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Genetics

Mutants defective in the energy-conserving NADH dehydrogenase of *Salmonella typhimurium* identified by a decrease in energy-dependent proteolysis after carbon starvation

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ABSTRACT **NADH dehydrogenase is the first component of the respiratory chain. It transfers electrons from NADH to ubiquinone and concomitantly establishes a proton motive force across the membrane. *Salmonella typhimurium* mutants defective in this enzyme were isolated in a screen for strains with increased expression of β-galactosidase from a *hemA-lacZ* protein fusion. This unexpected phenotype results from stabilization of the hybrid protein during carbon starvation and is apparently due to an energy requirement for proteolytic attack. Sequence analysis of DNA fragments cloned from an insertion mutant indicates that *S. typhimurium* has a large cluster of genes encoding the energy-conserving NADH dehydrogenase, similar to one recently described in *Paracoccus denitrificans*. These findings establish the potential for genetic analysis of a complex enzyme whose function, especially in proton efflux, is poorly understood.**

Electrons generated in many different catabolic reactions are first transferred to NADH and then donated to ubiquinone by the enzyme NADH dehydrogenase of the respiratory chain, which links catabolism to energy production. NADH dehydrogenases fall into two distinct classes: complex enzymes such as those purified from eukaryotic mitochondria or *Paracoccus denitrificans* (1–3) and simple FAD-linked enzymes such as the *ndh* gene product of *Escherichia coli* (4–7). Only the complex enzymes conserve energy by catalyzing proton efflux. The bovine energy-conserving (type I) NADH dehydrogenase has numerous subunits, with a total amino acid content greater than that of a prokaryotic ribosome (1). Genetic studies of this enzyme have been difficult because of its many subunits, including seven of mitochondrial origin.

Oxidation of NADH can generate a proton motive force in *E. coli*, indicating the presence of a type I NADH dehydrogenase (8, 9). Recently, the original *ndh* mutant of *E. coli* was shown to carry a second, previously unrecognized mutation inactivating the type I enzyme (10). A cluster including at least 14 genes that encode subunits of a type I NADH dehydrogenase has been identified and sequenced in *P. denitrificans* (3, 11), and a preliminary report suggests that *E. coli* has a similar locus (12). The relevant genes have been termed *nuo*, for NADH:ubiquinone oxidoreductase (10).

We recovered *Salmonella typhimurium nuo* mutants defective in the type I NADH dehydrogenase in a screen for strains with increased expression of a *hemA-lacZ* protein fusion. The *hemA* gene encodes glutamyl tRNA reductase, the first enzyme of the heme biosynthetic pathway. The mutant phenotype results from increased stability of the hybrid HemA-LacZ protein under certain conditions. It is not yet known whether there are effects on the stability of the native HemA protein. Apparently, *nuo* mutants cannot activate ATP-dependent proteolysis under starvation conditions. This simple genetic screen should facilitate analysis of an enzyme whose function is central to energy transduction.*

MATERIALS AND METHODS

Bacteria and Phage. All *S. typhimurium* strains were derived from the wild-type strain LT-2, which does not carry the *lac* operon. The *lac* fusion strains were constructed as described previously and are carried on the *S. typhimurium* chromosome in single copy (13). Both operon and protein fusion constructs extend from a *Bam*HI site 731 bp upstream of the *hemA* AUG codon to codon 18 of *hemA*. The sequence of the protein fusion joint from codon 16 of *hemA* to codon 9 of *lacZ* is TCG CTG CGG AAT TCC GAT CCC GTC. The predicted N-terminal sequence of the hybrid protein is MTL-LALGINHKTAPVSLRNSDPV; underlined amino acids are contributed by HemA. The *lon* mutant strain was isolated by its mucoid phenotype and its identity was confirmed by PCR mapping and DNA sequencing (T.E., unpublished data).

Media and Growth Conditions. Standard media and genetic techniques were as described or referenced (13–15). Indicator plates contained NCE medium (16) with 0.02% glucose, 50 μg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), and the appropriate antibiotic. The minimal medium used for liquid cultures (17) was supplemented with 10 mM NH_4Cl. All cultures were grown at 37°C. Construction, use, and nomenclature of defective transposons used in this work were as described or referenced (15).

Assay of β-Galactosidase. Cultures were grown in minimal medium with 2 mM glucose at 37°C with shaking, as described in *Results*. Portions of the culture were removed at various times and assays were performed as described (13, 18).

