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In Vitro Formation of Assimilatory Reduced Nicotinamide Adenine Dinucleotide Phosphate: Nitrate Reductase from a *Neurospora* Mutant and a Component of Molybdenum-Enzymes

(nitrogenases/sulfite oxidase/*E. coli*)

ALVIN NASON, KUO-YUNG LEE, SU-SHU PAN, PAUL A. KETCHUM*,
ANTONIO LAMBERTI, AND JAMES DEVRIES

McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218.

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ABSTRACT **An active *Neurospora*-like assimilatory NADPH-nitrate reductase (EC 1.6.6.2), which can be formed *in vitro* by incubation of extracts of nitrate-induced *Neurospora crassa* mutant *nit-1* with extracts of (*a*) certain other nonallelic nitrate reductase mutants, (*b*) uninduced wild type, or (*c*) xanthine oxidizing and liver aldehyde-oxidase systems was also formed by combination of the *nit-1* extract with other acid-treated enzymes known to contain molybdenum. These molybdenum enzymes included (*a*) nitrogenase, or its molybdenum-iron protein, from *Clostridium*, *Azotobacter*, and soybean-nodule bacteroids, (*b*) bovine liver sulfite oxidase, (*c*) respiratory formate–nitrate reductase from *Escherichia coli*, (*d*) NADH-nitrate reductase from foxtail grass (*Setaria faberii*), and (*e*) $FADH_2$- and reduced methyl viologen-nitrate reductase preparations from certain *Neurospora* mutants. Several molybdenum–amino-acid complexes, as possible catalytic models of nitrogenase, were inactive (as were some previously tested 20 nonmolybdenum enzymes) in place of the acid-treated molybdenum-containing enzymes. The results imply the existence of a molybdenum-containing component shared by the known molybdenum-enzymes.**

Assimilatory NADPH-nitrate reductase (NADPH: nitrate oxidoreductase, EC 1.6.6.2) from *Neurospora crassa* is an inducible, soluble, cytochrome *b*-containing, sulfhydryl molybdoflavoprotein of molecular weight 230,000. It also possesses inducible NADPH-cytochrome *c* reductase and reduced flavin-adenine dinucleotide ($FADH_2$)- and reduced methyl viologen (MVH)-nitrate reductase activities in a constant proportion (1–6). Incubation of a cell-free extract of the *Neurospora* nitrate reductaseless mutant, *nit-1*, that is induced by nitrate, with extracts of other nitrate reductase *Neurospora* mutants, that are nonallelic, or with uninduced wild type produced the active enzyme, presumably by a combination of at least two dissimilar protein subunits, coded for by different cistrons (7). One of the subunits was believed to be a nitrate-inducible component(s) of *nit-1* that is responsible at least for the early part of the electron transport sequence, as reflected by its inducible NADPH-cytochrome *c* reductase activity which is flavin-dependent. The second component was inferred to be a constitutive entity that is absent in *nit-1*, but present in all other mutants and uninduced wild type, and is responsible for the latter part of the nitrate reductase pathway that includes the molybdenum moiety. The second component could also be supplied by the individual molybdenum-enzymes bovine milk and intestinal xanthine oxidases, chicken liver dehydrogenase, and rabbit liver aldehyde oxidase (8), provided they were subjected to prior acidification, a treatment known to dissociate some proteins into their subunits (9–11). (However, sodium molybdate and some 20 different partially purified enzymes that do not contain molybdenum were inactive.) In all instances, *in vitro* formation of enzyme activity was accompanied by the conversion of cytochrome *c* reductase activity ($S_{20,w}$ = 4.5S) to the faster sedimenting form ($S_{20,w}$ = 7.9 S) which is associated with NADPH-nitrate reductase (suggestive of subunit assembly) and the appearance of NADPH-, $FADH_2$-, and MVH-nitrate reductase activities. The assimilatory NADPH-nitrate reductase thus formed is similar to the wild-type *Neurospora* enzyme. Moreover, *nit-1* is the only one of the nitrate reductase *Neurospora* mutants that also lacks xanthine dehydrogenase, and is unable to grow on hypoxanthine or nitrate as the sole nitrogen source (8), similar to certain *Aspergillus* mutants (12).

