bispecific nitrate reductase which is absent from normal tissue. Thus, multiple genes for nitrate reductases apparently exist in higher plants.

The total purification of higher plant nitrate reductase has required the application of affinity chromatography, the most prevalent matrix being blue-Sepharose. Rapid purification is essential since numerous higher plant proteases have been identified (Hageman and Reed, 1979). Proteinase inhibitors have been somewhat effective with leupeptin being the best inhibitor (Wray and Kirk, 1981).

E. Assimilatory Nitrite Reductases

Nitrite reductase, the second enzyme of the nitrate assimilatory pathway, catalyzes the six-electron reduction of nitrite to ammonium. The reduction proceeds as follows, apparently with no intermediates released in the reaction

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O$$

Although catalyzing the same reaction, the nitrite reductases from photosynthetic organisms (EC 1.7.7.1) are significantly different from those from nonphotosynthetic sources (EC 1.6.6.4), particularly in molecular weight and electron donor specificity. Nitrite reductases from several photosynthetic sources, including *Chlorella fusca*, spinach, squash, and *Porphyra yezoensis*, have been purified to homogeneity (Vennesland and Guerrero, 1979, and references therein). The nitrite reductase from the fungus *N. crassa* also has been purified to homogeneity (Greenbaum *et al.*, 1978; Prodouz and Garrett, 1981). The two best characterized nitrite reductases have been obtained from spinach leaves (Vega and Kamin, 1977; Lancaster *et al.*, 1979) and *N. crassa* (Garrett, 1978; Prodouz

and Garrett, 1981) and will be discussed as representative examples to compare nitrite reductases from photosynthetic and nonphotosynthetic organisms.

Assimilatory nitrite reductases have in common the possession of iron–sulfur center and siroheme prosthetic groups. The iron–sulfur center in spinach nitrite reductase is organized as a tetranuclear Fe_4S_4 cluster (Lancaster *et al.*, 1979); the *Neurospora* nitrite reductase is thought to contain two such Fe_4S_4 centers (Prodouz and Garrett, 1981). Siroheme is an iron tetrahydroporphyrin of the isobacteriochlorin type, containing eight carboxyl groups and having two adjacent pyrole rings reduced (Scott *et al.*, 1978). Siroheme is also found in assimilatory sulfite reductase (Siegel *et al.*, 1974). The siroheme function likely serves as the site of binding and reduction of nitrite in *Neurospora* (Vega *et al.*, 1975; Garrett, 1978) and spinach (Vega and Kamin, 1977). Siegel's laboratory (Wilkerson *et al.*, 1983) has evidence from Mössbauer spectroscopic studies that the Fe_4S_4 cluster and the siroheme of spinach nitrite reductase undergo exchange interactions, indicating that these two centers are chemically linked. It thus seems probable that the nitrite-reducing center of assimilatory nitrite reductases is the chemically coupled Fe_4S_4/siroheme prosthetic pair:

$$6e^- \rightarrow [Fe_4S_4/\text{siroheme}] \begin{cases} NO_2^- + 8H^+ \\ \hookrightarrow NH_4^+ + 2H_2O \end{cases}$$

This basic nitrite-reducing unit is found in association with a 61,000-MW protein in photosynthetic organisms where ferredoxin serves as the electron donor (Vega and Karmin, 1977). In contrast, the enzyme from nonphotosynthetic organisms as typified by *Neurospora crassa* is a 290,000-MW homodimeric flavoprotein of 140,000-MW subunits which utilizes reduced pyridine nucleotide (either NADPH or NADH) as electron donor (Lafferty and Garrett, 1974; Greenbaum *et al.*, 1978; Prodouz and Garrett, 1981). Thus, the following electron transfer schemes aptly represent the nitrite reductases of photosynthetic and nonphotosynthetic cells:

Spinach: $6\ Fd_{red} \rightarrow [Fe_4S_4/\text{siroheme}] \rightarrow NO_2^-$

Neurospora: $3NAD(P)H \rightarrow [FAD \rightarrow Fe_4S_4/\text{siroheme}] \rightarrow NO_2^-$

It is interesting to note that nonphotosynthetic cells apparently require a relatively larger and more complex nitrite reductase in order to utilize the reduced pyridine nucleotides as electron donors since they lack ferrodoxin and the photochemical means to reduce it (Garrett, 1978). The comparative properties of spinach and *Neurospora* nitrite reductase are summarized in Table II. Both classes of enzyme are also inhibited by sulfhydryl agents such as pHMB, the anions CN^- and sulfite which are substrate analogs, and CO which binds avidly to nitrite reductase in which the siroheme moiety is reduced (Greenbaum *et al.*, 1978).