Immunoprecipitation. Cultures were grown in minimal medium with 2 mM glucose at 37°C. Aliquots (5 ml) of the cultures were labeled with 0.5 mCi of [^{35}S]methionine (Tran^{35}S-label, ICN; about 1000 Ci/mmol; 1 Ci = 37 GBq); L-methionine was added to 0.05% for the chase. Samples were taken at various times as described in *Results* and analyzed (19) using 1 μg of a monoclonal antibody to β-galactosidase (Promega) and protein A-Sepharose CL-4B (Sigma) for precipitation of antigen–antibody complexes.

Cloning and DNA Sequence Analysis. Chromosomal DNA from a strain carrying the *nuoD9*::Tn*10d*-Cam insertion was digested with *Hin*dIII and *Pst* I and cloned into pBR322 to give pTE491. The insert was sequenced by the dideoxy method with Sequenase (version 2.0; United States Biochemical) as described by Strathmann *et al.* (20), with the use of γδ-specific primers. The *nuoD9*::Tn*10d*-Cam insertion site was sequenced from a PCR product of LT-2 (wild type) template. Sequence analysis was carried out using the Uni-

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Abbreviations: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; dNADH, deamino-NADH.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L22504).

versity of Wisconsin Genetics Computer Group sequence analysis programs (21).

NADH Oxidase Assays. Cells were grown overnight in N^-C^- medium containing 10 mM NH_4Cl, 0.3% lactate, and 0.1% Casamino acids. After cell disruption with a French press, crude membranes were prepared as described (9). Glycerol and dithiothreitol were then added to final concentrations of 10% and 1 mM, respectively. NADH oxidase assays were carried out in a total volume of 1 ml containing 50 mM potassium phosphate (pH 7.5), 5 mM $MgSO_4$, and membranes (9). The reaction was started by addition of NADH or deamino-NADH (dNADH; Sigma) to a final concentration of 250 μM. Activity was measured spectrophotometrically by determining the decrease in absorbance at 340 nm. A millimolar extinction coefficient of 6.22 was used in calculations.

RESULTS

Isolation of Mutants. As part of our effort to understand the regulation of heme biosynthesis in *S. typhimurium*, mutants were sought that alter the expression of β-galactosidase from a *hemA-lacZ* protein fusion. As explained below, two factors were important in the recovery of mutants: (*i*) use of a special medium with only a low concentration of glucose and (*ii*) employment of a hybrid HemA-LacZ protein as the reporter. A number of transposon Tn*10d*-Tet insertion mutations were identified in strain TE2685 (*putPA*::*hemA-lac*[pr]). This strain carries a protein fusion joining the N-terminal 18 codons of the *hemA* gene to a *lacZ* gene that has all but the first 8 codons, followed by the rest of the *lac* operon (13). Mutants were found that had a dark blue colony phenotype on minimal X-Gal plates containing 0.02% glucose as the carbon source. Other mutations conferring a similar phenotype were then isolated by mutagenesis with Tn*10d*-Cam, and the two collections were sorted into linkage groups by transductional crosses with phage P22. At least five different groups of mutations with a similar phenotype have been recognized. One of these groups is characterized here; this group (*nuo* mutants) is defective in the type I NADH dehydrogenase.

Assays of β-Galactosidase Activity After Carbon Starvation. The mutant phenotype was observed on minimal X-Gal plates containing low concentrations (0.02%) of glucose or glycerol as the carbon source, but not on plates with 0.2% of either compound. This suggested a role for carbon limitation in the phenotype. Accordingly, we measured the enzyme activity of β-galactosidase in an otherwise wild-type *hemA-lacZ*[pr] strain (TE2685) during growth in liquid minimal medium containing a limiting concentration of glucose (2 mM; Fig. 1*A*). Enzyme activity was seen to rise during exponential growth and then fall dramatically after the onset of carbon starvation. The apparent half-life of β-galactosidase in the decay phase is about 30 min, as determined from a plot of the logarithm of activity versus time. In contrast to the results with wild type, a *nuo* mutant strain (Fig. 1*B*) showed a slight decrease at early times after starvation but the β-galactosidase activity remained relatively high even after many hours. Similar results were observed when extracts were prepared by sonication, except that the small decrease for *nuo* mutant strains was eliminated (C.D.A., unpublished data). In other experiments, the activity during exponential growth was shown to be the same in *nuo* mutants as in the *nuo*$^+$ control. A starvation-induced decrease in β-galactosidase activity was not observed with a *hemA-lac* operon fusion (Fig. 1*C*), and the behavior of an operon fusion strain carrying a *nuo* mutation was the same as in *nuo*$^+$ (C.D.A., unpublished data). Finally, no effect of carbon starvation was observed in a *nuo*$^+$ strain when the protein fusion was provided on a multicopy plasmid (Fig. 1*D*).