In the present paper, we report that other acid-treated molybdenum-enzymes from diverse phylogenetic sources can also interact with the induced *Neurospora nit-1* extract to form a *Neurospora*-like assimilatory NADPH-nitrate reductase. The results suggest the existence of a molybdenum-containing component, perhaps in the form of a molybdenum cofactor, that is shared in common by the known molybdenum-enzymes among a diversity of organisms including microbes, plants, and animals. A preliminary account of a portion of this work has been published (13).

MATERIALS AND METHODS

N. crassa wild type and several nitrate reductase mutant strains including *nit-1* were maintained, grown, and induced with nitrate and crude extracts prepared as in an earlier study (7), unless otherwise noted. Sources of substrates, cofactors and various chemicals have been cited (7). Sulfite oxidase (EC 1.8.3.1) was purified from beef liver according to the procedure of MacLeod *et al.* (14). Cell-free, high-speed supernatant and pellet fractions of *Escherichia coli* respiratory nitrate reductase (designated S_{144} and P_{144}, respectively) that possesses formate-, and to a lesser extent, NADH-nitrate reductase activities were prepared by suspension of frozen *E. coli* K235 cells (15), kindly provided by Dr. S. Roseman, in an equal weight of cold 0.1 M phosphate buffer (pH 7.3)–1 mM 2-mercaptoethanol–500 μM

Abbreviations: $FADH_2$, reduced Flavin-adenine dinucleotide; MVH, reduced methyl viologen.

* Present address: Biology Department, Oakland University, Rochester, Mich.

TABLE 1. *In vitro formation of assimilatory NADPH-nitrate reductase by incubation of individual acid-treated molybdenum-enzymes with an extract of nitrate-induced Neurospora mutant nit-1*

Mo-enzymes and components	NADP → NO_3^- activity formed (pH before incubation with *nit-1*)			
	7.3	2.5	2.0	1.8
Nitrogenases	(*nmol NO_2^- produced/0.20 ml/60 min*)			
Expt. 1 (*Az. vinelandii*)				
Nitrogenase	4.2	4.7	54.4	—
Mo-Fe protein	0	1.0	1.6	7.0
Fe protein	0	0	0	—
Expt. 2 (*Az. vinelandii*)				
Nitrogenase	0	7.4	—	—
Mo-Fe protein	0	14.0	—	—
Expt. 3 (*Cl. pasteurianum*)				
Mo-Fe protein	12.6*	—	11.6	—
Fe protein	0.1*	—	0.1	—
Expt. 4 (*soybean nodule bacteroids*)				
Nitrogenase	4.0	—	61.7	—
Mo-Fe protein	4.8	—	62.4	—
	(pH 7.0)	(pH 2.4)	(pH 2.1)	(pH 1.4)
	(per 10 min assay)			
Bovine liver SO_2^- oxidase	0	5.3	83.5	26.1

