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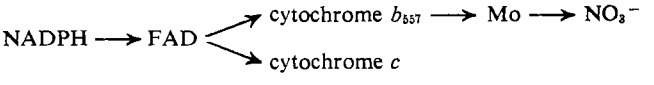
Purification and Properties of the Nitrate Reductase Isolated from *Neurospora crassa* Mutant *nit*-3. Kinetics, Molecular Weight Determination, and Cytochrome Involvement†

Alan D. Antoine

ABSTRACT: The nitrate reductase from *Neurospora crassa* mutant *nit*-3 exhibited reduced FAD–nitrate reductase and reduced methylviologen–nitrate reductase activities and the activities were nitrate inducible. The nitrate reductase was purified and characterized. The reduced FAD–nitrate reductase and reduced methylviologen–nitrate reductase activities of the mutant *nit*-3 enzyme remained associated following a 180-fold purification with the most highly purified fraction being homogeneous on disc gel electrophoresis. Both associated activities were relatively stable to treatment at 50° and inhibited by *p*-hydroxymercuribenzoate, had a pH optima between 7.0 and 8.0 depending on the type of buffer used and exhibited substrate affinities slightly different from the parent wild-type enzyme. The enzymatic activities reside in a protein complex having a sedimentation coefficient value of 6.8 ± 0.1 S, a Stokes radius of 56 ± 1 Å, and an estimated molecular weight of 160,000 ± 2000. The cytochrome associated with the homogeneous mutant enzyme exhibited a cytochrome b_{557} absorption spectrum. The properties of this mutant enzyme were compared to those of the partially purified enzyme from the parent wild-type *N. crassa* STA4.

Previous studies (Garrett and Nason, 1969) on the nitrate inducible nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.2) of wild-type *Neurospora crassa* strain 5297a, including the identification and sequence of action of its components, have postulated the following electron-transport scheme for this enzyme complex



It has been further shown by Garrett and Nason (1969) that the *Neurospora crassa* nitrate reductase exists as a single protein complex with a molecular weight of approximately 228,000 and exhibits four nitrate inducible associated en-

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zymatic activities, namely, FAD-dependent NADPH–nitrate reductase, FAD-dependent NADPH–cytochrome *c* reductase, reduced FAD–nitrate reductase, and reduced methylviologen–nitrate reductase. All four activities maintain a proportional relationship throughout a 500-fold purification of the enzyme and are sensitive to heat treatment and sulfhydryl inhibitors but to varying degrees.

Using nitrate reductase mutants of *N. crassa*, Sorger (1965, 1966) and Sorger and Giles (1965) have postulated that the native NADPH–nitrate reductase is an aggregate of at least two polypeptide chains, one that transports electrons from NADPH to FAD and then to cytochrome *c* and another which accepts electrons from reduced FAD and transfers them finally to nitrate. These investigators demonstrated that *N. crassa* mutant *nit*-1 exhibited an inducible NADPH–cytochrome *c* reductase activity following induction of *nit*-1 by nitrate. None of the other associated activities found in the induced wild-type were present in this mutant. The same authors suggested that the absence of these activities was due to the presence of a mutationally altered second polypeptide with no catalytic activity in this mutant. The function of the second polypeptide under the control of *nit*-1 would hence be to transport electrons from reduced FAD to nitrate *via* molybdenum. Cytochrome b_{557} (Garrett and Nason, 1967) would function in this portion of the electron-transport sequence in the wild-type enzyme. It was further shown by Sorger (1966) that mutant *nit*-3 contained a constitutive reduced benzylviologen–nitrate reductase activity but no NADPH–cytochrome *c* reductase nor NADPH–nitrate reductase activity, and was presumably synonymous with the second polypeptide, the terminal portion of the electron-transport sequence.

The present paper analyzes some of the structural and kinetic properties of the enzymatically active protein isolated from *N. crassa* mutant *nit*-3 and compares these properties to those of the wild-type nitrate reductase isolated from the parent strain of this mutant. A preliminary reporting of these results has been presented (Antoine, 1973).

