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Correlation Between Reduced Nicotinamide Adenine Dinucleotide Phosphate Levels and Morphological Changes in *Neurospora crassa*

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A colonial mutant of *Neurospora crassa*, previously shown to be altered in the structure of glucose-6-P dehydrogenase [a reduced nicotinamide adenine dinucleotide phosphate (NADPH) producing reaction], contained only 40% as much NADPH in extracts as did the wild type. A partial revertant strain, when grown at 23 C, had the same total NADPH content as the wild type, but, at 34 C, had lower levels of NADPH as well as a colonial morphology. A revertant with complete wild-type morphology had wild-type levels of NADPH. Two different colonial mutants, which have also been reported to be altered in NADPH-generating reactions, were found to have a lower content of NADPH, whereas other colonial mutants had wild-type levels. The wild-type strain, when grown under conditions in which it contained a lower total content of NADPH, had a morphology similar to that of a colonial mutant. The evidence indicates that lowered NADPH content leads to a dramatic alteration in the morphology of *Neurospora*, but not necessarily vice versa. The possible pleiotropic effects of the NADPH deficiency are discussed.

One operational approach for studying mutations which produce observed morphological changes is to try to determine the single biochemical cause for each change, i.e., which primary gene product has been altered in a given mutant. Once the specific enzymatic deficiency is known, then it might be possible to document some of the pleiotropic effects produced by this mutation, and thereby further the description of the morphological changes in a more specific biochemical manner. This paper deals mainly with a pleiotropic effect produced by a mutation at the *col-2* locus in *Neurospora crassa*. Mutation at the *col-2* locus led to a slow growth rate and compact mycelial form, as opposed to the rapid growth and filamentous form of the wild type. The primary biochemical effect of this mutation was a change in the structure of the enzyme, glucose-6-P dehydrogenase. The altered enzyme was present in normal amounts but had reduced affinities for its substrates, glucose-6-P and nicotinamide adenine dinucleotide phosphate (NADP). Another effect of this mutation was an accumulation of glucose-6-P in the *col-2* strain as compared to the wild-type strain (1, 3). The partial block in the pentose phosphate shunt because of the altered glucose-6-P dehydrogenase could not be alleviated by growth of *col-2* on certain pentoses as carbon sources (3). Therefore, attention was focused on a possible decrease in the rate of production of NADPH by the altered enzyme. This paper reports on the correlation between the total content of NADPH in a variety of strains and the morphology of these strains.

MATERIALS AND METHODS

Cultures. *RL3-8A* (wild type), *col-2*, *col-3*, *rg*, *col-2b₂ inosS1*, and *col-2b₂ inosR11* (revertant) have all been described previously (1, 2). Other morphological mutants (balloon, doily) were obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, N.H. The double mutant *bal col-2* was isolated from a cross of *bal* × *col-2*. This double mutant grows more slowly than either parental strain and was picked only from those ordered tetrads which had all recombinants, i.e., four wild types and four double mutants.

Reagents. Nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-P, lactic dehydrogenase type II, glutamic dehydrogenase type I, and glucose-6-P dehydrogenase type VI were all ob-

tained from Sigma Chemical Co., St. Louis, Mo. Alcohol dehydrogenase and α-ketoglutarate were obtained from Boehringer Mannheim. Triethanolamine-phosphate buffer (*p*H 6.0) was 0.5 M triethanolamine-hydrochloride, 0.4 M KH_2PO_4, and 0.1 M K_2HPO_4.

Growth conditions. All cultures were grown as liquid shake cultures in Vogel's minimal medium (9) containing 2% glucose at 23 C unless otherwise stated. Most of the cultures were grown in 50 ml of this medium in a 125-ml Erlenmeyer flask. A few very slow-growing strains were grown up in this way, briefly sheared in a sterile Waring blendor, and then the sheared inoculum was added to 300 ml of minimal medium in a 1-liter flask. Cultures inoculated in this way were grown for 2 days, at which point >90% of the dry weight was the result of fresh growth. In all cases, only actively growing cultures were extracted, i.e., those that could still double their dry weight when allowed to continue their growth.