The *Az. vinelandii* nitrogenase and its two metallo-protein fractions in Expt. 1 were kindly given by Drs. R. C. Burns and R. F. W. Hardy, and in Expt. 2 by Dr. W. A. Bulen. In Expt. 1, the nitrogenase fraction contained 35–40 mg of protein/ml, the Mo-Fe-protein fraction contained 35 mg protein/m', and the Fe-protein fraction contained 35 mg of protein/ml. All fractions were diluted 1:25 with 0.1 M NaCl solution at the indicated pH (acidified with HCl), maintained thus for 7 min at 0°C, mixed with an equal volume of crude extract of induced *nit-1* (about 10 mg protein/ml), and the resulting mixture was incubated at room temperature for 30 min. The NADPH-nitrate reductase formed was determined by a 60-min. assay. In Expt. 2, the nitrogenase fraction [in 25 mM Tris·HCl (pH 7.5)–0.1 mg dithiothreitol/ml] contained 34.3 mg of protein/ml, and the Mo-Fe-protein (in 0.27 M NaCl–25 mM Tris·HCl (pH 7.4)–0.1 mg of dithiothreitol/ml) contained 41.2 mg protein/ml; same conditions of processing (except 1:10 dilution at indicated pHs instead of 1:25). The *Cl. pasteurianum* nitrogenase Mo-Fe protein and Fe protein fractions in 0.05 M Tris (pH 8.0) were kindly provided by Dr. Leonard Mortenson and contained 30 mg of protein/ml and 2.7 mg of protein/ml, respectively; same conditions of incubation and assay as described for *Az. vinelandii*. The slight but definite activity of the Fe protein fraction is possibly due to a nitrogenase contamination. The bacteroid nitrogenase fractions from soybean nodules were kindly provided by Dr. H. J. Evans and Burton Koch. The nitrogenase was a crude extract [20–35% polypropylene glycol fract., 19.5 mg of protein/ml, in 0.05 M Tris (pH 8.0), containing 1 mg $Na_2S_2O_4$/ml with a specific activity of 7–9.5 nmol(N_2 reduced/min per mg of protein]. The Mo-Fe protein fraction was a DEAE-cellulose eluate, 25 mM Tris (pH 8.0) 0.15 M NaCl containing 1 mg $Na_2S_2O_4$/ml and 1.97 mg of protein/ml. Essentially, the same conditions of incubation (1:25 dilution at pH values 7.3 and 2.0 for 5 min and 0°C) and assay as described for Expt. 1 with *Az. vinelandii*. The partially purified bovine liver sulfite oxidase (EC 1.8.3.1) (4 mg of protein/ml) with a specific activity of 830 μmol cytochrome *c* reduced/min per mg of protein displayed no detectable xanthine oxidizing or aldehyde oxidase activities; same conditions as described for Expt. 1 with *Az. vinelandii* except that 1:10 dilution at the indicated pH values and, 10 min incubation with extract induced *nit-1* and a 10-min assay for nitrate reductase were used.

* Incubation with undiluted preparations at pH 7.3, but 1:25 dilution at pH 2.0.

EDTA ("preparation buffer"), rupture in a French pressure cell at 10,000 psi and centrifugation at 10,000 × *g* and 144,000 × *g* for 10 and 90 min, respectively. The supernatant solution (S_{144}) was used as such, whereas the pellet was washed three times by successive resuspension and centrifugation before final suspension in preparation buffer (about one-tenth of the original volume used for rupturing the cells) to constitute the P_{144} fraction. Partially purified preparations of assimilatory NADH-nitrate reductase from foxtail grass (*Setaria faberii*) and the multi-protein complex nitrogenase, including its two separate components (the molybdenum-iron protein and the iron protein) from *Azotobacter vinelandii* (16, 17), *Clostridium pasteurianum* (18), and soybean (*Glycine max.*) nodule bacteroids (19), were kindly provided by the indicated sources. Acid treatment of proteins and other substances was usually achieved by a 5- to 50-fold dilution at 0°C in 0.1 N NaCl adjusted with 1 N HCl to the desired pH. In some instances, more concentrated HCl solutions were used to minimize dilution. After 1–7 min, the acid-treated preparations were incubated with an equal volume of a crude extract of nitrate-induced *nit-1* (final pH of mixture 6.8) for 30–60 min at room temperature. The NADPH-nitrate reductase activity thus formed was assayed as described (5), generally for 30–60 min, and is expressed in nmoles of nitrite produced per assay period. $FADH_2$- and MVH-nitrate reductase activities were determined by the usual procedure (6), and formate- and NADH-nitrate reductase activities were determined by the same method as NADPH-nitrate reductase, except that 0.05 ml of 10 mM sodium formate and NADH, respectively, were used in place of NADPH. All assays were performed with two or more different aliquots and were directly proportional to enzyme concentration. Procedures for sucrose gradient sedimentation analysis (SW 39L rotor, 39,000 rpm, for about 20 hr), protein determination, estimation of Stokes radius by Sephadex G-200 gel filtration, and molecular weight calculation were the same as detailed elsewhere (6–8).