Experimental Section

Cultures. *N. crassa* nitrate reductase mutant *nit*-3 (FGSC 358) as well as the parent wild-type strain, STA 4 (FGSC 262), were obtained from the Fungal Genetics Stock Center, California University, Humbolt, Arcata, Calif.

Cultivation and Induction Methods. The parent wild-type *N. crassa* strain STA4 was maintained on Fries basal medium (Garrett and Nason, 1969) containing $NaNO_3$ (6.9 g/l.). *N. crassa* mutant strain *nit*-3 was routinely maintained on Fries basal medium containing NH_4NO_3 (3.3 g/l.) as the sole nitrogen source and was tested periodically for the inability to grow on nitrate-containing growth medium. Growth temperature in all cases was 28°. Conidia transferred aseptically from 1.5% agar slants to sterile distilled water were used for inoculation of all experiments. Kilogram amounts of mycelia were generally obtained by cultivation in 30-l. batches of basal medium containing NH_4Cl (4.2 g/l.) until growth achieved a wet weight of about 20 g/l. At this time, the mycelium was harvested by filtration, washed with distilled water and then induced for 3–4 hr in 30 l. of induction medium (0.1% $NaNO_3$–1.0% sucrose–0.1% KH_2PO_4). Following induction, the mycelium was washed with distilled water and stored at −10° until use. Aeration was maintained at 20 l./min during growth and induction procedures.

Purification of Enzymes. The NADPH–nitrate reductase from parent wild-type *N. crassa* STA4 was partially purified according to the procedure outlined previously for the wild-type strain 5297a (Garrett and Nason, 1969). The partially purified enzyme from STA4 used throughout these experiments had a specific activity (nanomoles of nitrite formed per 10 min per mg of protein) of 85,500 for the FAD-dependent NADPH–nitrate reductase activity, 47,000 for the reduced methylviologen–nitrate reductase activity and 22,000 for the reduced FAD–nitrate reductase activity. FAD-dependent NADPH–cytochrome *c* reductase activity was present (specific activity 98,800 nmol of cytochrome *c* reduced per min per mg of protein). The nitrate reductase synthesized by *nit*-3 (exhibiting reduced FAD–nitrate reductase and reduced methylviologen–nitrate reductase activities) was isolated and purified essentially in the same manner, except that the phase separation step was omitted and a final step was added involving preparative polyacrylamide gel electrophoresis (PAGE). The PAGE system reagents were adapted from the analytical systems of Davis (1964) and Clarke (1964). The polyacrylamide gel stock reagent had the following composition per 100 ml: acrylamide, 7.5 g; *N,N*-methylenebisacrylamide, 0.2 g; *N,N,N,N'*-tetramethylethylenediamine, 0.06 ml; Tris, 4.5 g; adjusted to final pH of 8.5 with 1 N HCl. Fifty milliliters of the above solution was rapidly mixed with solid ammonium persulfate (35 mg/50 ml) to initiate polymerization, added to the PAGE apparatus (Quickfit, Instrutec Corp., Fairfield, N. J.) and overlaid with 5 ml of distilled water. The gel solidified within 2 hr at 25° and was then chilled to 4°. The polymerized gel surface was then washed by aspiration with Tris–glycine buffer (0.6 and 2.9 g per l., respectively), pH 8.5. The same buffer was used in the upper and lower reservoirs as well as for enzyme elution. Protein samples ranging in concentration from 25 to 50 mg in a total volume of 5 ml were dialyzed against distilled water for 3 hr prior to electrophoresis. The dialyzed enzyme was mixed with 2 ml of a sucrose (10%)–Bromophenol Blue (0.1%) solution and added with a syringe through the upper reservoir buffer onto the top of the polymerized acrylamide gel. Electrophoresis was performed at 400 V with refrigeration and generally took a period of from 10 to 15 hr.