Extraction and assay procedures for NADPH and NADH. Mycelia were rapidly harvested by suction filtration, were continuously washed with distilled water, and were never completely dried on the filter paper. The entire process was completed in 30 sec or less. The washed cultures were then immediately immersed in 3 ml of a 0.5 N alcoholic (50% ethyl alcohol) KOH solution (preheated to 70 C) and stirred with a spatula for exactly 1 min in a water bath at 70 C (6). The tubes were removed and chilled with stirring in a water-ice bath for 30 sec, and the extracts were then neutralized to approximately *p*H 7 by the slow addition (with stirring) of 2.0 ml of cold triethanolamine-phosphate buffer. A second extraction with hot alcoholic KOH yielded little (less than 5%) additional NADH or NADPH and was therefore not employed. The supernatant solutions were assayed after centrifugation for 10 min at 27,000 × *g* (4 C). The final volume of the extract and the residual dry weight of the extracted mycelia (after a 95% ethyl alcohol wash and overnite drying at 37 C) were measured.

The assay procedure for NADH and NADPH was a spectrophotometric one, using highly purified enzymes. Cuvettes contained varied amounts of extract, 0.02 ml of neutralized α-ketoglutarate (32 mg/ml) solution, 0.02 ml of sodium pyruvate solution (50 mg/ml), 0.2 ml of 1 M ammonium chloride solution, and water to 2.0 ml. The optical density (OD) at 340 nm was measured, 20 μliters of a 1/100 fresh dilution of lactic dehydrogenase was then added with stirring, and the OD_{340} was measured until it reached a plateau (a few minutes). The total decrease in OD, after blank corrections, was taken as an indication of the amount of NADH present. Twenty μliters of a fresh 1/100 dilution of glutamic dehydrogenase was then added with stirring, and the subsequent corrected decrease in OD was used for calculation of the NADPH content.

NAD and NADP. Mycelia were harvested, washed, and immersed in a 2 to 3 ml of cold 10% trichloroacetic acid solution with gentle stirring. After centrifugation at 27,000 × *g* for 10 min at 5 C, the mycelia were reextracted with 2 to 3 ml of cold trichloroacetic acid, and the process was repeated. The second extract was not combined with the first, since it contained about 10 to 20% of the amounts in the first extract. Trichloroacetic acid was then removed from the supernatant solutions by repeatedly mixing with 8 to 10 ml of ether and removing the ether layer until the extract was between *p*H 5 and 6. The residual ether was removed with a brief bubbling of nitrogen, and the extracts were then assayed. NAD was assayed by measuring OD_{340} increase after the addition of 50 μliters of a fresh 1/100 dilution of alcohol dehydrogenase in a reaction mixture containing extract, 0.5 ml of 0.1 M sodium pyrophosphate buffer (*p*H 8.8), 0.1 ml of 95% ethyl alcohol, and water to a final volume of 2.0 ml. The amount of NAD found in wild type was approximately 20% higher than that found by other workers (8). NADP was separately measured by using 0.1 ml of glucose-6-P (5 mg/ml), 1.0 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.5), water to 2.0 ml, and 20 μliters of a 1/50 dilution of glucose-6-P dehydrogenase. In both assays, the observed increase in OD_{340} was adjusted for any slight changes in OD_{340} in the complete reaction mixture minus enzyme. In all four assays, two different amounts of extract were assayed simultaneously, the average values of which were used for subsequent calculations. In all four assays, the total error involved in individual determinations was estimated to be 3 to 5%.

Extraction and assay controls. Numerous control experiments were performed to determine the effects of various parameters on the extract and assay procedures, percentage of recovery of NADPH, time of growth of cultures, etc. Addition of known amounts of NADH and NADPH solutions to both *col-2* and wild-type mycelium, followed by immediate extraction, resulted in >85% recovery of both reduced coenzymes from both strains. In another control experiment, approximately equal amounts of mycelia of *col-2* and wild type were harvested, mixed, and then extracted. The predicted levels were found, thus indicating that the *col-2* extracts do not contain any agents which appreciably destroy NADPH or NADH. Alternate extraction procedures, such as the addition of cysteine to the alcoholic KOH solution (to prevent oxidation of NADPH) or extraction with an alcoholic Tris (*p*H 7.8) solution did not change the amounts found in either *col-2* or wild type. The time and temperature of extraction were found to be critical factors. By using known amounts of NADPH and NADH solutions, it was found that after 1 min of heating at 70 C, the NADPH and NADH began to be destroyed, or, at temperatures greater than 70 C, 1 min of heating also led to incomplete recovery. Therefore, these two variables were carefully controlled. The extracted NADH and NADPH were stable during the subsequent neutralization, centrifugation, and assay procedures. The enzymatic assays were calibrated with known amounts of the coenzymes, and the specificity of these indicator enzymes was determined. Lactic dehydrogenase did not appear to be active with NADPH, although glutamic dehydrogenase was active with NADH. Alcohol dehydrogenase appeared to be specific for NAD, and glucose-6-P dehydro-