RESULTS

The earlier findings (7) that the acid-treated xanthine oxidizing and liver aldehyde oxidase systems can interact with an extract of nitrate-induced *nit-1* to form assimilatory NADPH-nitrate reductase prompted a similar examination of other known molybdenum-enzymes. The data of Table 1 demonstrate that the same phenomenon occurs with nitrogenases of *Azotobacter vinelandii*, *Clostridium pasteurianum*, and soybean nodule bacteriods, and with bovine liver sulfite oxidase, recently reported to contain molybdenum (20). Short-term exposure of the molybdenum-enzymes to suitable acid pH values before mixing with an extract of induced *nit-1*, greatly enhances the appearance of nitrate reductase activity. In the case of the nitrogenases, the ability to interact with *nit-1* to form NADPH-nitrate reductase resides in the molybdenum-iron protein and not in the iron protein moiety

TABLE 2. *In vitro conversion of bacterial formate- and higher plant NADH-nitrate reductases to Neurospora-like NADPH-nitrate reductase by acid-treatment and incubation with an extract of nitrate-induced Neurospora mutant nit-1*

Preparation	Nitrate reductase activity			
	NADPH → NO_3^-	NADH → NO_3^-	Formate → NO_3^-	
	(*nmoles NO_2^- produced/0.2 ml/30 min*)			
Expt. 1				
S_{144}, pH 7.3 (1:5 dilution)	0	0	40.4	
S_{144}, pH 7.3 (1:20 dilution) + preparation buffer	0	0	1.1	
S_{144}, pH 7.3 (1:20 dilution) + *nit-1*	2.3	0	0.9	
S_{144}, pH 2.3 (1:20 dilution) + preparation buffer	0	0	0	
S_{144}, pH 2.3 (1:20 dilution) + *nit-1*	12.0	0	0	
Expt. 2				
P_{144}, (undiluted)	0	19.5	40.6	
P_{144}, pH 7.3 (1:10 dilution) + *nit-1*	5.2	0	0	
P_{144}, pH 2.3 (1:10 dilution) + prepartion buffer	0	0	0	
P_{144}, pH 2.3 (1:10 dilution) + *nit-1*	9.6	0	0	
			$FADH_2$ → NO_3^-	RMV → NO_3^-
	(*nmoles NO_2^- produced/0.2 ml/30 min*)			
Foxtail grass				
Enzyme, pH 7.3 (1:4 dilution)	0	17.2	91.6	130.4
Enzyme, pH 2.5 (1:1 dilution)	0	0	0	0
	(*nmoles NO_2^- produced/0.2 ml/60 min*)			
Enzyme, pH 2.5 (1:1 dilution) + *nit-1*	10.7	0	5.9	2.3
Enzyme, pH 7.3 (1:1 dilution) + *nit-1*	0	—	—	—

E. coli fractions (S_{144}, 41.3 mg of protein/ml; P_{144}, 15.5 mg protein/ml) were acid-treated by 1:10 or 1:20 dilutions with HCl solutions (pH 2.3), maintained thus for 5 min at 0°C, combined with an equal volume of crude extract of nitrate-induced *nit-1* (about 10 mg of protein/ml) or pH 7.3 preparation buffer as indicated, and incubated at room temperature for 30 min. Nitrate reductase activities were determined by 30-min assays. Foxtail grass (*Setaria faberii*) NADH-nitrate reductase in lyophilized powder form, was a gift of Dr. R. H. Hageman. It was purified an additional 15-fold to a specific activity of about 175 (0.71 mg of protein/ml) by DEAE-cellulose column chromatography, mixed with an equal volume of HCl solution (pH 1.25) to give a final pH of 2.5, and maintained thus for 1–2 min at 0°C before incubation for 30 min at room temprature with an equal volume of extract of induced *nit-1*. Nitrate reductase activities were determined by 30- and 60-min assays.