Assay and Analytical Procedures. Sources of substrates, cofactors, and other chemicals and all standard enzyme assays have been previously cited (Garrett and Nason, 1969). A unit of enzyme activity, regardless of the electron donor source, is expressed as the formation of one nanomole of nitrite from the substrate, nitrate, in 10 min. Protein was determined according to the method of Lowry *et al.* (1951) or by reading the absorbance at 260 and 280 nm. Colorimetric readings were made using a Gilford Model 300 spectrophotometer. Sedimentation analyses were performed using linear sucrose gradients (15.5–33.0% w/v) according to the procedure of Martin and Ames (1961). Determination of molecular weights were done by the method of Siegel and Monty (1966). Absorption spectra were determined with a Cary Model 14 recording spectrophotometer. Analytical polyacrylamide gel disc electrophoresis was according to the procedure of Clarke (1964).

Results

Enzyme Induction. The use of the induction technique (Figure 1) had the effect of producing much higher specific activities in the crude extracts of *N. crassa* mutant *nit*-3, generally 10-fold greater specific activity levels, when compared to growth alone in Fries medium containing NH_4NO_3 as the growth substrate and inducer. The presence of high initial

TABLE I: Summary of Purification Scheme.[a]

Fraction No.	Procedure	Vol (ml)	Protein (mg)	Reduced FAD–Nitrate Reductase Act. (Units/ml)	Reduced FAD–Nitrate Reductase Sp Act. (Units/mg)	Reduced FAD–Nitrate Reductase Recov (%)	Reduced Methylviologen–Nitrate Reductase Act. (Units/ml)	Reduced Methylviologen–Nitrate Reductase Sp Act. (Units/mg)	Reduced Methylviologen–Nitrate Reductase Recov (%)
1	Crude extract	5100	35,000	198	29	100	6,120	890	100
2	pH 5.1 supernatant	4850	16,400	173	51	83	5,240	1,550	81
3	First 50% ammonium sulfate precipitate	610	9,340	1,640	107	98	47,400	3,100	93
4	DEAE-cellulose column	765	2,850	829	222	63	24,700	6,640	61
5	Second 50% ammonium sulfate precipitate	96	2,400	5,910	232	56	167,000	6,420	51
6	35–40% ammonium sulfate precipitate	48	1,270	9,520	356	45	277,000	10,500	43
7	Hydroxylapatite column	52	189	4,500	1,220	23	135,000	37,200	23
8	Third 50% ammonium sulfate precipitate	12	165	18,100	1,280	21	502,000	36,500	19
9	Sephadex G-200 column	6	29	19,400	3,950	12	576,000	119,000	11
10	PAGE column	15	12	4,250	5,220	6	124,000	152,000	6

[a] Procedures of the purification were outlined by Garrett and Nason (1969). Washed mycelia of nitrate (1260 g) induced *N. crassa nit*-3 were used in this purification scheme. The buffer generally present was 0.1 M phosphate buffer (pH 7.3), 0.001 M 2-mercaptoethanol, and 0.0005 M EDTA. Fractions 3 and 6 were dialyzed 4 hr *vs.* a lower phosphate buffer concentration (*i.e.*, 0.001 M) before proceeding to the next step. The procedure for fraction 10 is given in the section on experimental procedures. Fractions 9 and 10 were concentrated to these volumes by vacuum dialysis. One unit of activity is defined as the formation of 1 nmol of nitrite in 10 min assay time.

levels of activity greatly facilitated the purification of the enzyme. The same technique also enhanced the initial specific activities of the four associated nitrate reductase activities present in the parent wild-type, *N. crassa* STA4. Low levels of enzyme activity were present in mutant *nit*-3 prior to induction and averaged 1 and 18 (specific activity), respectively, for the reduced FAD–nitrate reductase and reduced methylviologen–nitrate activities.

