A Comparison of Nitrate Reductase from *Neurospora crassa* Wild-Type and Mutant *nit-3*

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The assimilatory nitrate reductases (EC 1.6.6.1~3), which catalyze the two-electron reduction of nitrate to nitrite, the first step of nitrate assimilation, have been isolated from a number of different sources, including fungi, green algae, and higher plants.\(^1,2\) The enzymes accept electrons from pyridine nucleotides, and contain FAD, cytochrome b-557, and molybdenum as prosthetic groups.\(^3,4\) Besides the NAD(P)H-linked nitrate reduction, they also catalyze the reduction of cytochrome c by NAD(P)H and the reduction of nitrate by reduced flavin or reduced viologen dyes.\(^3,4\)

Usually assimilatory nitrate reductases are extremely heat-labile, and upon mild heat treatment NAD(P)H-linked activities, but not two other activities (reduced flavin- and reduced viologen dye-dependent nitrate reductase activities), decrease rapidly.\(^5\) Enzyme-bound FAD, one of the components of the intramolecular electron transport system, shows a protective role against the heat inactivation of the enzyme.\(^5\)

In this paper, we report on the assimilatory nitrate reductase of *Neurospora crassa*, which has been highly purified and studied extensively.\(^6\) In *N. crassa*, there are six mutants known to have defects in nitrate assimilation.\(^7\) Partial purification and some characterization have been reported on the nitrate reductase from one of the mutants, *nit-3*.\(^8\) The enzyme cannot accept electrons from reduced pyridine nucleotides, but accepts from reduced flavins and viologen dyes, so that it is thought that this enzyme does not play a physiological role in assimilatory nitrate reduction, and it has been pointed out that substrate affinities of the *nit-3* nitrate reductase are slightly different from the wild-type enzyme but are very similar to those of the heat-modified preparation of wild-type enzyme.\(^9\) Our results described here strongly suggest that the heat-modified enzyme seems to be identical to *nit-3* nitrate reductase.

*N. crassa* strains (wild-type: FGSC 987, *nit-3*: FGSC 358) obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, were cultivated at 30°C according to the method of Antoine.\(^8\) The mycelia (100 g), collected by filtration, were suspended in 200 ml of 200 mM potassium phosphate buffer (pH 7.5) containing 20 μM FAD, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, mixed with quartz sand (50 g), and ground in a mortar with a pestle for 30 min. The crude homogenate was centrifuged at 150,000 × g for 20 min to get a supernatant fraction. This crude extract was further purified by ammonium sulfate fractionation (33 ~ 50% saturation), followed by DEAE-cellulose, Sepharose 6B, and hydroxylapatite column chromatography. The specific activities of the final preparations are around 10 μmol nitrite formed/min/mg protein (NADPH-nitrate reductase activity of wild-type enzyme and reduced methyl viologen (MVH)-nitrate reductase activity of *nit-3* enzyme). Although neither preparation was yet electrophoretically homogeneous, their molecular properties were practically the same as in previous studies.\(^7,9\) NADPH- and MVH-nitrate reductase activities, sedimentation coefficients, and Stokes radius were determined by our method.\(^5\) Analytical slab gel electrophoresis was carried out according to the method of Davis,\(^10\) and MVH-nitrate reductase activities were stained by the method described by Amy and Garrett.\(^11\)

As shown in Fig. 1, incubation of the purified wild-type

![Fig. 1. Effects of Heating at 43°C on the Nitrate Reductase Activities of Wild-type and *nit-3*.

A 0.02 ml sample of each enzyme was diluted to 0.2 ml with 50 mM potassium phosphate buffer (pH 7.4) with or without 20 μM FAD, and placed in a water bath at 43°C. Some was withdrawn at each indicated time and assayed for the nitrate reductase activities. The results were compared with those obtained with the untreated enzyme sample. The symbols are: O, NADPH-nitrate reductase activity of wild-type enzyme; MVH-nitrate reductase activity of wild-type enzyme (Δ); and *nit-3* enzyme (□). Each open or closed symbol demonstrates the enzyme activities treated without or with addition of 20 μM FAD, respectively.
TABLE I. MOLECULAR SIZE AND Km VALUES OF WILD-TYPE, HEAT-MODIFIED, AND nit-3 NITRATE REDUCTASES

Heat-modified enzyme was prepared by the incubation of wild-type enzyme at 43°C for 30 min without addition of FAD. Determination of the Stokes radius and sedimentation coefficient was carried out as described in the text.

<table>
<thead>
<tr>
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<th>Wild-type</th>
<th>Heat-modified</th>
<th>nit-3</th>
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<tbody>
<tr>
<td>$s_{20,w}$</td>
<td>8.0</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Stokes radius</td>
<td>7.0</td>
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<td>6.2</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>228,000</td>
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<td>172,000</td>
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<tr>
<td>Km for MVH</td>
<td>20.0</td>
<td>8.4</td>
<td>8.0</td>
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<tr>
<td>Km for NO$_3^-$</td>
<td>0.23</td>
<td>1.0</td>
<td>1.1</td>
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Figure 2 shows the results of polyacrylamide slab gel electrophoresis of these three nitrate reductase preparations. Upon the detection of nitrite formation there appeared purple bands indicating the position of MVH-nitrate reducing activities. The mobility of the heat-modified enzyme clearly differs from the native wild-type enzyme and seems to be identical to that of nit-3 enzyme. The decrease in the molecular size and changes in catalytic properties and in mobility of slab gel electrophoresis described above strongly suggest that the heat treatment caused a marked change in protein conformation and a dissociation of a part of the native wild-type enzyme. Further, the remaining nitrate-reducing molecule seems to be identical to nit-3 enzyme. Although the data are not shown, the absorption spectrum of heat-modified enzyme closely resembles that of nit-3 enzyme, indicating the lack of enzyme-bound FAD in contrast to the wild-
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...type enzyme. And both heat-modified and *nit*-3 enzymes exhibited no affinities for the Blue Dextran Sepharose 4B column in the conditions where wild-type enzyme was completely absorbed, suggesting that they do not have pyridine nucleotide binding sites. From these observations, the NADPH-binding moiety including the FAD portion might have dissociated from the native enzyme upon heat-treatment.

The *nit*-3 nitrate reductase is considered to be either: (a) a dimer of a cryptic gene product (deletion or nonsense mutation) of approximately 80,000 to 90,000 molecular weight which can still interact with the molybdenum-containing cofactor and cytochrome *b*-557 to give a partially functional nitrate reductase molecule or (b) a dimer of a proteolytic fragment generated by the protease action of the abnormal *nit*-3 gene product, which nevertheless retains the ability to participate in the partial enzyme functions. A similar mutant enzyme has been found in *Chlamydomonas reinhardii* mutant 305, and a heteromultimeric structure for the native nitrate reductase complex has been proposed. Although two identical subunits composing *N. crassa* wild-type nitrate reductase have been reported by Pan and Nason, there is still some possibility of a heteromultimeric nature from our observations.

REFERENCES