of the nitrogen-fixing multi-protein complex (Table 1). The seemingly similar effect with the *Cl. pasteurianum* system at pH values of 2.0 and 7.3 reflects in reality a considerably greater activity at the lower pH, since 1:25 dilutions were used at pH 2.0 by contrast to undiluted preparations at pH 7.3. The results of various control experiments (not shown) including incubation of the acid-treated fractions with pH-7.3 preparation buffer, instead of the *nit-1* extract, as well as incubation of *nit-1* extract alone or with 10 mM–10 μM sodium molybdate, were negative. With the sole exception of a single *Azotobacter* nitrogenase fraction (Expt. 1) that exhibited slight xanthine dehydrogenase activity, neither the nitrogenase preparations, nor the liver sulfite oxidase contained detectable NADPH- or NADH-nitrate reductase, xanthine oxidase, xanthine dehydrogenase, or aldehyde oxidase. The sucrose density gradient profile of liver sulfite oxidase activity ($S_{20,w}$ = 6.3) coincided with its ability (generated by subsequent acidification to pH 2.1) to interact with a *nit-1* extract to form NADPH-nitrate reductase (not shown). Moreover, the sucrose density gradient profile and Stokes radius of the NADPH-nitrate reductase thus formed, were similar to those of the wild type *Neurospora* enzyme.

Cell-free *E. coli* extracts possess a molybdenum-containing (21), membrane-bound respiratory nitrate reductase complex, in which formate is the most effective electron donor for nitrate reductase (21–24). Such preparations interacted (after adjustment to pH 2.3) with *nit-1* extract to form a soluble assimilatory NADPH-nitrate reductase, which is like that of *Neurospora* (Table 2). Thus, both the high-speed supernatant and pellet fractions of *E. coli* exhibiting formate- and, to a lesser extent NADH-nitrate reductase activities, yielded a soluble, highly specific NADPH-nitrate reductase with a sucrose density gradient profile (not shown, $S_{20,w}$ = 7.8S) and Stokes radius (7.4 nm, 74 Å) similar to those of wild-type *Neurospora* enzyme. The NADH-specific nitrate reductase of a higher plant (foxtail grass) by successive acid treatment and incubation with *nit-1* extract also produced a soluble *Neurospora*-like NADPH-nitrate reductase (Table 2). Acid treatment eliminated formate-nitrate reductase in *E. coli* and NADH-nitrate reductase in foxtail grass (despite restoration of the pH to neutrality), but subsequent incubation with *nit-1* extract led to the appearance of NADPH-nitrate reductase activity.

The wild-type NADPH-nitrate reductase itself also can serve as a source of the molybdenum component (Table 3). Although adjustment of the wild-type enzyme to pH 2.0 caused a complete loss of all three nitrate reductase activities, they were partially restored by mixing with an extract of induced *nit-1*, but not with the preparation buffer alone. Neither uninduced *nit-1* nor uninduced or induced *nit-2* and

nit-3 extracts could be used instead of induced *nit-1*. Also, mildly heat-treated enzyme (which typically undergoes a complete loss of NADPH-nitrate reductase activity, and a 3-fold increase in MVH-nitrate reductase, while $FADH_2$-nitrate reductase remains unchanged, ref. 6) was partially restored by mixing with induced *nit-1* extract, provided the heated enzyme was adjusted to pH 2.0 (Table 3).

Neurospora mutant *nit-3* characteristically lacks inducible NADPH-nitrate and NADPH-cytochrome *c* reductase activities, but possesses small endogenous levels of $FADH_2$- and MVH-nitrate reductase activities that are strikingly increased by nitrate induction (7). Most of the inducible $FADH_2$- and MVH-nitrate reductase activities are confined to the soluble fraction (not shown), while the ability to form NADPH-nitrate reductase—*without* benefit of prior acid treatment, as in the case of uninduced wild-type and other mutants (7)—resides solely in the particulate fraction. A similar pattern was obtained with the mutant *nit-25376*. In both mutants, the early portion of the nitrate reductase chain has apparently been genetically altered, as reflected by the lack of inducible cytochrome *c* reductase activity, while the rest of the sequence is functional. It might be expected by analogy with the wild-type enzyme that suitable acid treatment of the induced, soluble $FADH_2$- and MVH-nitrate reductase system of *nit-3* and *nit-25376* could also serve as a source of the molybdenum component for the *in vitro* assembly of NADPH-nitrate reductase. This expectation was experimentally borne out (Table 4). Prior acid treatment of the soluble preparation was required with the optimal pH occurring at 3.5, by contrast to the lower pH values that are required for the other molybdenum-proteins indicated in Tables 1–3.

