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In Vitro Formation of Nitrate Reductase Using Extracts of the Nitrate Reductase Mutant of *Neurospora crassa*, *nit-1*, and *Rhodospirillum rubrum*

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In vitro formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-nitrate reductase (NADPH: nitrate oxido-reductase, EC 1.6.6.2) has been attained by using extracts of the nitrate reductase mutant of *Neurospora crassa*, *nit-1*, and extracts of either photosynthetically or heterotrophically grown *Rhodospirillum rubrum*, which contribute the constitutive component. The in vitro formation of NADPH-nitrate reductase is characterized by the conversion of the flavin adenine dinucleotide (FAD) stimulated NADPH-cytochrome *c* reductase, contributed by the *N. crassa nit-1* extract from a slower sedimenting form (4.5*S*) to a faster sedimenting form (7.8*S*). The 7.8*S* NADPH-cytochrome *c* reductase peak coincides in sucrose density gradient profiles with the NADPH-nitrate reductase, $FADH_2$-nitrate reductase and reduced methyl viologen (MVH)-nitrate reductase activities which are also formed in vitro. The constitutive component from *R. rubrum* is soluble (both in heterotrophically and photosynthetically grown cells), is stimulated by the addition of 10^{-4} M Na_2MoO_4 and 10^{-2} M $NaNO_3$ to cell-free preparations, and has variable activity over the pH range from 3.0 to 9.5. The activity of the constitutive component in some extracts showed a threefold stimulation when the pH was lowered from 6.5 to 4.0. The constitutive activity appears to be associated with a large molecular weight component which sediments as a single peak in sucrose density gradients. However, the constitutive component from *R. rubrum* is dialyzable and is insensitive to trypsin and protease. These results demonstrate that *R. rubrum* contains the constitutive component and suggests that it is a low molecular weight, trypsin- and protease-insensitive factor which participates in the in vitro formation of NADPH nitrate reductase.

Assimilatory reduced nicotinamide adenine dinucleotide phosphate (NADPH)-nitrate oxido-reductase (EC 1.6.6.2) is a soluble, heteromeric enzyme with a molecular weight of 230,000 which contains flavin, cytochrome b_{557}, and molybdenum as components of its electron transport sequence (4, 15, 19, 20, 21). NADPH-nitrate reductase and its associated flavin-stimulated NADPH-cytochrome *c* reductase are induced by nitrate in both *Aspergillus nidulans* (24) and *Neurospora crassa* (5). The *Neurospora* enzyme also contains reduced methyl viologen (MVH)-nitrate reductase and reduced flavin adenine dinucleotide ($FADH_2$) nitrate reductase (5).

Genetic studies of nitrogen metabolism in *A. nidulans* led Pateman et al. (23) to postulate the presence of a cofactor, common to both NADPH-nitrate reductase and xanthine dehydrogenase. The subsequent development of a procedure for the in vitro formation of NADPH-nitrate reductase (8, 14, 16) led Nason et al. (16) to postulate the involvement of a molybdenum cofactor in the in vitro formation of NADPH-nitrate reductase. NADPH-nitrate reductase is formed in vitro when extracts of the nitrate-induced *N. crassa* nitrate-reductase mutant, *nit-1*, which specifically contains the FAD-stimulated NADPH-cytochrome *c* reductase, are mixed with extracts of uninduced wild-type *N. crassa*. Current evidence suggests that the FAD-stimulated NADPH-cytochrome *c* reduc-

tase activity contributed by the *nit-1* extract combines with the constitutive component present in uninduced extracts of wild-type *N. crassa* to form NADPH-nitrate reductase which is essentially identical to the wild-type enzyme (14). This in vitro assembly of NADPH-nitrate reductase has also been used as an assay to demonstrate the presence of the constitutive component in molybdenum-containing enzymes (8, 16). Acid-treated preparations of purified xanthine-oxidizing enzymes, aldehyde-oxidyzing enzymes, nitrogenase, sulfite oxidase, and nitrate reductase are active in the in vitro formation of NADPH-nitrate reductase when mixed with extracts of nitrate-induced *N. crassa, nit-1* (8, 16). A similar approach has been used to demonstrate that extracts of wild-type *Escherichia coli* contain the constitutive component (11, 16). These data support the hypothesis that the constitutive component contains molybdenum and is common to all molybdenum-containing enzymes (8, 16). However, no biochemical evidence has been reported which directly implicates the involvement of a low molecular weight cofactor (23) in the in vitro formation of NADPH-nitrate reductase.

Preliminary investigations in our laboratory demonstrated that a soluble form of the constitutive component, which is active in the in vitro formation of NADPH-nitrate reductase, is present in relatively high concentrations in extracts of *Rhodospirillum rubrum*. This investigation reports the partial characterization of the constitutive component isolated from *R. rubrum* and presents biochemical evidence which suggests that the constitutive component is a low-molecular-weight factor.

