TPNH-CYTOCHROME C REDUCTASE AND NITRATE REDUCTASE IN MUTANT AND WILD TYPE NEUROSPORA AND ASPERGILLUS

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Nitrate reductase has been shown (Nicholas and Nason, 1954) to be an electron transfer system in which the initial electron donor is usually reduced triphospho-pyridine nucleotide (TPNH) and the final acceptor is nitrate. The sequence of transfer of the electron is from TPNH to flavine adenine dinucleotide (FAD) to molybdenum to nitrate.

Cytochrome c reductase and nitrate reductase of Neurospora crassa have been shown to be intimately associated during purification (Kinsky and McElroy, 1958), the ratio of the two activities remaining constant beyond the first ammonium sulphate precipitation up to a seventy-fold concentration of nitrate reductase by either of two different methods. Both activities are adaptive on nitrate; this further suggests that they are functionally associated.

In the present studies, a very similar picture has emerged. Both specific activities increase simultaneously when a mycelial pad is transferred from a medium with no nitrate to one in which nitrate, or nitrite, is the sole source of nitrogen; both simultaneously decrease when the pad is transferred from a medium containing nitrate as the sole source of nitrogen to a medium containing no nitrate. Sucrose density gradient

centrifugations of crude extracts from nitrate adapted Neurospora show nearly identical activity profiles for the two activities.

The evidence just described does not rule out the possibility that the entire catalysis is carried out by an aggregate of units of which each carries out part of the reduction, but of which each individual component is incapable of effecting the entire electron transport from TPNH to nitrate.

It seems quite reasonable to accept Kinsky and McElroy's interpretation that the cytochrome c reduction is a branch function of nitrate reductase. That cytochrome c is not an intermediate in the main path of the redox chain is suggested by the fact that if one reduces cytochrome c with TPNH using nitrate reductase, the cytochrome cannot be reoxidized anaerobically with nitrate in the presence of this enzyme (Kinsky and McElroy, 1958).

Forty mutants lacking nitrate reductase and able to grow on nitrite but not on nitrate, have recently been reported in Aspergillus (Cove and Pateman, 1963). In that study, at least six cistrons have been implicated in the control of the reductase. Previous investigations with Neurospora have demonstrated the existence of two classes of nitrate reductaseless mutants (Silver and McElroy, 1954): the first has an inhibitor of nitrate reductase, the second grows on nitrate at pH7.0 but not at pH5.6. Complementation studies with these mutants were complicated by the varied genetic backgrounds of the auxotrophs, and the mutants were not mapped by recombination. In other studies (Barratt et al., 1954; Houlahan et al., 1949; Perkins, 1959), three different genes controlling the utilization of nitrate nitrogen have been identified, but no enzymological studies were performed.

It is pertinent at this point to ask which gene controls which step in the reduction sequence. To attempt to answer this question a comparative

genetic and enzymological study was carried out with the three genetically mapped nitrate non-utilizing strains and with 35 new mutants of Neurospora crassa strain 74A produced in this laboratory. These mutants are all characterized by a) having no detectable nitrate reductase; b) their lack of growth on nitrate as a sole source of nitrogen; c) their growth like wild type on ammonia or nitrite; d) their inability to accumulate measurable amounts of nitrite when grown on a one percent nitrate medium (Sorger, 1963).

Three genes have been shown to be concerned with nitrate reductase by forced complementation studies; these genes have been found by recombination studies to correspond to the three known loci controlling the utilization of nitrate nitrogen. One mutant (nr 15), however, has thus far defied classification into one of these three categories, and may represent a fourth locus (Sorger, 1963).

Each mutant locus is associated with a different phenotype. The first (nit-1) lacks the overall reductive function, but retains the nitrate adaptive cytochrome c reductase. The second (nit-2) has neither adaptive activity. The third (nit-3) lacks both adaptive reductases and behaves differently from the others in crude extract mixtures (Table 1). When a volume of cell-free extract from a nitrate-adapted or unadapted nit-1 or nit-2, or from unadapted wild type is mixed with an equal volume of nitrate-adapted wild type, nitrate reductase activity is greater than if one dilutes with the same volume of buffer. If one does the same experiment with a crude extract from nit-3, nitrate reductase activity is equal to or less than for a similar dilution with buffer. Cytochrome c reductase activity is halved in all such crude extract mixtures. The one genetically mapped Aspergillus nidulans mutant, ni-3, (Käfer, 1958) has been studied as a comparison, and it has been found to resemble nit 1. It has no nitrate reductase, but possesses

Table 1

Nitrate (NR) and cytochrome c (CR) reductases in wild type and mutant Neurospora

Strain	Chromosomal Location	Complementation Group	N Source *	Mean specific activity ± σ CR**	NR***
WT	-	-	NO_3^-	87 ± 12	7.2 ± 0.7
"	-	-	-N	23 ± 1	0
nit-1	IR	A	NO_3^-	86	0
"	IR	A	-N	36	0
nit-2	IL	B	NO_3^-	20 ± 5	0
"	IL	B	-N	21 ± 5	0
nit-3	IVR	C	NO_3^-	29 ± 5	0
"	IVR	C	-N	18 ± 3	0

* An inoculum was grown to a mycelial pad in a standing culture in which NH_3 was the sole source of nitrogen. The pad was then cut into nearly equal sectors, and each sector treated as follows: NO_3^- means the sector was shaken for sixteen hours in the dark on a rotary shaker in a medium resembling Fries but containing sodium potassium tartrate and sodium nitrate instead of ammonium tartrate and ammonium nitrate; -N means the sector was shaken in a medium resembling the above, but without a nitrogen source.