Enzyme Purification. Purification of the reduced FAD–nitrate reductase activity and the reduced methylviologen–nitrate reductase activity of *N. crassa* mutant *nit*-3 resulted in a 180-fold purification of both activities. A typical purification scheme is shown in Table I. Both enzymatic activities remained in relatively constant proportions throughout the purification and the reduced FAD–nitrate reductase activity averaged 3–4% of the reduced methylviologen–nitrate reductase activity. This average was up to 40 or 50% in the STA4 enzyme. Both *nit*-3 activities exhibited coincident profiles from the DEAE-cellulose (DE-22) and Sephadex G-200 gel filtration columns. In addition, sucrose density centrifugation of an aliquot of fraction 10 showed that both activities were coincident. Analytical polyacrylamide gel disc electrophoresis in 7.5% gels at pH 9.4 of an aliquot of enzyme obtained from the PAGE system showed the presence of a single protein band with coincident activities (Figure 2A, section 7).

pH Optimum and Proportionality. The two associated activities exhibited by the *nit*-3 enzyme were stimulated to a greater extent by phosphate buffering than by Tris buffering and both activities were generally optimal between pH 7.0 and 8.0 (Figure 3). The rate of nitrate reduction was linear with time and proportional to protein concentration for both associated *nit*-3 enzyme activities. The same pH optima and proportionality were exhibited by the parent wild-type enzyme for the corresponding enzyme activities.

Substrate Affinities. From the reduced methylviologen sat-

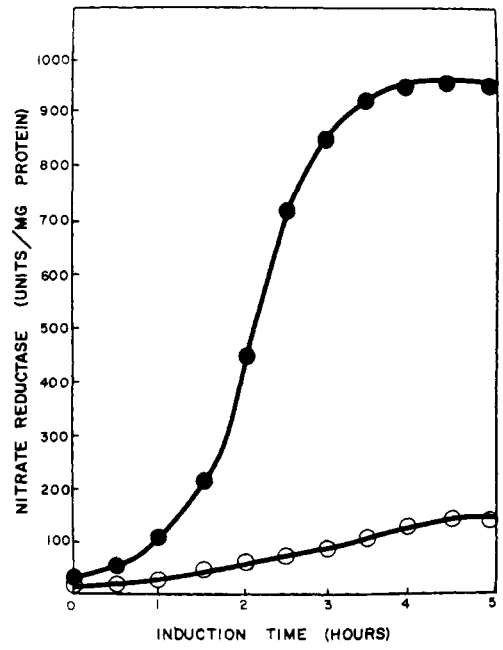

FIGURE 1: *N. crassa* mutant *nit*-3 was grown in 30 l. of NH_4Cl basal medium to a wet weight of 22 g/l., harvested, washed, and resuspended in the same volume of induction medium. Samples were harvested at various time intervals, homogenized in a tissue grinder, and centrifuged at 20,000*g* for 20 min. The supernatant fluid was tested for reduced FAD–nitrate reductase activity (○) and reduced methylviologen–nitrate reductase activity (●). The pellets retained less than 5% of the total activity.

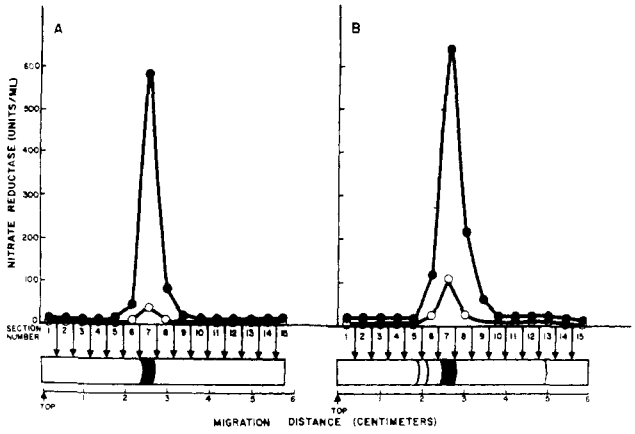

FIGURE 2: (A) Disc gel electrophoresis of *N. crassa* mutant *nit*-3 at pH 9.4 in 7.5% gels according to Clarke (1964). Fraction 10 (75 μg of protein), sucrose (2.5 mg) and Bromophenol Blue (2.5 μg) in a total volume of 0.1 ml were added to the top of replicate gel tubes and electrophoresis was performed at 6 mA/tube until the emergence of the tracking dye. Gels were then removed and either stained for protein with Naphthol Blue Black (0.1% in 7.5% acetic acid) followed by destaining with 7.5% acetic acid or sectioned (4 mm thick), homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.3), and assayed to locate the reduced FAD–nitrate reductase activity (○) and reduced methylviologen–nitrate reductase activity (●). (B) Same procedure for 100 μg of protein of partially purified wild-type STA4 nitrate reductase following exposure to 50° for 10 min. NADPH–nitrate reductase activity was zero.