MATERIALS AND METHODS

Cultures and growth. *R. rubrum* strains S_1 and ATCC 9791 were grown on the malate, glutamate, ammonium chloride medium of Ormerod et al. (22). Cells were grown heterotrophically in Fernbach flasks shaken on a New Brunswick rotary shaker at room temperature or photosynthetically at 30 C in 1-liter screw-cap bottles which were illuminated by a 100-W tungsten bulb. Cultures were normally harvested in the late exponential or stationary phase of growth and washed in 0.02 M phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 6.5. *N. crassa, nit-1*, was grown in Fries basal medium containing ammonium chloride and then induced for 3 h at pH 4.5 (9) in medium containing 0.69% (wt/vol) $NaNO_3$ as the sole nitrogen source.

Purification of the constitutive component from R. rubrum. Washed *R. rubrum* cells were suspended in a ratio of 1 g (wet weight) to 3 ml in 0.02 M phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 6.5, containing (unless otherwise stated) 10^{-2} M $NaNO_3$, 10^{-4} M Na_2MoO_4, and broken in a French pressure cell at 20,000 psi. The supernatant fluid resulting from centrifugation of the broken cell suspension at 20,000 $\times$ *g* for 20 min was designated as the crude extract. Centrifugation of the crude extract at 144,000 $\times$ *g* for 1 h yielded the high-speed supernatant fraction containing the constitutive activity which was stable at -20 C for up to 2 months. Protamine sulfate (2.5% wt/vol) was added with stirring in a ratio of 1 volume to 6 volumes of the high-speed supernatant fraction. The resulting precipitate was removed by centrifugation at 20,000 $\times$ *g* for 20 min. Protein was determined by the modified Folin-Lowry procedure (10). Bacteriochlorophyll (BChl) concentration was determined spectrophotometrically after extraction with acetone: methanol (7:2 vol/vol) and calculated by using the specific absorbancy reported by Clayton (2).

Preparation of cell-free extracts of N. crassa, nit-1. Cell-free extracts of *N. crassa, nit-1* were prepared in 0.1 M phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 7.3, 5×10^{-3} M ethylenediaminetetraacetic acid (EDTA), 1% (wt/vol) NaCl by the method previously described (14). The complementing activity in extracts of *N. crassa, nit-1* lost 48% of its activity during storage at -20 C for 54 days when prepared in the above buffer. At +4 C the same complementing activity had a half-life of 24 h. Preparations of *nit-1* used in certain experiments were purified further, by ultracentrifugation at 175,000 $\times$ *g* for 1 h followed by concentration using ammonium sulfate fractionation (14). The 0 to 35% ammonium sulfate fraction was suspended in 0.1 M phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 7.2, containing 0.1% (wt/vol) NaCl and 5×10^{-4} M EDTA. NADPH was shown to stabilize this fraction (unpublished data), and was therefore added to the *nit-1* preparations during the assay for the constitutive component.

Enzymatic assays. Xanthine oxidase, xanthine dehydrogenase, and sulfite oxidase were assayed according to previously described procedures (8). NADPH-nitrate reductase, $FADH_2$-nitrate reductase, MVH-nitrate reductase, and NADPH-(FAD)-cytochrome *c* reductase were assayed by the procedure of Garrett and Nason (5) and units were expressed in nanomoles of nitrite formed per 10 min or nanomoles of cytochrome *c* reduced per minute. Nitrite reductase was assayed by using the procedure previously described (18). The constitutive component was measured by incubating increasing concentrations of the *R. rubrum* preparation at room temperature with an excess amount (between 150 and 280 μg of protein) of the nitrate-induced *N. crassa, nit-1* extract. The in vitro formation of NADPH-nitrate reductase was linear with increasing concentrations of the *R. rubrum* preparation and with increasing time (up to 30 min). After incubating for 10 to 45 min, the amount of NADPH-nitrate reductase formed was determined by the normal assay procedure. The amount of the constitutive component is expressed as units of NADPH-nitrate reductase formed per 10 min (unless otherwise noted) when samples of *R. rubrum* preparations are incubated with an excess amount of the *nit-1* extract at room temperature. Trypsin, protease type V from *Streptomyces griseus*,

alcohol dehydrogenase, FAD, and NADPH were obtained from Sigma Chemical Co.

Sucrose density gradients. NADPH-nitrate reductase was sedimented in a 15.5 to 33% (wt/vol) sucrose density gradient prepared in 0.1 M phosphate (K_2HPO_4/KH_2PO_4) buffer, pH 7.2, 10^{-3} M mercaptoethanol, 5×10^{-4} M EDTA. Sucrose density gradients of the constitutive component from *R. rubrum* were prepared by using 15.5 to 33% (wt/vol) sucrose solutions in 0.02 M phosphate (K_2HPO_4/KH_2PO_4) buffer, pH 6.5. All sucrose density gradients were centrifuged at 40,000 rpm in the Beckman SW 50.1 rotor at +4 C for 18 h. Estimation of the sedimentation coefficient was made by the method of Martin and Ames (12) with yeast alcohol dehydrogenase as the marker protein.