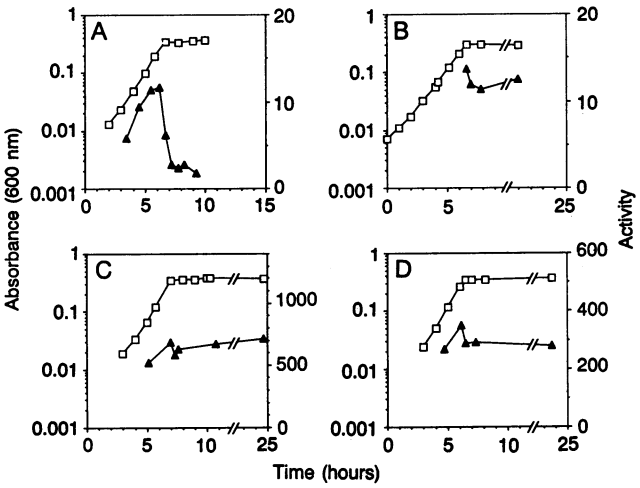

FIG. 1. Assay of β-galactosidase activity in *hemA-lac* strains after carbon starvation. □, Growth (A_{600}) in minimal medium containing 2 mM glucose; ▲, β-galactosidase activity for aliquots withdrawn at various times. (*A*) TE2685 *hemA-lacZ* [pr]. (*B*) TE5076 *hemA-lacZ* [pr] *nuoA2*::Tn*10d*-Tet. (*C*) TE2859 *hemA-lac* [op]. (*D*) TE5097 LT-2 (wild type) carrying pTE328 (*hemA-lacZ* [pr]). Note the variation in scale for β-galactosidase activity.

Pulse–Chase and Immunoprecipitation of HemA-LacZ Fusion Protein. A pulse–chase experiment was used to determine whether effects on proteolysis account for the higher levels of β-galactosidase activity in *nuo* mutant strains after carbon starvation. Cultures growing exponentially in minimal medium with limiting glucose were labeled with [^{35}S]methionine starting at a time ≈30 min before the onset of starvation. A 15-min labeling period was followed by a chase with unlabeled methionine. Samples were taken after 5 min of chase and at various times after starvation; these were analyzed by immunoprecipitation using a monoclonal antibody to β-galactosidase (Fig. 2). HemA-LacZ protein labeled during exponential growth was rapidly lost from the *nuo*$^+$ strain after carbon starvation. In contrast, the HemA-LacZ protein was apparently stable in the *nuo* mutant strain.

Genes Encoding Subunits of the NADH Dehydrogenase. Fragments of genomic DNA isolated from strain TE5077 (*nuoD9*::Tn*10d*-Cam) were cloned into pBR322, by selecting for the chloramphenicol resistance encoded by the transposon. The complete DNA sequence was determined for a *Pst* I–*Hin*dIII fragment (3950 bp in wild type) that is disrupted by the Tn*10d*-Cam insertion in strain TE5077. Analysis of the sequence suggested that this fragment encodes subunits of the *S. typhimurium* energy-conserving NADH dehydrogenase, similar to the *P. denitrificans NQO* genes sequenced by Yagi and collaborators (refs. 3 and 11 and references therein).

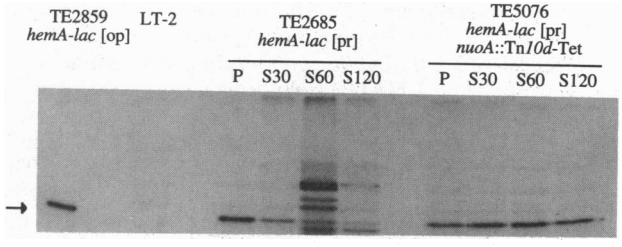

FIG. 2. Pulse–chase and immunoprecipitation with anti-β-galactosidase. Details of the experiment are described in the text; strains are described in the legend to Fig. 1. P, sample withdrawn after 5 min of chase and ≈5 min before the onset of carbon starvation; S, samples withdrawn at 30, 60, or 120 min after the onset of carbon starvation. The arrow marks the position of native β-galactosidase (116 kDa), produced by strain TE2859.