genase for NADP. In some assays of extracts of *col-2* and wild type, the NADPH levels were additionally determined (and confirmed) by assaying with NADPH-specific glutathione reductase. Additional control experiments on the effect of the phase of the growth cycle showed that early or mid-phase cultures of wild type were identical in their reduced nicotinamide coenzyme content. Also, shake cultures of *col-2* can be sheared and used as an inoculum for larger scale growth, and these new cultures (after 2 days, >90% new growth) had essentially the same NADH and NADPH levels as *col-2* cultures grown for 10 days. A similar series of control experiments was performed for the proper extraction of NAD and NADP.

RESULTS

Table 1 indicates that there were lower steady state levels of NADPH and NADH in *col-2* than in the wild type. At 23 C, the *col-2* strain had only approximately 40% of the wild-type NADPH level and approximately 60% of the NADH level, although, in absolute amounts (moles/g), the loss of NADH resulting from the *col-2* mutation was greater than the reduction in the amount of NADPH. All *col-2* isolates from a variety of crosses that were tested exhibited the lowered levels of both reduced coenzymes, and all wild-type isolates that were tested had the same range as the wild type shown in Table 1. A wild-type-like revertant, indistinguishable from wild type by the criteria used (growth rate and morphology), also appeared to be similar to wild type (Table 1). This indicates that the lowered levels are related to the *col-2* mutation and not to the genetic background. A temperature-sensitive partial revertant strain (*col-2b*$_2$ *inosS1*), which had almost the wild-type morphology at 23 C, had wild-type levels of NADH and NADPH. At 34 C, at which temperature it exhibited a colonial morphology, it had a lower level of NADPH, thus strengthening the correlation between the colonial morphology produced by mutation at the *col-2* locus and lower total amounts of NADPH.

In Table 2 it can be seen that balloon, *col-3*, and *bal col-2* had reduced NADPH levels when compared to the wild type. *Col-3* has been reported to be a mutation of 6-phosphogluconic acid dehydrogenase, a NADPH-producing reaction (J. F. Lechner and K. E. Fuscaldo, Bacteriol. Proc., p. 120, 1969). Balloon has been reported to be another mutation affecting glucose-6-P dehydrogenase (W. Scott and E. L. Tatum, Fed. Proc., p. 468, 1969). Therefore, it would appear that there are mutations at loci other than the *col-2* locus which can produce the same net effect, i.e., decreased amounts of NADPH and colonial morphology.

The change in the amounts of reduced nicotinamide coenzymes was not only because of a very slow growth rate or some peculiar internal environment caused by a morphological mutation (Table 2). The slow-growing morphological mutants, ragged and doily, which are genetically easily distinguishable from *col-2*, do not have any obvious alterations in their total NADH or NADPH content. This finding adds to the belief that a change in the morphology of the organism does not necessarily produce changes in the NADPH levels, but rather vice versa.

Table 3 reports that the wild-type strain can be made to grow with a colonial morphology by at least two different methods. Since one method yielded a mycelium with lowered NADPH content, and the other method did not, two possible conclusions might be inferred. First, there are many ways to alter the morphology of *Neurospora*, one of which is via lowering of the NADPH level. Secondly, lowering the amount of total NADPH probably has as an unavoidable

TABLE 1. *Amount of nicotinamide coenzymes in various strains*

Strain	Growth temp	NADH[a]	NADPH[a]	NAD[a]	NADP[a]	Morphology
	C					
RL3-8A	23	0.88, 1.00, 1.05, 0.78, 0.80	0.29, 0.28, 0.26, 0.23, 0.25	1.60, 1.85	0.15, 0.13	Wild type
RL3-8A	34	0.73, 0.90, 0.86	0.24, 0.30, 0.22	0.86, 1.06		Wild type
col-2	23	0.67, 0.42, 0.68 0.54, 0.42	0.12, 0.10, 0.12, 0.11, 0.10	1.40, 1.20	0.077, 0.076	Colonial
col-2	34	0.43, 0.22, 0.29	0.10, 0.08, 0.12			Colonial
col-2b$_2$ *inosS1*	23	1.08, 0.81, 0.97	0.30, 0.28, 0.30	1.40		Semicolonial
col-2b$_2$ *inosS1*	34	0.71, 1.05	0.13, 0.16	0.78, 0.87		Colonial
col-2b$_2$ *inosR11*	23	0.91, 0.93	0.24, 0.30			Wild type