RESULTS

Cell-free extracts of both photosynthetically and heterotrophically grown *R. rubrum* strain S_1 contain a soluble constitutive component which reacts with extracts of nitrate induced *nit-1* to form NADPH-nitrate reductase (Table 1). In photosynthetically grown cells all of the constitutive component remained in the supernatant fraction after centrifugation at 144,000 × *g* for 1 h, whereas the chromatophores, as indicated by specific content of bacteriochlorophyll, were sedimented in the pellet. This ultracentrifugation step resulted in a 1.8-fold purification of the constitutive component. Similar results were obtained for the heterotrophically grown cells during 1-h centrifugation at 144,000 × *g* and resulted in a 2.4-fold increase in the specific activity of the constitutive component in the high-speed supernatant fluid. The extracts used for the data presented in Table 1 were prepared in 0.02 M (Na_2HPO_4/NaH_2PO_4) buffer, pH 6.5, without added molybdenum and nitrate. This preparation procedure resulted in specific activities which are significantly lower than those reported in Table 3. Extracts of *R. rubrum* did not contain any NADPH-nitrate reductase, $FADH_2$-nitrate reductase, MVH-nitrate reductase, or NADPH-nitrite reductase. Furthermore, measurable levels of nitrite were not detected in extracts of *R. rubrum* even after the extracts were heated in a boiling-water bath for 10 min. *R. rubrum* strain ATCC 9791 was also shown to contain the constitutive component demonstrating that this property of *R. rubrum* is not strain specific. The remainder of the experiments reported here are on preparations of *R. rubrum* strain S_1.

TABLE 1. *Distribution of the constitutive component in photosynthetically and heterotrophically grown R. rubrum*

Fraction	Constitutive Component			
	Total mg of Protein[b]	Total U[a]	Sp Act[b]	BChl (μg/mg) Protein
Photosynthetic				
20,000 × *g* (20 min) Sup.	111.6	1,139	10.2	
144,000 × *g* (1 h) Sup.	55.8	1,025	18.3	0.32
144,000 × *g* (1 h) Pellet	56.0	0		13.40
Heterotrophic				
20,000 × *g* (20 min) Sup.	131.9	1,355	10.3	
144,000 × *g* (1 h) Sup.	74.3	1,786	24.0	
144,000 × *g* (1 h) Pellet	66.0	132	2.0	

[a] The amount of nitrite measured can be calculated from the information provided.

[b] The protein concentration in the 20,000 × *g* supernatant fluid of the *R. rubrum* extracts was 24.8 and 29.3 mg per ml, respectively. The pellets were resuspended in 1 ml of buffer. Between 0.02 and 0.10 ml of each fraction was used in the determination of the constitutive component.

During the in vitro formation of NADPH-nitrate reductase using extracts of *R. rubrum* and nitrate-induced *nit-1*, both $FADH_2$- and MVH-nitrate reductase were formed. These three nitrate reductase activities cosedimented in a single peak in the sucrose density gradient (Fig. 1) with $s_{20,w}$ equal to 7.8*S*. The NADPH-(FAD)-cytochrome *c* reductase contributed from the *nit-1* extract appears in two peaks: the 7.8*S* peak which coincides with the profile of the three nitrate reductase activities and a slower sedimenting 4.5*S* peak which is the activity from *nit-1* (14) which did not participate in the NADPH-nitrate reductase formation reaction. The sucrose density gradient profile for this NADPH-nitrate reductase formed in vitro (Fig. 1) is identical to those profiles observed for the wild-type *N. crassa* NADPH-nitrate reductase (14) and for the other previously reported NADPH-nitrate reductases formed in vitro (8, 14) except for the ratio of associated activities. The apparent K_m values for the partially purified preparation of NADPH-nitrate reductase formed in vitro by using extracts of *R. rubrum* and *nit-1* were determined for NADPH, FAD, and nitrate by the procedures of Garrett and Nason (5), and were shown to be 6.7×10^{-5} M, 8.8×10^{-8} M, and 5.3×10^{-4} M, respectively. These K_m values are similar to those reported for the wild-type *N. crassa* enzyme (5).