The *S. typhimurium* genes have been named *nuo*, following the terminology of Calhoun and Gennis (10).

In a central segment of the *P. denitrificans* cluster the gene order is *NQO4–NQO2–NQO1–NQO3*, including the *NQO1* gene encoding the NADH-binding subunit of NADH dehydrogenase. We have found *S. typhimurium* homologues for each of these subunits encoded in the same order in the *Pst* I–*Hin*dIII fragment. The complete *nuoE* and *nuoF* genes are contained within the *Pst* I–*Hin*dIII fragment, as are a C-terminal segment of *nuoD* and an N-terminal segment of *nuoG*. (The letters chosen for *S. typhimurium* genes correspond to the proposed *E. coli* nomenclature; K. Rudd, personal communication.)

The *S. typhimurium nuoF* gene probably encodes the NADH-binding subunit of NADH dehydrogenase, based on the homology of its predicted product not only to the *P. denitrificans NQO1* gene product but also to the bovine and *Neurospora* NADH-binding proteins (Fig. 3). The predicted functions of various segments of NuoF homologues have been reviewed (1–3). Of particular interest is the conservation of three segments of the protein with the following predicted functions: binding of NADH (NuoF residues 57–76), binding of the FMN cofactor (NuoF residues 174–193), and an Fe/S center (CGWCTPCRDGX$_{37}$C, NuoF residues 351–398). Similarly, three clusters of cysteine residues that are predicted to form Fe/S centers are conserved in NuoG (data not shown; sequence in GenBank, accession no. L22504). Analysis of the predicted protein sequences revealed that the *P. denitrificans* NQO proteins are more similar to their bovine and *Neurospora* counterparts than are the corresponding *S. typhimurium* proteins. For example, NuoF has 43% identity with the bovine NADH-binding subunit, whereas NQO1 has 65% identity in a similar comparison (Fig. 3).

Preliminary results from analysis of cloned fragments flanking the sequenced region indicate that the region upstream of *nuoDEFG* includes *S. typhimurium* genes homologous to the *NQO7–NQO6–NQO5* genes of *P. denitrificans*. PCR was used to map *nuo* insertions (T.E., unpublished data). Of those that have been physically located, the *nuoD9*::Tn*10d*-Cam insertion that was cloned lies the farthest downstream. A number of insertions lie in genes upstream of *nuoD*—e.g., the Tn*10d*-Tet insertion in strain TE5076 has been assigned to *nuoA*. Genetic analysis indicates that other insertions, not yet accurately mapped, lie downstream of the sequenced region.

Map Position and Orientation of the *nuo* Genes. A 3469-bp *Bgl* II–*Hin*dIII fragment of the cloned *nuo* segment was used to probe phage from the Kohara λ library of *E. coli* (22) as well as Southern blots of genomic DNA digests from *E. coli* W3110 (C.D.A. and T.E., unpublished data). The *nuo* probe hybridized to phages 4C8 (402) and E9B9 (403), and analysis of the genomic hybridization patterns indicates that the probe corresponds to a segment from 2410 to 2414.5 kbp of the Kohara map (22, 23). This map position (49.3 min in *E. coli*, ≈46 min in *S. typhimurium*) is consistent with genetic mapping by phage P22 transduction placing *nuo* insertions between *menB* and *ack-pta*. The map position was also confirmed by the DNA sequence of a region located 4.5 kb

