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Evidence for Two NAD Kinases in *Salmonella typhimurium*

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The electron-carrying cofactor NADP is formed by phosphorylation of NAD. A strategy for the isolation of NAD kinase mutants revealed two classes of temperature-sensitive mutations, *nadF* and *nadG*, mapping at min 13 and 72 of the *Salmonella* chromosome. Both mutant types grew on nutrient broth at both 30 and 42°C but on minimal medium showed a temperature-sensitive growth defect which was not corrected by any of the single nutritional supplements tested. A *nadF* deletion mutant grew on nutrient broth but not on minimal medium. A double mutant with the *nadF* deletion and a *nadG*(Ts) mutation showed temperature-sensitive growth on all media. We propose that *Salmonella typhimurium* has two NAD kinases, one encoded by the *nadF* and one by the *nadG* gene. This is supported by the fact that temperature-sensitive mutants of both genes produce kinase activity with altered heat stability. Results suggest that either one of two NAD kinases is sufficient for growth on rich medium, but that both are needed for growth on minimal media. Enzyme assays show that the *nadF* gene is responsible for about 70% of total NAD kinase activity, and that the *nadG* gene dictates the remaining 30%. While testing nutritional phenotypes of *nadF* and *nadG* mutants, we found that the biosynthetic intermediate, quinolinic acid (QA) inhibited growth of *nadF* mutants on nutrient broth. This suggested that the NadG enzyme might be inhibited by QA. Enzyme assays demonstrated that QA inhibits the NadG but not the NadF enzyme. This suggests the existence of a regulatory mechanism which controls NADP levels.

The electron carrier NADP, involved in most reductive biosynthetic reactions, is synthesized by the ATP-mediated phosphorylation of NAD (15, 20, 27). The cofactors NAD and NADP are known to participate in more than 300 different oxidation-reduction reactions (9, 24). In addition, NAD is split (to nicotinamide mononucleotide [NMN] and AMP) by the essential DNA ligase activity of the enteric bacteria *Salmonella typhimurium* and *Escherichia coli* (16, 22). Since so many enzymes use these cofactors, the levels of NAD and NADP are likely to be tightly regulated.

The current understanding of NAD metabolism in *S. typhimurium* is summarized in Fig. 1 and reviewed in references 9 and 24. The first two enzymes of the de novo synthetic pathway are encoded by the *nadB* and *nadA* genes (7, 9, 24). The level of NAD appears to signal transcriptional repression of the *nadA* and *nadB* genes by the NadI protein (12, 13). The first biosynthetic enzyme (NadB) is feedback inhibited by NAD^+(H) but not by $NADP^+$(H) (9, 24, 25). Thus the *nadB* gene is the primary site of regulation of the de novo NAD synthetic pathway. When an *E. coli nad* auxotroph is starved of nicotinic acid, its pool of NADP remains high, while the NAD pool drops precipitously (18). This suggests regulation of the NAD-NADP interconversion.

The cofactors NAD and NADP have distinct metabolic functions. Although both transfer electrons, cofactor NAD is used primarily in catabolic reactions that oxidize substrates and produce NADH. In contrast, cofactor NADPH is most often used to provide electrons for the reductive reactions of biosynthesis (19).

While the NAD kinase of bacteria has not been intensively studied, the enzyme was purified 180-fold from *Bacillus licheniformis* and 93-fold from *E. coli*. The *E. coli* enzyme was found to have a pH optimum of 7.2 and an apparent K_m for NAD and ATP of 1.9 and 2.1 mM, respectively (29, 30). We describe here the isolation and characterization of mutants of *S. typhimurium* deficient in NAD kinase.

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MATERIALS AND METHODS

Materials. Labeled [carbonyl-^{14}C]NAD and [carbonyl-^{14}C] nicotinic acid (NA) were purchased from Amersham Corp. All other chemicals were purchased from Sigma.

Bacterial strains. All strains used in this study are derived from *S. typhimurium* LT2 and are listed in Table 1. Bacteriophage MudA refers to a conditionally transposition-defective derivative (11) of the original Mu d1(Lac Ap^r) phage of Casadaban and Cohen (3), which forms operon fusions. The MudJ element is the transposition-defective mini-Mu phage, Mu d1-1734(Lac Kn^r), constructed by Castilho et al. (4). This MudJ element lacks transposition functions and carries kanamycin resistance. The Tn*10*d(Tc) element is a small transposition-defective derivative of transposon Tn*10*, constructed by Way et al. (28). The Tn*10*d(Cm) element is a transposition-defective derivative of transposon Tn*10*, encoding chloramphenicol resistance (7).