The constitutive component in both whole

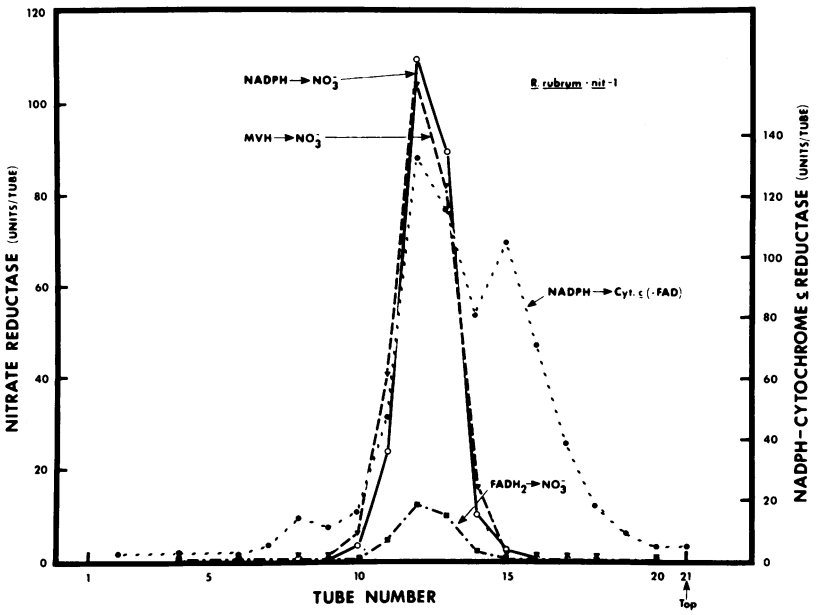

FIG. 1. *Sucrose density gradient of NADPH-nitrate reductase formed in mixtures containing crude extracts of R. rubrum and nitrate-induced N. crassa, nit-1. The NADPH-nitrate reductase formed was partially purified by using protamine sulfate and ammonium sulfate fractionations resulting in a specific activity of 196 U/mg of protein. A sample containing 1.99 mg of protein was sedimented in a 15.5 to 33% (wt/vol) sucrose density gradient at 175,000 × g for 18 h.*

cells and cell-free extracts of *R. rubrum* was stable at −20 C for up to 2 months; however, thawing and refreezing markedly decreased the effective time of storage. When nitrate and molybdenum were added to the *R. rubrum* extract, the activity of the constitutive component increased with time of storage at −20 C (Table 2). The constitutive activity in cell-free extracts of heterotrophically grown cells ruptured in 0.02 M buffer (Na_2HPO_4/NaH_2PO_4), pH 6.5, was stimulated 3.4-fold by the addition of 10^{-2} M $NaNO_3$ and 10^{-4} M Na_2MoO_4 when stored at −20 C for 18 and 42 h. When molybdenum and nitrate were added together, the stimulation was more than the sum of the stimulation observed when identical concentrations of these compounds were added separately. The variation in the units of constitutive activity per milliliter of the control sample (Table 2) may be due to variations in the complementing activity of the nitrate-induced *nit-1* extract used in the assay. Molybdenum and nitrate do not stimulate the in vitro formation of NADPH-nitrate reductase when they are added directly to extracts of *nit-1*. When the pH of the *R. rubrum* extract was lowered from an initial pH of 6.7 to 5.7, there was a twofold increase in the constitutive activity (Table 2).

TABLE 2. *Effect of nitrate and molybdenum on the constitutive component in extracts of R. rubrum*[a]

Additions to crude extract	Time of storage at −20 C (h)		
	1	18	42
No additions	100 (750)	100 (375)	100 (550)
10^{-2} M $NaNO_3$, pH 6.5	77	178	145
10^{-3} M $NaNO_3$, pH 6.5	83	93	82
10^{-4}M Na_2MoO_4, pH 6.5	117	121	127
10^{-5} M Na_2MoO_4, pH 6.5	117	107	114
10^{-2} M $NaNO_3$, 10^{-4} M Na_2MoO_4, pH 6.5	100	336	340
Acid treated to pH 5.7	247	414	281
Acid treated to pH 5.7 plus 10^{-2} M $NaNO_3$, 10^{-4} M Na_2MoO_4	250	457	250

[a] Results are expressed as the percentage of the amount of constitutive component observed in the control (no additions). The amounts of the constitutive component in the crude extracts are reported in parentheses and are expressed as units of NADPH-nitrate reductase formed per milliliter of *R. rubrum* extract. The activity of the constitutive component was shown to be linear with increasing concentrations in each of the above determinations. Neither nitrite nor NADPH-nitrate reductase was detected in the *R. rubrum* extracts before or after storage at −20 C in the presence of 10^{-2} M $NaNO_3$ and 10^{-4} M Na_2MoO_4.

At pH 5.7 the constitutive activity was not affected by the addition of 10^{-2} M $NaNO_3$ and 10^{-4} M Na_2MoO_4. The explanation of the differential effect of $NaNO_3$ and Na_2MoO_4 at these different pH is not apparent at this time; however, the data does suggest that nitrate and molybdenum together exert a specific chemical effect on the constitutive component at or near neutral pH.

When crude extracts of *R. rubrum* were adjusted to various pH (Fig. 2), the constitutive component was active over the pH range from 3.0 to 9.0. No activity was observed below pH 2.5. *R. rubrum* was stable at pH between 4.5 and 5.5 for several weeks at −20 C and for 24 h at +4 C. The stimulation of the constitutive activity between pH 4.0 and 5.0 varied from preparation to preparation and therefore two experiments are shown.