The *Neurospora* nitrite reductase, like the nitrate reductase in this organism,

TABLE II
COMPARATIVE PROPERTIES OF ASSIMILATORY NITRITE REDUCTASES

Property	Spinach[a]	*N. crassa*[b]
Molecular weight	61,000	290,000
Subunits	1	2 (α_2-type homodimer)
Subunit molecular weight	61,000	140,000
Electron donor	Ferredoxin	NAD(P)H
Flavoprotein	No	Yes (FAD)
Iron–sulfur clusters	One Fe_4S_4	Two Fe_4S_4
Siroheme	Yes	Yes
Specific activity (μmol NO_2^- reduced/mg protein)	108	27

[a]Vega and Kamin (1977) and Lancaster *et al.* (1979).
[b]Greenbaum *et al.* (1978) and Prodouz and Garrett (1981).

has the capacity to catalyze a number of partial electron transfer activities *in vitro*. These include a FAD-dependent NAD(P)H–diaphorase activity for which cytochrome *c*, ferricyanide, or dichlorophenol indophenol serve as electron acceptor, and a FAD-independent dithionite-nitrite reductase activity (Lafferty and Garrett, 1974; Vega, 1976). Further this enzyme can catalyze the two-electron reduction of hydroxylamine to ammonium in a reaction using NAD(P)H and requiring FAD. This NADPH-hydroxylamine reductase activity has no physiological significance because of the high K_m for NH_2OH, namely 3 m*M*.

F. Biochemical Regulation of Nitrate Assimilation

As previously mentioned, nitrate assimilation is an energetically expensive process, requiring four equivalents of reduced pyridine nucleotide per nitrate reduced. Consequently, regulation of this pathway, particularly by inactivation when sufficient ammonium levels are available, would be economically advantageous. Further, the efficient site of regulation would be the first step, nitrate reductase, effectively halting assimilation and preventing accumulation of the toxic metabolite, nitrite. Ammonium is not a direct feedback inhibitor of nitrate assimilation, nor are any of the primary amino compounds such as glutamine or glutamate. However, biochemical regulation of nitrate reductase activity through oxidation–reduction interconversion of the enzyme has been suggested (Losada, 1974). Losada demonstrated that algal and higher plant nitrate reductases could be inactivated *in vitro* by preincubation with reductants such as NADH or dithionite and then reactivated by oxidants such as ferricyanide. Losada suggested nitrate reductase could therefore exist in two interconvertible forms, an oxidized, active form and a reduced, inactive species. The generality of this mechanism is not universal; the *N. crassa* nitrate reductase is not subject to reductive inactiva-

tion *in vivo*. Using purified nitrate reductase from *Chlorella vulgaris*, Lorimer *et al.* (1974) demonstrated that two components were required for rapid inactivation of nitrate reductase, NADH and cyanide. Cyanide reacted stoichiometrically with NADH-reduced enzyme to give a stable enzyme–cyanide complex having an association constant to 10^{19} *M*. Further, stoichiometric amounts of cyanide were released when physiologically inactivated nitrate reductase, isolated from cells exposed to ammonium, was reactivated. This observation suggested a physiological role for cyanide in regulating nitrate reductase activity. These observations were expanded into a model for the metabolic regulation of nitrate assimilation (Solomonson and Spehar, 1977) in which cyanide was recognized as the simplest carbon–nitrogen compound and postulated to be pivotal in integrating and regulating carbon and nitrogen assimilation. Cyanide *in vivo* was hypothesized to arise from hydroxylamine and glyoxylate, hydroxylamine in turn being an intermediate product of nitrite reduction by nitrite reductase. The origin of CN^- remains unclear and thus the model, though attractive, remains speculative. It is clear however that nitrate reductase is present in an inactive, cyanide-bound state *in vivo* in algal cells treated with ammonium or in nitrate-grown cells in late log phase.

Several high-molecular-weight inhibitors have been reported to affect higher plant nitrate reductases. These inhibitors can be classified as either proteolytic enzymes or binding proteins. Nitrate reductase proteases have been implicated in wheat leaves and maize roots (Wallace, 1974, 1975; Sherrard *et al.*, 1979; Yamaya *et al.*; 1980). Both inhibitors were found to be heat and EDTA sensitive. Other reports have implicated two nitrate reductase inactivators in *N. crassa* (Walls *et al.*, 1978; Horner, 1983). These inactivators which were apparently proteolytic and could be inhibited by EDTA and/or phenylmethylsulfonyl fluoride may be involved in the turnover of nitrate reductase (Section III.E). Inhibitors which bind nitrate reductase have been reported for a number of plant species (Yamaya *et al.*, 1980, and references therein; Jolly and Tolbert, 1978). Although proteases and other proteinaceous inhibitors do affect nitrate reductase activity, their functions remain generally uncharacterized and their roles in the regulation of physiological nitrate reductase activity thus are unclear. These agents increase the difficulty in obtaining an unaltered nitrate reductase protein during purification and thereby complicate the characterization of the native enzyme form.

Reversible inactivation of nitrate reductase from higher plants, algae, or fungi has been achieved by either dialysis or gel filtration, with reactivation possible by EDTA or various amino acids (Smarrelli and Campbell, 1983; Ketchum *et al.*, 1977). The biological significance of these effects is uncertain. In addition, higher plant nitrate reductase was found to have an affinity for heavy metals (Smarrelli and Campbell, 1983; Nason and Evans, 1953). However, this inhibi-