Media. The E medium of Vogel and Bonner (26), supplemented with 0.2% glucose, was used as minimal medium. Difco nutrient broth (NB) (8 g/liter) was used as rich medium with added NaCl (0.5%). Difco agar was added at a final concentration of 1.5% to make solid media. Nutrients were added at the concentrations described by Davis et al. (6); exceptions are indicated in the text. Antibiotics were added to media at the following final concentrations (in μg/ml): ampicillin (sodium salt), 30 in NB and 15 in E minimal; tetracycline hydrochloride, 20 in NB and 10 in E medium; and kanamycin sulfate, 50 in NB and 125 in E medium. Media containing ampicillin were always prepared fresh before use. The chromogenic β-D-galactosidase substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was dissolved initially in *N,N*-dimethyl formamide (20 mg/ml) and was added to media at a final concentration of 25 μg/ml.

Transductional methods. The high-frequency, generalized transducing bacteriophage P22 HT 105/1 *int-201* mutant was used for all transductional crosses. This phage was derived by G. Roberts (22a) from the P22 HT 105/1 phage of Schmieger (23a). To select for inheritance of the Kn^r marker of MudJ and

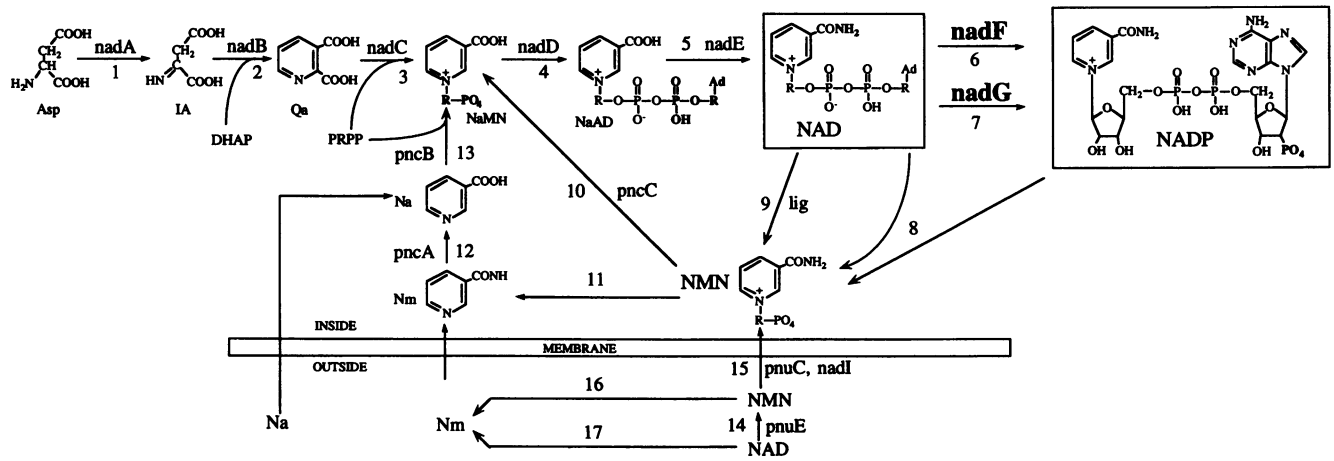

FIG. 1. The NAD metabolic pathways of *S. typhimurium*. The reactions (numbered) and enzymes included are as follows: 1, L-aspartate (Asp) oxidase; 2, QA synthetase; 3, QA phosphoribosyl transferase; 4, NaMN adenylyl transferase; 5, NAD synthetase; 6 and 7, NAD kinase; 8, NAD(P)ase; 9, DNA ligase; 10, NMN deamidase; 11, NMN glycohydrolase; 12, nicotinamide (Nm) deamidase; 13, NA phosphoribosyl transferase; 14, NAD pyrophosphatase; 15, NMN transporter; 16, NMN glycohydrolase; 17, NAD glycohydrolase. Abbreviations: DHAP, dihydroxyacetone phosphate; IA, iminoaspartate; PRPP, 5-phosphoribosyl-1-pyrophosphate; Na, nicotinic acid; NaMN, nicotinic acid mononucleotide; Nm, nicotinamide; NMN, nicotinamide mononucleotide. Genetic loci corresponding to enzymatic steps are indicated above the reaction arrows.

the Cm^r marker of Tn*10*d(Cm), the mixture of cell and phage was spread on NB plates and incubated overnight before replica printing to selective plates. In all other crosses, selective plates were spread directly with 2×10^8 cells and 10^8 to 10^9 phage. Transductants were purified, and phage-free colonies were isolated by streaking nonselectively on green indicator plates (5).