Crude extracts lost 50% of their constitutive activity when heated at +44 C for 5 min (Fig. 3). The activity lost during heating was not recoverable by acid treatment. In sucrose density gradients, the constitutive activity isolated from photosynthetically grown and heterotrophically grown (not shown) *R. rubrum* cells sediments in a single peak (Fig. 4) with $s_{20,w}$ ranging from 4.5 to 5.2S.

The specific activity of the constitutive component in crude extracts of heterotrophically grown *R. rubrum*, which have been stored in 10^{-2} M $NaNO_3$ and 10^{-4} M Na_2MoO_4 at −20 C for at least 24 h, can be increased approximately twofold by ultracentrifugation and protamine sulfate treatment (Table 3). This procedure results in 90% or greater recovery of the starting activity. The most stable preparations with the highest observed specific activities were observed routinely in the protamine-sulfate supernatant fluid. Other attempts at purification have demonstrated that the constitutive component in extracts of *R. rubrum* can be precipitated by 50% saturated ammonium sulfate, by 80% acetone, or by adjusting the pH to 3.5 with trichloroacetic acid. These procedures often result in total recovery of the constitutive activity but this activity is unstable after these treatments and is often lost during dialysis.

Dialysis experiments were designed to determine if there is an active dialyzable form of the constitutive component. When extracts of nitrate-induced *nit-1* (inside) were dialyzed against extracts of *R. rubrum* (outside), NADPH-nitrate reductase was formed inside the dialysis sacs (Fig. 5). The rate of formation of NADPH-nitrate reductase inside the

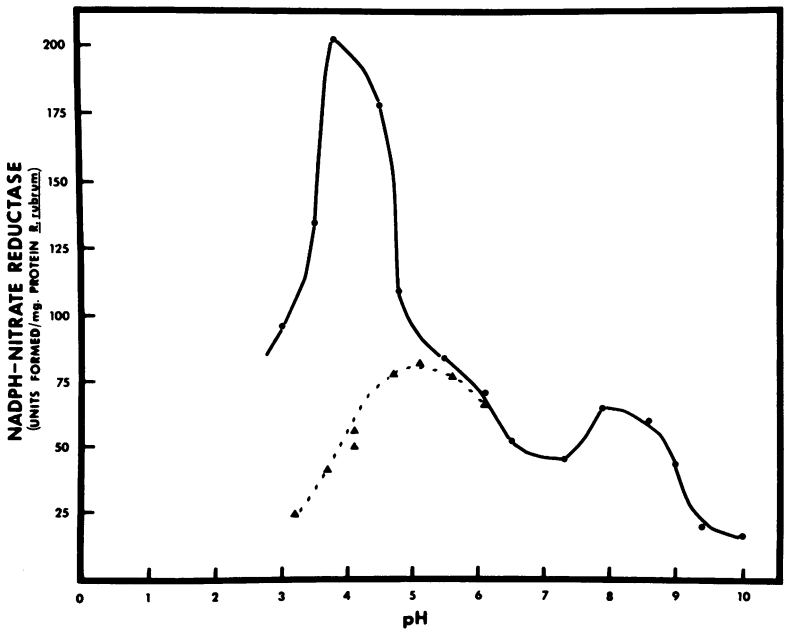

FIG. 2. *Effect of pH on the constitutive component in extracts of R. rubrum. The 144,000 × g supernatant fluid of the R. rubrum extract in 0.02 M (Na_2HPO_4/NaH_2PO_4) buffer, pH 6.5, containing 10^{-4} M Na_2MoO_4 and 10^{-2} M $NaNO_3$ was adjusted to the pH indicated by using NaOH or HCl. Samples were incubated with an excess amount of an extract of nitrate induced N. crassa, nit-1 for 10 min at room temperature and then assayed for NADPH-nitrate reductase. Two experiments are shown.*

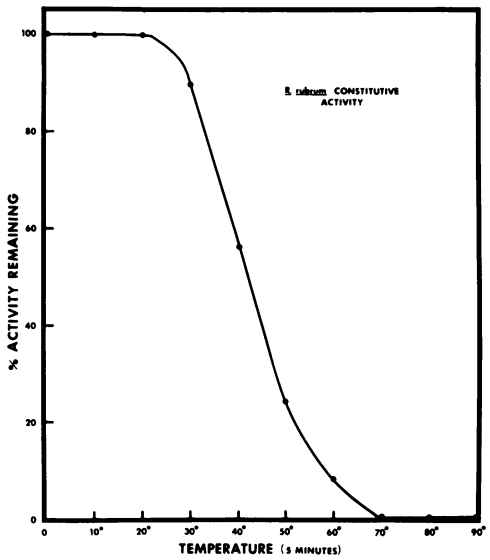

FIG. 3. *Heat stability of the constitutive component from R. rubrum. Samples of the 20,000 × g supernatant fluid in 0.02 M (Na_2HPO_4/NaH_2PO_4) buffer, pH 6.0, containing 10^{-4} M Na_2MoO_4 and 10^{-2} M $NaNO_3$ were heated at the indicated temperature for 5 min, cooled in an ice bath, incubated with an extract of nitrate-induced N. crassa, nit-1 for 10 min at room temperature, and assayed for NADPH-nitrate reductase. The amount of the constitutive component in the R. rubrum fraction incubated at 0 C was 25 U of NADPH-nitrate reductase formed per 10 min.*