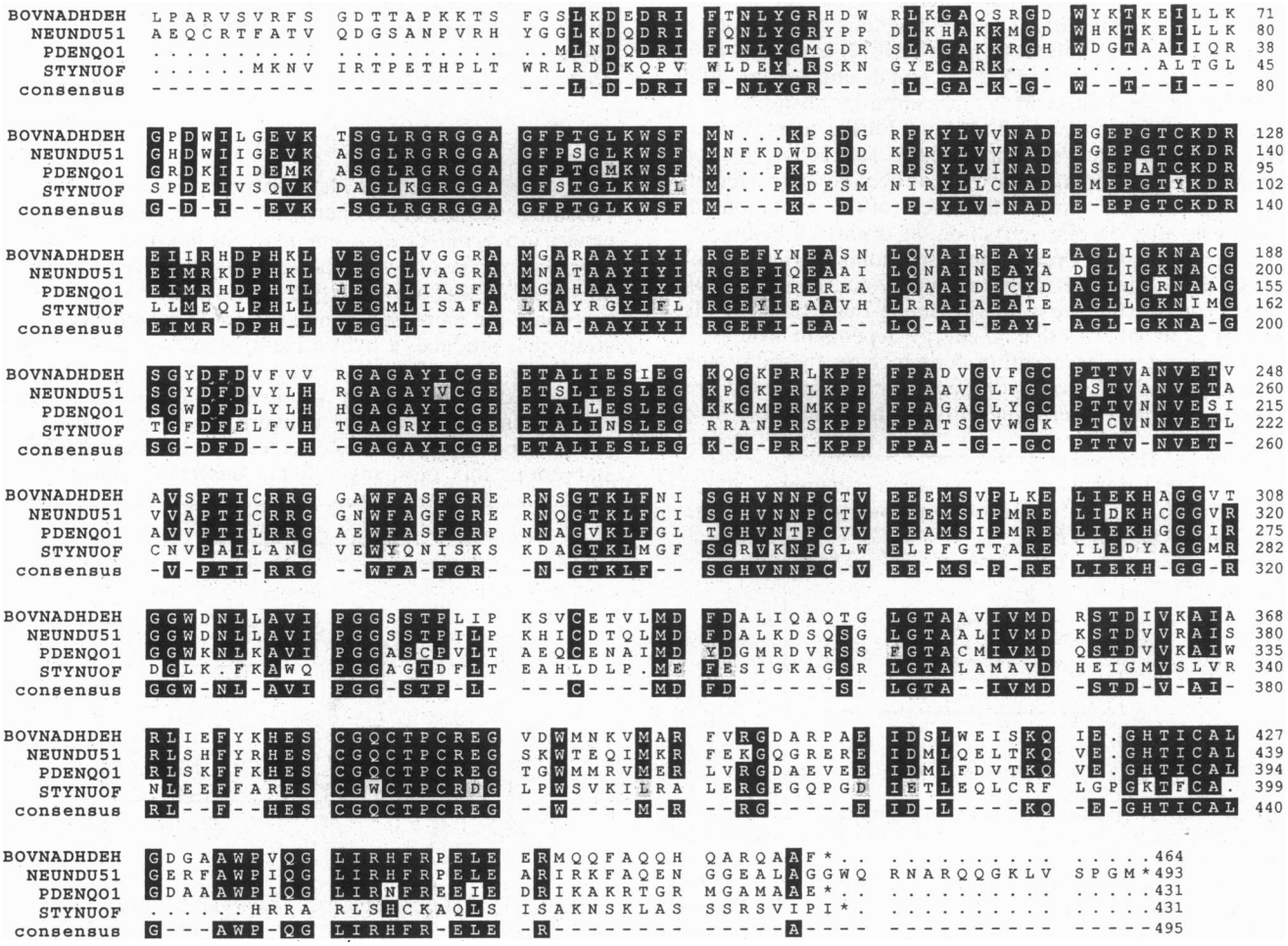

FIG. 3. Comparison of the predicted protein sequences for *nuoF*-related genes. Protein sequences of NADH-binding proteins were generated by translation of the following GenBank DNA sequence files: BOVNADHDEH, NEUNDU51, and PDENADH. The comparison was made with PILEUP and the results were displayed using PRETTYBOX (21).