TABLE 3. *Partial restoration of acid-treated and heat-treated wild type Neurospora NADPH-nitrate reductase by an extract of nitrate-induced Neurospora mutant nit-1*

Treatment of wild-type enzyme	Nitrate reductase activities: NADPH → NO_3^-	$FADH_2$ → NO_3^-	RMV → NO_3^-
	(*nmol of NO_2^- produced/0.10 ml/15 min*)		
0°			
pH 7.3	56.6*	22.2*	24.4*
pH 2.0	0*	0*	0*
pH 2.0 + buffer	0	0.2	0.6
pH 2.0 + *nit-1*	10.3	0.8	2.3
45°, 10′,			
pH 7.3	0*	24.7*	80.0*
pH 7.3 + *nit-1*	0.3	18.3	78.2
pH 2.0	0*	0*	0.5*
pH 2.0 + buffer	0	0	0.6
pH 2.0 + *nit-1*	23.2	3.8	5.4

The wild type, partially purified NADH-nitrate reductase (about 4 mg of protein) was diluted 1:7 in either the preparation buffer (pH 7.3) or 0.1 N NaCl–HCl solution (to a final pH of 2.0, for less than 3 min at 0°C) as indicated. Heat treatment (45°C, 10 min) always preceded acid treatment when both treatments were applied. Incubation with an equal volume of crude extract of induced *nit-1* (8.7 mg of protein/ml) or preparation buffer as indicated was for 15 min at room temperature, and nitrate reductase assays for 15 min.

* nmol of NO_2^- produced/0.05 ml per 15 min instead of per 0.10 ml/15 min.

TABLE 4. *Effect of prior pH treatment of high-speed supernatant fractions of induced Neurospora mutants nit-3 and nit-25376 on their ability to form NADPH-nitrate reductase by incubation with an extract of nitrate-induced mutant nit-1*

Incubation mixture	NADPH → NO_3^- activity formed (pH treatment before incubation with *nit-1*): 7.0	4.0	3.5	3.0	2.6	2.2
	(*nmol of NO_2^- produced/0.10 ml/30 min*)					
nit-3, S_{144} + *nit-1*	0	8.8	16.1	11.4	7.2	3.9
	(*nmol of NO_2^- produced/0.10 ml/60 min*)					
nit-25376, S_{144} + *nit-1*	0	8.2	18.9	—	15.3	3.7

The S_{144} fractions are the supernatant solutions obtained by centrifuging the crude extracts (10,000 × *g* for 10 min, supernatant solutions) of nitrate-induced mutants *nit-3* and *nit-25376* at 144,000 × *g* for 3 hr. *nit-3*, S_{144} (5.3 mg of protein/ml) was acid-treated for 4 min at 0°C, incubated for 30 min at 23°C with $^1/_{10}$ volume of an ammonium sulfate fraction of induced *nit-1* (22.1 mg of protein/ml) and assayed for NADPH-nitrate reductase for 30 min. Prior pH adjustment was attained with 0.05 N HCl (about 0.8–2.0 ml/7.0 ml of extract). *nit-25376*, S_{144} (2.9 mg of protein/ml) was acid-treated for 1 min at 0°C, incubated for 30 min at 23°C with $^1/_5$ volume of induced *nit-1* extract (8.4 mg of protein/ml) and assayed for NADPH-nitrate reductase for 60 min. Prior pH adjustment was attained with 0.5 N HCl (about 0.25 ml per 3 ml of extract).

Preliminary attempts to utilize several crystalline molybdenum-amino-acid complexes† (originally prepared as possible catalytic models of nitrogenase) as a source of the molybdenum component with *nit-1* extract for the formation of NADPH-nitrate reductase were fruitless.