dialysis sac was linear for the first 40 min, and appeared to reach a maximum in the control experiment (A) after 120 min of dialysis. When increasing amounts of trypsin were added to the *R. rubrum* extract at zero time, the initial rates of formation were similar to the control, however; the amount of NADPH-nitrate reductase formed after 120 min of dialysis increased with increasing concentrations of trypsin (B), (C). At 0.33 mg of trypsin per ml of *R. rubrum* extract (C), the formation of NADPH-nitrate reductase was stimulated 1.8-fold over the untreated control. After 120 min of dialysis the amount of trichloroacetic acid soluble protein (estimated from absorbancies at 280 and 260 nm) was increased by 0, 4.5, and 9.5% of the zero time control in experiments A, B, and C, respectively. When trypsin was added directly to the *nit-1* extracts (0.01 mg of trypsin per ml) immediately prior to the addition of the extract

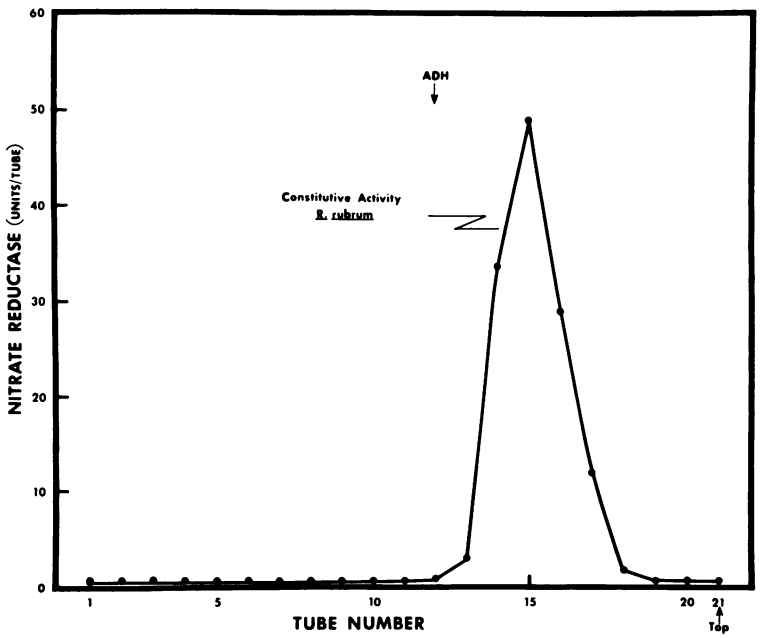

FIG. 4. *Sucrose density gradient of the constitutive component from R. rubrum. A crude extract of photosynthetically grown R. rubrum was centrifuged at 144,000 × g for 1.5 h. The complementing activity was concentrated eightfold in the 0 to 50% saturated ammonium sulfate fraction. A sample of this fraction was centrifuged at 175,000 × g for 17 h in a 15.5 to 33% (wt/vol) sucrose density gradient in 0.02 M (K_2HPO_4/KH_2PO_4) buffer, pH 6.5. One tenth of a milliliter of each fraction was incubated with nitrate induced N. crassa, nit-1 for 45 min and assayed 10 min for NADPH-nitrate reductase. The ordinant represents units of nitrate reductase formed per 45 min. Recovery is equal to 45% of the activity placed on the gradient.*

TABLE 3. *Purification of the constitutive component from extracts of R. rubrum*

Exp	Fraction	Total U	Total Protein[a]	Sp Act
1	20,000 × *g* supernatant[b]	7,224	52.8	137
	144,000 × *g* supernatant	4,326	40.2	108
	Protamine sulfate supernatant	6,225	26.4	236
2	20,000 × *g* supernatant[b]	7,030	96.2	73
	144,000 × *g* supernatant	11,285	51.8	218
	Protamine sulfate supernatant	10,453	29.6	353
3	20,000 × *g* supernatant[b]	10,588	56.0	189
	144,000 × *g* supernatant	12,250	32.2	380
	Protamine sulfate supernatant	12,688	28.7	434

[a] The 20,000 × *g* supernatant fluid containing 26.4, 30.0, and 16.0 mg of protein per ml in experiment 1, 2, and 3, respectively.