Table 1. Assay of NADH dehydrogenase

Strain	Genotype	Substrate	Exp. 1 Activity	Exp. 1 dNADH/NADH	Exp. 2 Activity	Exp. 2 dNADH/NADH	Exp. 3 Activity	Exp. 3 dNADH/NADH
LT-2	Wild type	dNADH	0.082	0.15	0.077	0.17	0.037	0.23
		NADH	0.54		0.46		0.16	
TE2685	*hemA-lac* [pr]	dNADH	0.097	0.22	0.099	0.17	0.023	0.13
		NADH	0.44		0.59		0.18	
TE5076	*hemA-lac* [pr]	dNADH	2.4×10^{-3}	0.006	3.1×10^{-3}	0.005	1.5×10^{-3}	0.006
	nuoA::Tn*10d*-Tet	NADH	0.39		0.60		0.26	
TE5077	*hemA-lac* [pr]	dNADH	2.4×10^{-3}	0.006	2.7×10^{-3}	0.006	1.8×10^{-3}	0.006
	nuoD::Tn*10d*-Cam	NADH	0.43		0.42		0.28	

Activity is given in μmol of substrate consumed per min per mg of protein.

upstream of the *nuoD9*::Tn*10d*-Cam insertion (T.E., unpublished data), which is nearly identical to the appropriate sequence from *E. coli* (24). The *nuo* genes are transcribed counterclockwise with respect to the genetic map.

***nuo* Mutants Are Defective in Oxidation of dNADH.** The two classes of NADH dehydrogenase can be distinguished by their different substrate specificities. dNADH is a substrate for the type I but not the type II enzyme (9); a *nuo* mutant of *E. coli* is completely defective in dNADH oxidase activity (10). Assays of NADH oxidation by crude membrane preparations are shown in Table 1. Although the activity observed with NADH was somewhat variable, in the *nuoA* and *nuoD* mutants oxidation of the specific substrate dNADH was decreased 20- to 40-fold compared to wild type. These data confirm the predicted enzymatic defect of the *nuo* mutants.

Growth of *nuo* Mutants. The doubling time of *nuo* mutants in minimal glucose is about 60 min (TE5077) compared to 50 min for a wild-type strain (TE2685), and in glucose starvation experiments the final yield (as measured by absorbance) is only about 10% less than for wild type. Furthermore, the *nuo* mutants are able to grow on glycerol and lactate as sole carbon and energy sources. Growth on acetate was extremely poor, and this may explain the effect of *nuo* mutations on proteolysis after carbon starvation, as discussed below.

Energy-Dependent Proteolytic Degradation of HemA-LacZ Protein. How can a defect in the energy-conserving NADH dehydrogenase stabilize a hybrid HemA-LacZ protein? The simplest explanation is that an energy-dependent protease is active during carbon starvation, for which the hybrid protein is a substrate. NADH dehydrogenase mutations could prevent proteolysis by interfering with the energy metabolism of carbon-starved cells. If so, the loss of β-galactosidase activity seen after carbon starvation should be dependent on continued ATP synthesis via respiration. We tested this prediction by using sodium azide to poison respiration. When sodium azide was added to cultures at intermediate times after the onset of carbon starvation, no further loss of β-galactosidase activity occurred (Fig. 4).

Only two energy-dependent proteases are known in *E. coli*: Lon and ClpP (25). We isolated a *lon* insertion mutant of *S. typhimurium* and tested the effect of the *lon* mutation on the stability of the HemA-LacZ protein following carbon starvation (Fig. 5). The initial rate of loss of enzyme activity after carbon starvation was substantially decreased in the *lon* mutant (Fig. 5 *Inset*). In addition, the β-galactosidase activity observed in the *lon* mutant was higher than in the *lon*$^+$ parent strain at all times. Thus, Lon apparently contributes to degradation of the fusion protein, but an additional protease may also be involved.

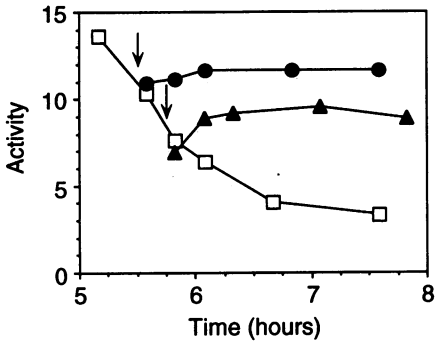

FIG. 4. Loss of β-galactosidase activity after carbon starvation is sensitive to azide. Strain TE2685 *hemA-lacZ* [pr] was grown in minimal medium with 2 mM glucose, and samples were taken for β-galactosidase assays at various times. At the times indicated by the arrows, portions of the culture were treated with 5 mM sodium azide to inhibit respiration and ATP synthesis. As determined from A_{600} measurements, carbon starvation commenced at 5 hr 20 min, 10 min after the first sample was taken. Azide was added after 10 or 25 min of starvation. □, No azide; ●, azide added at first arrow; ▲, azide added at second arrow.