uration curve for the purified *nit*-3 enzyme an apparent K_m of 8×10^{-6} M was calculated for this electron donor. Reduced benzylviologen produces almost identical saturation curves with the *nit*-3 enzyme and has an apparent K_m of 5×10^{-6} M. A lower affinity for reduced FAD was determined for the *nit*-3 enzyme and it was found that 20-fold greater concentrations of reduced FAD were required for enzyme saturation when compared to the saturating concentration of reduced

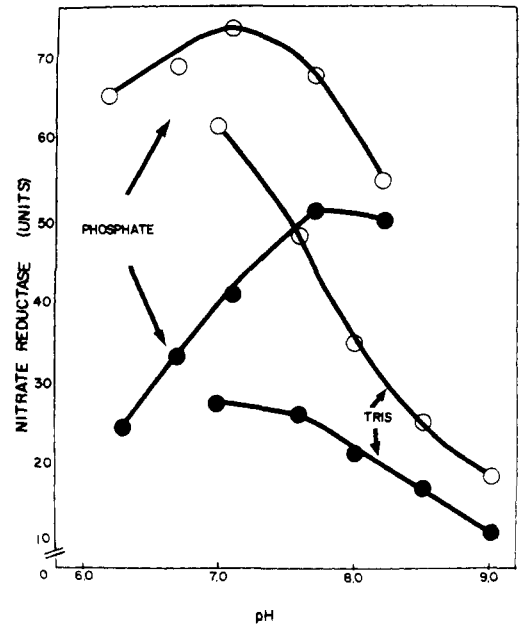

FIGURE 3: pH optimum curves of *N. crassa* mutant *nit*-3 nitrate reductase activities in 0.1 M phosphate or 0.1 M Tris buffer. Fraction 9 was used at a concentration of 20 μg of protein/assay for the reduced FAD–nitrate reductase activity (○) and 0.5 μg of protein per assay for the reduced methylviologen–nitrate reductase activity (●).

TABLE II: Kinetics of the Nitrate Reductase Associated Activities from *Neurospora crassa* mutant *nit*-3 and Wild-Type STA4.[a]

Activity	Substrate	App K_m Value (M) Enzyme Source *Nit*-3	STA4
Reduced FAD–nitrate reductase	$FADH_2$	2×10^{-4}	3×10^{-4}
	Nitrate	3×10^{-4}	4×10^{-4}
Reduced methylviologen–nitrate reductase	Reduced methylviologen	8×10^{-6}	2×10^{-5}
	Nitrate	9×10^{-4}	2×10^{-4}

[a] K_m values were calculated from Lineweaver–Burk plots of substrate saturation curves. The activities of the STA4 enzyme are cited under Experimental Section. Values represent the average of three determinations of three replicates each for all substrate concentrations tested.

methylviologen. From Lineweaver–Burk plots of the effect of nitrate concentration on the reduced methylviologen– and reduced FAD–nitrate reductase activities of the *nit*-3 enzyme, K_m's of 9×10^{-4} and 3×10^{-4} M, respectively, were calculated. Table II summarizes the data on the substrate affinities of the enzymatic activities of *nit*-3 and compares them to the same activities of the partially purified wild-type (STA4) enzyme. It is of interest that the reduced methylviologen–nitrate reductase activity of the *nit*-3 enzyme had a greater affinity for reduced methylviologen and a lesser affinity for nitrate when compared to the same activity of the wild-type enzyme.