[b] Cells suspended in 0.02 M (Na_2HPO_4/NaH_2PO_4) buffer containing 10^{-2} M $NaNO_3$ and 10^{-4} M Na_2MoO_4 were ruptured and then adjusted to pH 6.0 before centrifugation.

of *R. rubrum*, the amount of NADPH-nitrate reductase formed was reduced 95% when compared to a control without trypsin. Therefore, the sensitivity of the *nit-1* preparation to trypsin (23,000 daltons) represents an internal experimental control for the permeability of the dialysis membrane. Dialysis experiments were also performed by using protease (Fig. 6). When protease was added to the *R. rubrum* extract (outside) in final concentrations of 0.33 and 0.66 mg/ml, the amount of NADPH-nitrate reductase formed inside the dialysis sac containing *nit-1* was not greatly increased over the control after 120 min of dialysis. After 120 min, the amount of trichloroacetic acid-soluble protein was increased by 0, 22, and 23.8% of the zero time control, respectively, in experiments A, B, and C. When 0.01 mg of protease was added to 1 ml of the *nit-1* extract, which in turn was immediately used for the in vitro formation of NADPH-nitrate reductase with samples of a *R. rubrum* extract, there was a 50% reduction in the amount of NADPH-nitrate reductase formed when compared to a control without protease. Neither nitrite nor the constitutive component was detected inside the dialysis sac during 1 h of dialysis of extracts of *R. rubrum* against buffer in place of the *nit-1* extract. Similarly, when extracts of *nit-1* were dialyzed for 1 h against buffer, no nitrite was detected and no NADPH-nitrate reductase was formed inside the dialysis sac.

DISCUSSION

Extracts of *R. rubrum* contain the constitutive component which reacts in vitro with the nitrate reductase mutant of *N. crassa*, *nit-1* to form NADPH-nitrate reductase which is essentially identical to the *N. crassa* wild-type enzyme. The constitutive component is present in both photosynthetically grown and heterotrophically grown cells, is soluble after centrifugation at 144,000 × *g* for 1 h, and is heat sensitive.

The physiological role of the constitutive component in *R. rubrum* is not known. Contrary to previous reports (6, 7, 25), we have been unable to grow *R. rubrum* on nitrate as the sole nitrogen source or to demonstrate nitrate-reductase activity in cell-free extracts of *R. rubrum*. Moreover, extracts of *R. rubrum* grown on glutamate and ammonium chloride do not contain xanthine oxidase, xanthine dehydrogenase, or sulfite oxidase. It is unlikely that these extracts contain nitrogenase since the above conditions of growth (ammonia and glutamate) are known to repress the synthesis of the nitrogenase which is present in *R. rubrum* (1, 13); however, the constitutive component may be a precursor for the synthesis of nitrogenase in *R. rubrum*. The above molybdenum-containing enzymes can contribute the constitutive component to the in vitro formation of NADPH-nitrate reductase (8, 16) after they are acid treated to a pH below 2.6. Acid treatment is known to dissociate multimeric enzymes into their protein subunits (3, 17). Since the *R. rubrum* extracts did not contain measurable amounts of xanthine oxidase, xanthine dehydrogenase, or sulfite oxidase and since acid treatment was not a prerequisite for the observation of the constitutive activity in extracts of *R. rubrum*, the constitutive component in *R. rubrum* exists as an entity which is independent of these molybdenum-containing enzymes.

Purification of the constitutive component has been difficult due to its instability in dilute solutions and due to its inactivation after treatments with ammonium sulfate, acetone, or trichloroacetic acid. Nevertheless, the specific activities of the partially purified constitutive component from *R. rubrum* approach the reported values (487 U per mg of protein) for the same activity in acid-treated preparations of