DISCUSSION

This study describes the identification of a cluster of genes encoding subunits of the energy-conserving NADH dehydrogenase in *S. typhimurium*, similar to one previously described in *P. denitrificans* (3, 11). The serendipitous discovery of the *nuo* mutants that are defective in this enzyme occurred by screening for increased expression of β-galactosidase in a strain that produces a hybrid HemA-LacZ protein. Proteolysis limits accumulation of this protein. During carbon starvation or growth on plates with low amounts of glucose, the energy-conserving NADH dehydrogenase is required for proteolysis of HemA-LacZ. At least in the case of carbon starvation, this probably reflects inefficient utilization of acetate accumulated during growth on glucose (26, 27) and

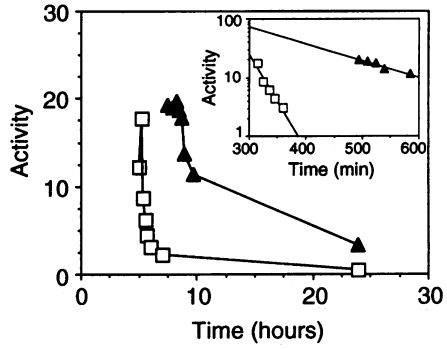

FIG. 5. Loss of β-galactosidase activity after carbon starvation is partially *lon*-dependent. Strains were grown and assayed as for the experiment shown in Fig. 1. □, TE2685 *hemA-lacZ* [pr]; ▲, TE5202 *hemA-lacZ* [pr] *lon*::Mud-Cam. (*Inset*) Semilogarithmic plot of the data from the initial period of starvation; for each strain the first point coincides with the beginning of starvation.

subsequent failure to activate ATP-dependent proteolysis. A mutation in *lon*, which encodes a known ATP-dependent protease, reduced but did not eliminate this proteolytic activity.

We have no information yet on the rate of synthesis or decay of the HemA-LacZ protein during exponential growth. The rate of synthesis is difficult to determine due to the low abundance of the protein, and its stability during exponential growth has not yet been measured. The loss of β-galactosidase activity after starvation as compared to exponential growth might be caused by either decreased synthesis or increased proteolysis, or both. No loss of activity was seen after carbon starvation when the hybrid protein was expressed from a high copy plasmid (Fig. 1*D*); this result could be reconciled with either mechanism but is simply explained by titration of limiting amounts of a protease.

As discussed elsewhere (28, 29), hydrolysis of newly formed peptide bonds is substantial in growing cells and presumably functions to recycle abortive products of various translational mishaps. Carbon starvation results in a decrease in protein synthesis and, likely, a lower rate of recycling. This might free newly unemployed proteases to attack lower affinity targets. Thus, changes in proteolysis may not require a specific inductive mechanism. Competition among proteolytic substrates also leads to the possibility that for some unstable proteins the effect of a *lon* mutation is indirect; increased amounts of a Lon-sensitive competitor could affect reactions catalyzed by other proteases.

In addition to *nuo* mutants, several other classes of mutants were recovered. These have not yet been identified but some are predicted to be involved in catabolism or energy production based on their growth properties: poor growth on acetate but good growth on glucose or glycerol. Since *nuo* mutants grow well (except on acetate), the type II NADH dehydrogenase is sufficient for growth (10). This is perhaps less surprising in view of the fact that rapidly growing organisms such as *Bacillus subtilis* and *Saccharomyces cerevisiae* contain only a type II enzyme. Apparently, metabolic adjustments can be made to generate the additional energy normally provided in *S. typhimurium* by the type I enzyme.

Some questions about the genetic screen remain unanswered. For example, several other hybrid β-galactosidase proteins that we have tested are also affected by a *nuo* mutation on indicator plates, but enzyme assays show that the activity is unchanged after carbon starvation in liquid culture and is not affected by a *nuo* mutation. Also, additional classes of mutants with a similar colony phenotype were recovered, which do not affect the loss of HemA-LacZ β-galactosidase activity after carbon starvation in liquid medium. The inconsistency between plate phenotypes and enzyme assays may be due to artifacts inherent in the X-Gal method; alternatively, it may reflect the different sensitivities or physiological contexts of the two tests. Regardless of its precise mechanism, a simple screen should be of great value in the genetic analysis of NADH dehydrogenase function.

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