[a] All values are given in micromoles per gram residual dry weight. Abbreviations: nicotinamide adenine dinucleotide (NAD), reduced form (NADH); nicotinamide adenine dinucleotide phosphate (NADP), reduced form (NADPH).

consequence a drastic alteration in morphology. This statement is consistent with all of the facts in the preceding three tables, and probably can only be disproved by discovery of conditions in which lowered NADPH levels and the wild-type morphology prevail simultaneously.

The level of NADH was also changed by the *col-2* mutation, even though changes in temperature (Table 1) and carbon source (Table 3) do not appear to affect this level in wild type. The *col-2* mutation also appeared to lower the amount of reduced glutathione when assayed by the method of Klotzsch and Bergmeyer (7), to a level approximately half that found in the wild-type or *rg* strains (Brody, *unpublished data*). It is not apparent how this one mutation leads to lowered levels of all three reduced forms of these coenzymes. Nor is it apparent how the altered metabolism of the *col-2* strain has adjusted itself to keep the ratios of NAD:NADH (2:1) and NADPH:NADP (1.8:1) similar to that of the wild type (Table 1). The finding that the ratios remained the same, although the absolute levels were considerably reduced, is an intriguing one and will require a more extensive analysis of the factors that regulate NADP and NAD formation.

TABLE 2. *Amount of NADH and NADPH in various colonial mutants*[a]

Strain	NADH	NADPH	Morphology
Balloon	0.55, 0.57,0.75	0.11, 0.14, 0.13	Colonial
bal col-2	0.74	0.12	Extreme colonial
col-3	0.42, 0.33	0.15, 0.08	Slow colonial
rg	0.77, 0.90	0.27, 0.20	Colonial
Doily	0.79, 0.97	0.25, 0.26	Colonial

[a] All cultures were grown at 23 C. All values are given in micromoles per gram residual dry weight. Abbreviations: NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

DISCUSSION

In certain respects, it is not surprising to find that the product of an enzymatic reaction (NADPH) was decreased when the enzyme (glucose-6-P dehydrogenase) was partially inactivated by mutation. In general, this is what one expects. The fact that it has occurred in this instance can be interpretated to mean two things. First, the decreased amount of NADPH in *col-2* is an independent confirmation of the original finding that the *col-2* mutation affected the structure and function of glucose-6-P dehydrogenase. Secondly, that glucose-6-P dehydrogenase (or the pentose phosphate shunt) appears to be the principal supplier of reduced NADP in *Neurospora* grown on glucose, since a partial block in glucose-6-P dehydrogenase leads to a partial loss of NADPH. Although this conclusion is also not totally unexpected, it is interesting that there does not appear to be sufficient metabolic flexibility, either by enzyme induction or by decreased feedback inhibition, to completely compensate for the partial enzymatic block. The exact extent of the partial block is not clear, since the decreased amount of NADPH in *col-2* may already reflect some compensation by other NADPH-producing or NADPH-utilizing reactions. In this respect, it is also possible that the residual amount of NADPH found in *col-2* may result almost exclusively from other enzyme reactions [i.e., the pentose phosphate shunt may have been operating at 5% of the wild-type level, rather than the 50 to 70% level derived from radiorespirometry measurements (3)]. Possibly, only measurement on glucose-6-P dehydrogenase deletion mutants (if viable) will be able to distinguish between the amount of NADPH contributed by the pentose phosphate shunt and the amount produced via other reactions.