Stability and Effect of Heat Treatment. The activities of the nitrate reductase from *N. crassa* mutant *nit*-3 were stable for periods of 6 months or longer under storage conditions at −20° with only a 5% loss of activity. The same activities of the wild-type enzyme were also stable under the same conditions of storage; however, the NADPH–nitrate reductase and NADPH–cytochrome *c* reductase activities of this enzyme were considerably more labile. The reduced FAD–nitrate reductase activity of the *nit*-3 enzyme lost 20% of the original activity after 30 min of heat treatment at 50°. The reduced methylviologen–nitrate reductase activity was unaffected by this treatment. Both activities were lost, however, by heat treatment of the *nit*-3 enzyme at 60° for 2 min. The stability of the *nit*-3 reduced methylviologen–nitrate reductase activity is of interest since the same activity of the parent wild-type enzyme exhibited a rapid two- to threefold activation following comparable heat treatment. At the same time, the FAD-dependent NADPH–nitrate reductase and NADPH–cytochrome *c* reductase activities of the wild-type enzyme are lost. The same results were found for the wild-type strain 5297a (Garrett and Nason, 1969). Retesting the heat-treated STA4 enzyme for substrate affinities showed a shift in K_m from 2×10^{-5} to 9×10^{-6} M for reduced methylviologen following heat treatment, the K_m for the same substrate of the *nit*-3 enzyme. The K_m values of the *nit*-3 enzyme were unaffected by this heat treatment. In addition, the relative migration rate of the heat-denatured wild-type enzyme reduced methylviologen–nitrate reductase activity corresponds to that of the *nit*-3 protein on disc gel electrophoresis at pH

TABLE III: Determination of Molecular Weights.[a]

Enzyme	Sedimentation Coef ($s^{0.725}_{20,w} \times 10^{13}$ sec)	Stokes Radius (Å)	Mol Wt (g)
$FADH_2$, reduced methylviologen–nitrate reductase from *nit*-3	6.8 ± 0.1	56 ± 1	160,000 ± 2000
NADPH–nitrate reductase from STA4	7.9 ± 0.1	70 ± 2	235,000 ± 3000

[a] Sedimentation coefficients were determined from sucrose density centrifugation experiments according to the method of Martin and Ames (1961). Measurement of the Stokes radii by Sephadex G-200 gel filtration chromatography and calculation of molecular weights were described in Siegel and Monty (1966) and Garrett and Nason (1969). Catalase and yeast alcohol dehydrogenase were used as standards. Results shown are the average of three determinations. Fraction 10 of purified *nit*-3 enzyme was used in all experiments.

9.4 (Figure 2B). The same activity of the untreated wild-type enzyme normally migrates to section 6 in this gel system.

Inhibition by p-Hydroxymercuribenzoate. The reduced methylviologen–nitrate reductase activity of the parent wild-type STA4 enzyme undergoes a 2-fold increase in activity following incubation with low concentrations (10^{-5} M final concentration) of *p*-hydroxymercuribenzoate while the NADPH-associated activities are completely inhibited. Similar findings were previously reported (Garrett and Nason, 1969). Under the same experimental conditions the reduced methylviologen–nitrate reductase activity of *nit*-3 is not similarly affected by *p*-hydroxymercuribenzoate (Figure 4). In addition, significant inhibition of the reduced FAD– and reduced methylviologen–nitrate reductase activities of the *nit*-3 and the STA4 enzymes was found only at high concentrations of *p*-hydroxymercuribenzoate.

Sedimentation Coefficient and Molecular Weight Determination. The techniques of sucrose density gradient centrifugation and gel filtration were applied for the determination of molecular weights of the *nit*-3 and STA4 enzymes. The molecular weight of a macromolecule can be estimated from the sedimentation coefficient, *s*, and the Stokes radius, *a*, obtained by these techniques. Table III summarizes the results for the sedimentation coefficient, Stokes radius and molecular weight of the *nit*-3 enzyme as compared to the same measurements of the parent wild-type STA4 enzyme. From these measurements a molecular weight of 160,000 ± 2000 for the reduced FAD– and reduced methylviologen–nitrate reductase of *nit*-3 was calculated. The molecular weight of 235,000 for the parent wild-type enzyme is slightly higher than that previously estimated for the wild-type *N. crassa* 5297a nitrate reductase (Garrett and Nason, 1969).