DISCUSSION

Of several possible hypotheses originally advanced to account for the *in vitro* formation of assimilatory NADPH-nitrate

† The following model molybdenum–amino acid complexes, a gift of Dr. R. W. F. Hardy, were tested as freshly prepared stock solutions: a 1:1 crystalline molybdenum (V)-L-histidine complex of structure 1 (25), about 3 mg/ml in 0.1 N HCl; a 1:1 crystalline molybdenum (V)-L-cysteine complex [sodium cysteinatomolybdenum (V)] of structure 3b (26), 4 mg/ml in aqueous solution; a 1:2 crystalline molybdenum (VI)-methyl cysteinate complex [Bis(methyl cysteinato) dioxomolybdenum (VI)] of structure 6a (26), 1.2 mg/ml in 20% acetone. Incubation periods up to 30 min at room temperature of each of the above stock solutions with equal volumes of *nit-1* extract, followed by the standard assay for NADPH-nitrate reductase with 0.1 ml and 0.2 ml of the incubation mixture failed to show enzyme activity. The above molybdenum–cysteine and -cysteinate complexes were able to catalyze (*a*) the reduction of nitrate to nitrite by dithionite with or without added FAD or methyl viologen, and (*b*) the reduction of nitrate to nitrite to only a slight extent by NADH and NADPH. The molybdenum–histidine complex was inactive in all of the above tests.

reductase in mixtures of *Neurospora* extracts, the concept of a complementation phenomenon that is based on the interaction of at least two dissimilar subunits coded for by different cistrons was favored (7). The viewpoint received particularly strong support from profile data of sucrose density gradients showing the conversion of a slower sedimenting inducible NADPH-cytochrome *c* reductase (contributed by the *nit-1*) to a faster sedimenting form, characteristically associated with NADPH-nitrate reductase. The same data were also used to argue against a second hypothesis, namely activation of a proenzyme in one of the extracts by a component of the second extract.

The present experimental evidence, however, exhibits two features that raise the liklihood of a third interpretation based on a combination of the above two hypotheses.

(*a*) The data point to the existence of a similar, if not indistinguishable, molybdenum-containing component in all molybdenum-enzymes, whether from prokaryotic or eukaroytic cells. The known highly specific phenomena of recognition, interaction, and assembly of protein subunits in the formation of multimeric enzymes would suggest that the molybdenum-containing component occurring throughout the phylogenetic scale is an identical and immutable molybdenum-polypeptide chain, a highly unlikely possibility. A more reasonable explanation would be that the molybdenum moiety exists as part of a relatively small organic molecule or cofactor, perhaps even as a small polypeptide. A 6S subunit (molecular weight, about 150,000) arising from acid-treated xanthine oxidase (8) and a 4.6S subunit (molecular weight, about 55,000) from acid-treated liver sulfite oxidase (unpublished) each possesses the ability to interact with *nit-1* to form NADPH-nitrate reductase; these may be "protein carriers" that contain the presumed molybdenum cofactor. The molybdenum cofactor then may be released upon interaction with *nit-1* extract. In the case of *Neurospora* preparations, the molybdenum cofactor may be more easily accessible, thus accounting for its ability to interact with *nit-1* extract without prior acidification (7). Several additional lines of evidence beyond the scope of the present paper also lend support to the notion of a molybdenum cofactor‡.

(*b*) The properties of the enzyme resulting from the *in vitro* reaction are obviously determined or "programmed" by the induced *nit-1* extract. For no matter what the source of the molybdenum component, ranging from microbes to mammals, the *in vitro* formation of the system invariably yields a wild-type assimilatory NADPH-nitrate reductase which is like that of *Neurospora*.

It therefore seems feasible that *in vitro* assembly and activation of *Neurospora* assimilatory NADPH-nitrate reductase can be ascribed to the combination of protein subunits from induced *nit-1* by linkage to the presumed molybdenum-containing component or cofactor furnished by *Neurospora* extracts or by acid-treated molybdenum-enzymes from various sources. According to this hypothesis, the molybdenum cofactor serves both as a link that binds the enzyme subunits to yield the active enzyme and as an electron carrier. Possibly both roles are universal in all molybdenum enzymes.

‡ Manuscript in preparation.

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