In any event, there are sufficient reasons not to make too many physiological conclusions based only on a decreased total amount of NADPH. For one, there is excellent evidence for compartmentalization of small molecules in *Neurospora* (4). Secondly, it is not known how NADPH is distributed within the mycelial mass of *Neurospora*, i.e., how much is at the actively growing tip

TABLE 3. *Amount of NADH and NADPH in wild type under various growth conditions*

Strain	Growth conditions	Morphology	NADH[a]	NADPH[a]
RL3-8A	Minimal-glucose, 23 C	Wild type	0.88, 1.00, 1.05, 0.78, 0.80	0.29, 0.28, 0.26, 0.23, 0.25
RL3-8A	Minimal-glucose + 10% sorbose, 23 C	Colonial	0.73, 0.96	0.26, 0.25
RL3-8A	Minimal + 2% acetate, 23 C	Wild type	0.90	0.32
RL3-8A	Minimal + 2% acetate, 34 C	Semicolonial	0.80, 0.88	0.16, 0.16

[a] All values are given in micromoles per gram residual dry weight. NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

regions, etc. A third factor to consider is the distribution among the organelles in any given area, such as mitochondria, nuclei, etc. A fourth factor is the possible equilibrium between a pool of NADPH and enzyme-bound NADPH. Measurement of total NADPH, therefore, could easily represent the sum total of six or eight different size pools of NADPH, and a lower total amount could result from a decrease in the size of every one of these pools. On the other hand, the finding of a lower total might mean that there was almost a complete reduction of NADPH in some areas, such as enzyme-bound NADPH at the tip regions, and little effect in other areas. These are just some of the reasons why it may be physiologically meaningless to present the levels of metabolic intermediates as an average value, and downright misleading to express these values in mM.

The lowering of the overall NADPH content probably has many pleiotropic effects, and possibly only a few of them are actually involved in the alteration of the morphology. Since NADPH participates in numerous enzymatic reactions (5), it is difficult to try to choose which of these many NADPH-dependent reactions might actually be affected by a lowered level of substrate, and to what extent. Of those affected, it is not clear which ones play a causal role in the observed morphological change, and which a trivial role. It should also be pointed out that other types of pleiotropic effects could be visualized. The lowered overall NADPH content could affect the steady-state level of certain enzymes, if NADPH were involved as a corepressor. A second possible effect might be on coenzyme-regulated polymerization of enzyme monomers, such as the NADP concentration-dependent polymerization of yeast glucose-6-P dehydrogenase subunits (10). Obviously, the state of aggregation of any enzyme might easily affect its physiological activity or its sensitivity to metabolic regulation (feedback). A third possibility is that the NADPH produced by *Neurospora* glucose-6-P dehydrogenase is preferentially used in only one or a few other reactions, and is not freely diffusible or in true equilibrium with other pools of NADPH. Therefore, the partial loss of NADPH produced by glucose-6-P dehydrogenase could have serious effects on any enzyme system directly coupled to glucose-6-P dehydrogenase, with few effects on other NADPH-dependent reactions.

This last possibility is intriguing, since it has been reported that the glucose-6-P dehydrogenase of *Neurospora* appears to be a complex protein under multigenic control (W. Scott and E. L. Tatum, Fed. Proc., p. 468, 1969). It is possible that the protein complex which catalyzes glucose-6-P dehydrogenase also catalyzes other reactions, and that mutation in any one of the subunits leads to a partial loss of more than just glucose-6-P dehydrogenase activity. If this were the case, then the lowered level of total NADPH would only be one of the initial pleiotropic effects produced by mutation in this postulated enzyme complex. However, no other enzyme activities have been found to be associated with the purified complex at this time.

Attempts to reverse the morphological consequences of the *col-2* mutation by a combination of genetic and environmental techniques were performed. All of these were directed towards either increasing the intracellular levels of NADPH or decreasing its utilization. Partial success was obtained [i.e., slightly faster and more filamentous growth of *col-2*, when glutamic acid was substituted for glucose as sole carbon source (Brody, *unpublished data*)]. A double mutant, *am col-2*, which had little or no activity in the NADP-dependent glutamic dehydrogenase reaction because of the *am* mutation, did not show any response to glutamic acid. One possible explanation for these results is that growth of *col-2* on glutamic acid generated additional NADPH via the NADP-dependent glutamic dehydrogenase and therefore produced the fluffier appearance, whereas the *am col-2* was incapable of doing so. Although this interpretation is still favored, the situation appears to be more complex and will be reported in greater detail at some later date.

The effects of the NADPH deficiency on the amounts and types of fatty acids in the neutral lipids and phospholipids of *Neurospora* were investigated and will also be reported (Brody and Nyc, *manuscript in preparation*).

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