** Cytochrome c reductase was measured by the method of Kinsky and McElroy (Kinsky and McElroy, 1958). One unit specific activity is defined as a change in absorbance of 0.01/minute at 550 mμ in a 1 cm light path per mg. of protein at room temperature.

*** Nitrate reductase was measured by a modified version of the method described by Nicholas and Nason (Nicholas and Nason, 1954). The assay mixture was: 0.05 ml 2×10^{-3} M TPNH; 0.05 ml 10^{-5} M FAD; 0.10 ml 10^{-1} M KNO_3; 0.20 ml 2×10^{-1} M Pyrophosphate buffer pH7.0; 0.1 to 0.2 ml extract, and 10^{-1} M phosphate buffer pH7.0 (which is the extraction buffer) to compensate for the difference in volume due to the addition of varied amounts of extract. The reaction was stopped by adding 0.1 ml 25.5 percent barium acetate to precipitate the pyridine nucleotides and subsequently 2.5 ml 95 percent ethyl alcohol to precipitate the proteins. The suspension was then centrifuged and the 3 ml of clear supernatant assayed for nitrite colorimetrically with 1 ml each of 1 percent sulfanilamide in 3.2 M HCl and 0.02 percent naphthyl ethylene diamine. Colour was allowed to develop for 30 minutes before reading. Activity was measured as the difference between samples with and without TPNH in the assay mixture, one unit being defined as a difference in absorbance at 540 mμ of 0.01 in a 1 cm light path after 15 minutes reaction time at room temperature. Specific activity is expressed as units per mg. protein.

a nitrate-adaptive cytochrome c reductase (Table 2).

Table 2

Nitrate (NR) and cytochrome c (CR) reductases in wild type and mutant Aspergillus *

Strain	Exp.**	Chromosomal Location	N Source	Mean Specific Activity ± σ CR	NR***
WT	1	-	NO_3^-	120	12.7
"	1	-	-N	40	0.7
"	2	-	NO_3^-	66	7.5
"	2	-	-N	30	0.4
"	3	-	NO_3^-	77 ± 16	25.0 ± 1.9
"	3	-	-N	43 ± 15	0.8 ± 1.1
ni-3	1	IIR	NO_3^-	70	0.8
"	1	"	-N	35	0.0
"	2	"	NO_3^-	120	0.3
"	2	"	-N	25	0.0
"	3	"	NO_3^-	63 ± 6	0.0
"	3	"	-N	51 ± 3	0.0

* Provided by E. Käfer as a complex stock and outcrossed by Jean Foley. Its genotype is y ni-3.

** Experiments 1, 2 and 3 differ in the conditions used for adaptation on nitrate.

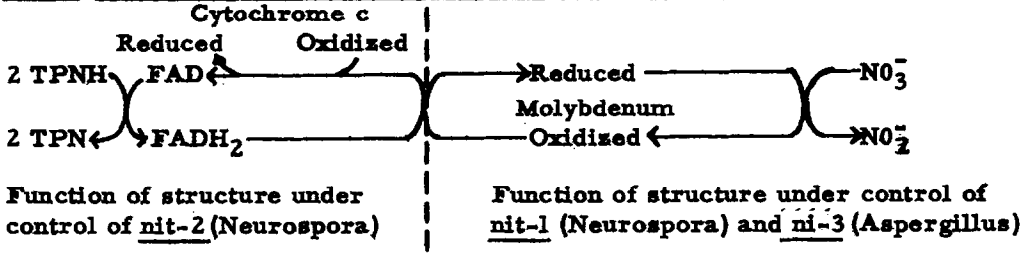
*** Nitrate reductase was measured as before, except for the incubation time which is 30 minutes and the quantity of extract added which is 0.4 ml. Specific activity is therefore defined as difference in absorbance at 540 mμ in 30 minutes per mg. of protein in the 3.2 ml. of clear supernatant.

The above evidence provides a working model for nitrate reductase in Neurospora and Aspergillus; two structures, at least, must co-operate to carry out the entire reduction from TPNH to nitrate. The first carries the electron from TPNH to FAD, and if supplied with cytochrome c under the right conditions, will transfer the electron to it. This structure is probably under the control of nit-2, because when this locus is altered, no nitrate adaptive cytochrome c reductase is produced. The second structure

carries the electron from FAD to molybdenum and then to nitrate and is probably under the control of nit-1, since in this mutant class the entire reductase cannot function; however, the nitrate adaptive cytochrome c reductase still can. (In E coli it has already been shown that an enzyme which carries the electron from cytochrome b to molybdenum and thence to nitrate can be separated from the remainder of the electron transport chain.) (Taniguchi et. al., 1959). When both structures are associated under the right conditions, they are capable of co-operating to carry out the entire electron transfer. The fact that nit-3 represents a mutant class not included in the hypothesis and that nr 15 represents perhaps another one suggests the model is an oversimplification. At this point, however, it is not very useful to include these mutants in the hypothesis.

The hypothesis is summarized schematically below in Figure 1.

Fig. 1. Functional Model of Nitrate Reductase in Neurospora and Aspergillus



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