Cytochrome Content. The visible absorption spectrum of the purified nitrate reductase of *nit*-3 (fraction 10) has a major peak at 413 nm (Figure 5). The sodium hydrosulfite reduced difference spectrum, however, shows an α peak at 557 nm, a β peak at 530 nm, and a γ (Soret) peak at 425 nm. Similar absorption maxima also appeared on a spectrum of the wild-type STA4 enzyme. These spectra are typical of a *b*-type cytochrome and appear to be identical with the cytochrome b_{557} of the partially purified nitrate reductase from wild-type *N. crassa* 5297a (Garrett and Nason, 1967). The cytochrome component of the *nit*-3 enzyme was not reduced by NADPH in the presence of FAD as was the case with the *b*-type cytochrome of the parent wild-type STA4 enzyme.

Discussion

The wild-type *N. crassa* assimilatory NADPH–nitrate reductase is a discrete enzyme system and exists as a single protein complex under physiological conditions. It is noteworthy that the NADPH-associated activities of the wild-

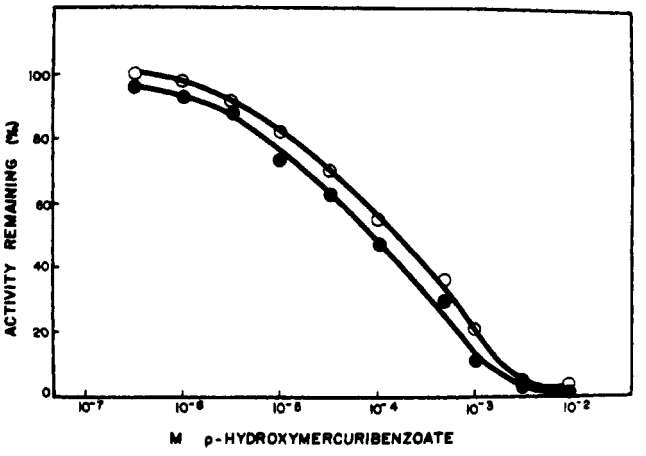

FIGURE 4: Effect of *p*-hydroxymercuribenzoate on the reduced FAD–nitrate reductase activity (○) and reduced methylviologen–nitrate reductase activity (●) of *N. crassa* mutant *nit*-3 enzyme. Assay tubes contained 20 and 0.8 μg of protein (fraction 9) for the respective activities and were preincubated for 10 min prior to assay in the presence of the indicated *p*-hydroxymercuribenzoate concentrations. Results were compared to enzyme activities without inhibitor under standard assay conditions.

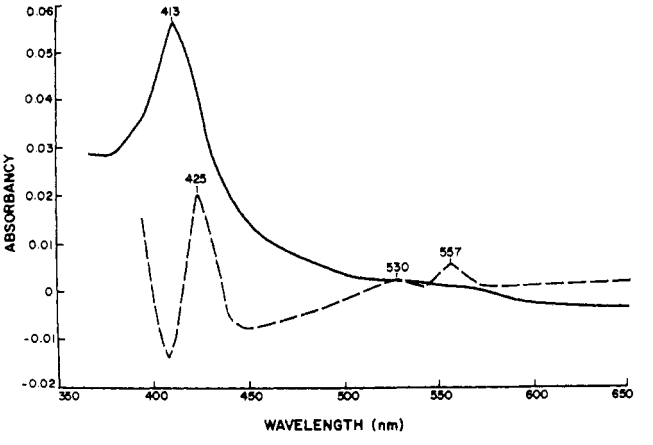

FIGURE 5: Oxidized (——) and reduced (— —) difference spectra of the purified nitrate reductase (fraction 10) from *N. crassa* mutant *nit*-3. The sample cuvet of the oxidized form contained 100 μg of protein/ml, using a reference cuvet of distilled water, while the cuvet of the reduced form contained 100 μg of protein/ml plus a few crystals of sodium hydrosulfite with a reference cuvet of enzyme alone.

type enzyme essentially disappear with heat treatment, inhibition with *p*-hydroxymercuribenzoate and following prolonged storage conditions, whereas the remaining two associated activities are stable and one (the reduced methylviologen–nitrate reductase activity) actually undergoes activation. These results support the hypothesis for the presence of at least two different protein subunits in the wild-type enzyme complex. Apparently one protein subunit is sensitive to denaturation while another is relatively stable and retains its two associated activities. It is also apparent from these results that wild-type nitrate reductase enzymes obtained from two different strains of *N. crassa* are similar if not identical. The properties of the parent wild-type STA4 enzyme used in these studies for comparative purposes are in close agreement with those previously published for strain 5297a (Garrett and Nason, 1969).