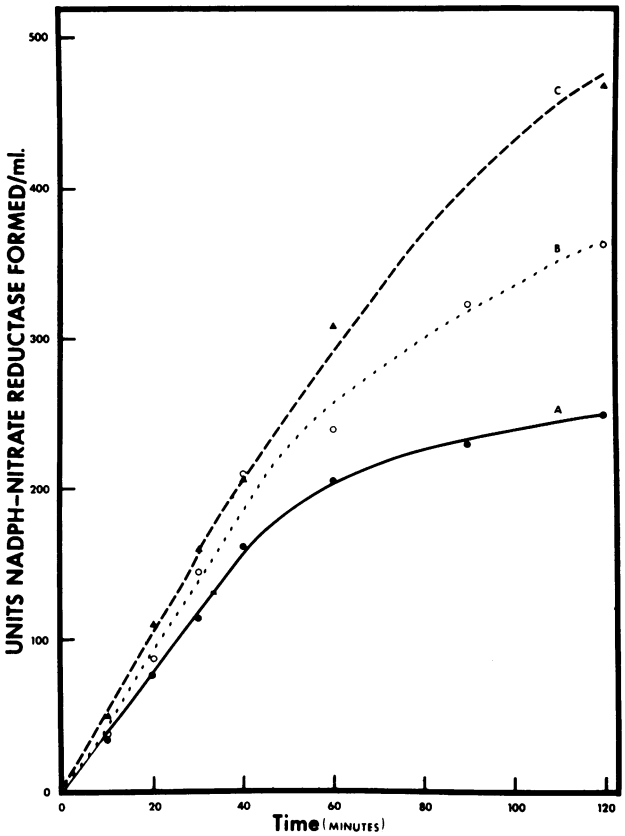

FIG. 5. *Dialysis of nitrate-induced N. crassa, nit-1 extracts against trypsin-treated extracts of R. rubrum. Crude extracts of nitrate-induced nit-1 (1.5 ml) were placed in dialysis sacs and dialyzed against 1.5 ml of the 144,000 × g supernatant fluid of R. rubrum; A, with no additives; B, containing 0.25 mg of trypsin, and C, containing 0.5 mg of trypsin. The dialysis was performed at room temperature. Samples were removed from inside the dialysis sac at timed intervals and assayed for the presence of NADPH-nitrate reductase.*

purified xanthine oxidase (8). Experiments are currently in progress to purify further the constitutive component from *R. rubrum*.

The constitutive component is presumed to contain molybdenum. This hypothesis is supported by the observation (8, 16) that all known molybdenum-containing enzymes can contribute the constitutive component to the in vitro formation of NADPH-nitrate reductase. Furthermore, our data show that molybdenum as well as nitrate stimulates the activity of the constitutive component in extracts of *R. rubrum* at or near neutral pH.

The sedimentation characteristics of the constitutive component suggest that this component is associated with a large molecular weight moiety (probably protein). However, the dialyzability of the constitutive component suggests that this component is a low molecular weight entity. The constitutive component is also insensitive to trypsin and protease treatment. The formation of NADPH-nitrate reductase during dialysis was observed to increase substantially when increasing amounts of trypsin were added to the *R. rubrum* extract. These experiments suggest that the active constitutive component is bound to a protein carrier molecule(s) in extracts of *R. rubrum*, and that the active constitutive component dissociates from the large molecular weight protein carrier prior to passage through the dialysis membranes. Trypsin treatment of the constitutive component may increase the rate of dissociation of the active constitutive component by altering the protein carrier molecule(s). Therefore, it is probable that *R. rubrum* contains a low molecular weight molybdenum-containing component that is required for the activity and structure of the NADPH-nitrate reductase formed in vitro in extracts of *N. crassa, nit-1*. Evidence re-

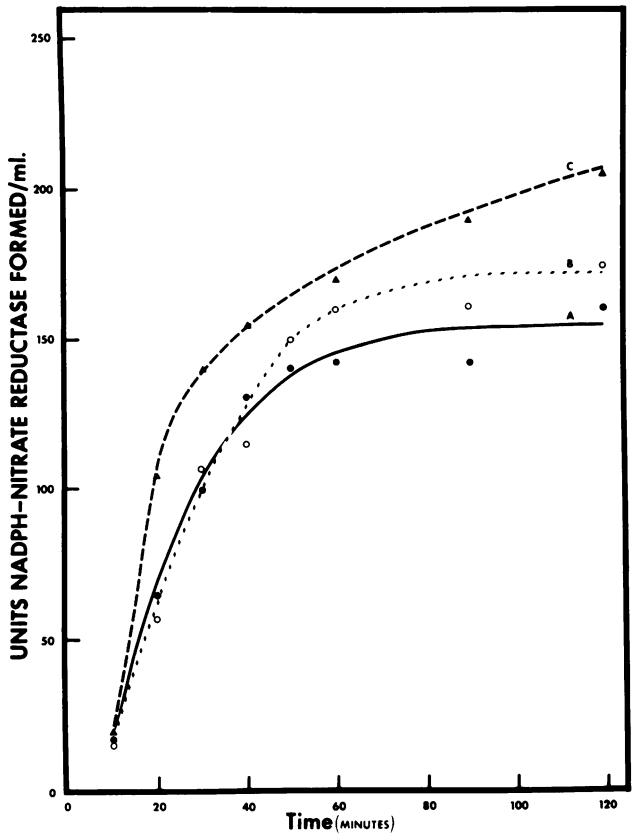

FIG. 6. *Dialysis of nitrate-induced N. crassa, nit-1 extracts against protease-treated extracts of R. rubrum. Crude extracts of nitrate-induced nit-1 (1.5 ml) were placed in dialysis sacs and dialyzed against 1.5 ml of the 144,000 × g supernatant fluid of R. rubrum; A, with no additions; B, containing 0.5 mg of protease; and C, containing 1.0 mg of protease. The dialysis was performed at room temperature. Samples were removed from inside the dialysis sac at timed intervals and assayed for NADPH-nitrate reductase.*

ported previously (8, 16, 23) suggests that this component is also a functional part of all the known molybdenum-containing enzymes.

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ADDENDUM IN PROOF

The presence of the constitutive component in selected bacteria other than *R. rubrum* and its dialyzable nature have been reported by P. A. Ketchum and R. S. Swarin (Biochem. Biophys. Res. Commun. **52**:1450–1456).

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