The present findings indicate that *N. crassa* mutant *nit*-3 exhibits a reduced FAD–nitrate reductase activity in addition to the reduced methylviologen–nitrate reductase activity. In addition, both activities are (a) present at low levels in the absence of the inducer, nitrate, and are nitrate inducible in *nit*-3; (b) coincident with each other following a 180-fold purification and upon sucrose density centrifugation indicative of an association in a single protein moiety; (c) represent two of the activities commonly associated with wild-type *N. crassa* nitrate reductase although the relative proportions of the two activities are different. In addition to the presence of the two associated activities in *nit*-3, the purified enzyme from this mutant also exhibited oxidized and reduced absorption spectra which are comparable to if not identical with the partially purified parent wild-type nitrate reductase. Both enzymes exhibit α, β, and γ (Soret) peaks indicative of a *b*-type cytochrome and which has been designated as cytochrome b_{557} (Garrett and Nason, 1967). The importance of this finding is that it reconfirms the association of this *b*-type cytochrome with the assimilatory nitrate reductase complex and also that it locates the cytochrome b_{557} component with the active *nit*-3 protein which may be synonymous with the protein subunit of the native enzyme complex exhibiting the activities of the terminal portion of the electron-transport sequence.

The determination of the K_m's for the various substrates of the *nit*-3 nitrate reductase has emphasized the similarities and differences between the kinetics of the mutant and the parent wild-type associated activities. No greater than fivefold differences were observed between the mutant and parent wild-type reduced methylviologen–nitrate reductase activity.

The results obtained following heat treatment deserve further comment. The nitrate reductase activities of *nit*-3 are stable to heat treatment at 50°. The same is nearly true for the comparable wild-type activities except that in this case the reduced methylviologen–nitrate reductase activity was activated two- to threefold by heat treatment. It would appear that the initial portion of the electron-transport scheme resides in a structure that is extremely labile to heat inactivation with a probable loss of tertiary structure. Concomitant with this loss of tertiary structure the accessibility of reduced methylviologen to its active site is increased either by an increase in turnover number or by one or more additional active sites becoming operational that were previously restricted. The site of reduced methylviologen–nitrate reductase activity of the *nit*-3 enzyme is not similarly restricted since the controlling protein subunit (the *nit*-1 locus) is absent. The reduced methylviologen–nitrate reductase activity of the *nit*-3 enzyme exists at maximum levels which partially explains the observed differences between the relative proportions of the comparable activities of the *nit*-3 and STA4 enzymes.

The inhibitory pattern exhibited by *p*-hydroxymercuribenzoate on the activities of the STA4 and *nit*-3 enzymes indicates the involvement of freely accessible sulfhydryl groups in the initial portion of the electron-transport chain and less accessible sulfhydryl groups in the terminal portion of the electron-transport chain. Since inhibition of the wild-type enzyme by low concentrations of *p*-hydroxymercuribenzoate was accompanied by a stimulation of the reduced methylviologen–nitrate reductase activity (similar to the effect of heat treatment), but not in the *nit*-3 enzyme, it is suggested that sulfhydryl groups may participate in the binding of the protein subunits that comprise the complete nitrate reductase complex.

Finally, molecular weight determinations show that the *nit*-3 enzyme represents a major fraction of the native nitrate reductase complex. Construction of the subunit structure of this enzyme complex will be aided by the purification of the NADPH–cytochrome *c* reductase from *N. crassa* mutant *nit*-1 and by the resolution of the native wild-type enzyme into its structural subunits.

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