TABLE 1. *S. typhimurium* LT2 strains used in this study

Strain	Genotype
TT10738	*nadB449*::MudJ
TT17510	*nadB449*::MudJ *nadF548*(Cs)
TT17511	*nadB449*::MudJ *nadG550*(Ts)
TT17512	*nadB449*::MudJ *nadF549*(QA^s)
TT17513	*zbc-3767*::Tn*10*d(Tc)
TT17514	*zhd-3769*::Tn*10*d(Tc)
TT17516	*nadB449*::MudJ *zbc-3768*::Tn*10*d(Tc)
TT17517	*nadB449*::MudJ *nadF548*(Cs) *zbc-3767*::Tn*10*d(Tc)
TT17518	*nadB449*::MudJ *nadG550*(Ts) *zhd-3769*::Tn*10*d(Tc)
TT17526	*nadB449*::MudJ *nadF549*(QA^s) *zbc-3768*::Tn*10*d(Tc)
TT15340	*nadB449*::MudJ *pnuA126*::Tn*10*d(Tc)
TT17533	*nadB449*::MudJ *zhd-3775*::Tn*10*d(Cm) *nadG550*(Ts)
TT17534	*nadB449*::MudJ *zhd-3775*::Tn*10*d(Cm)
TT17535	*nadB449*::MudJ *zbc-3776*::Tn*10*d(Cm) *nadF548*(Cs)
TT17536	*pnuE84*::MudJ *zbc-3769*::Tn*10*d(Tc)
TT17537	*pnuE84*::MudJ *zhd-3769*::Tn*10*d(Tc) *nadG550*(Ts)
TT17538	*pnuE84*::MudJ *zbc-3767*::Tn*10*d(Tc)
TT17539	*pnuE84*::MudJ *zbc-3767*::Tn*10*d(Tc) *nadF548*(Cs)
TT17540	Δ*nadF553*
TT17541	*nadB449*::MudJ Δ*nadF553*
TT17542	*nadB449*::MudJ *nadF551*(Ts) *zbc-3767*::Tn*10*d(Tc)
TT17543	*nadB449*::MudJ *nadG552*(Ts) *zbc-3769*::Tn*10*d(Tc)
TT17544	Δ*nadF553 nadG550*(Ts) *zhd-3769*::Tn*10*d(Tc)
TT17545	*nadB449*::MudJ Δ*nadF553 nadG550*(Ts) *zhd-3769*::Tn*10*d(Tc)
TT15340	*nadB449*::MudJ *pncA278*::Tn*10*d(Cm) *nadI579*::Tn*10*d(Tc)
TT17560	*nadB449*::MudJ *nadF548*(Cs) *nadI579*::Tn*10*d(Tc)
TT17561	*nadB449*::MudJ *nadG550*(Ts) *nadI579*::Tn*10*d(Tc)
TT17562	*nadB449*::MudJ *nadF549*(QA^s) *nadI579*::Tn*10*d(Tc)
TT17568	*nadB449*::MudJ *zbc-3776*::Tn*10*d(Cm)

Mutagenesis. Diethyl sulfate mutagenesis of cells was done as described by Roth (23). Hydroxylamine mutagenesis of P22-transducing phage was performed as described by Davis et al. (6). The general method for localized mutagenesis is that of Hong and Ames (10).

Isolation of Tn*10*d(Tc) and Tn*10*d(Cm) insertions linked to the *nadF* or *nadG* gene. A *nadG* and a *nadF* mutant were transduced to tetracycline resistance by using P22 phage grown on a random pool of 10^4 Tn*10*d(Tc) or 10^4 Tn*10*d(Cm) insertion mutants (28). Transductants (Tc^r) were screened for loss of the recipient auxotrophic phenotype, indicative of coinheritance of a *nadF*$^+$ or *nadG*$^+$ allele with the selected Tn*10* element. Linked Tn*10* insertions were then transduced back into the original *nad* mutant to test linkage of the Tn*10*d(Tc) element to the mutant gene (*nadF* or *nadG*).

Mapping of *nadF* and *nadG* mutations. The location of the *nadF* and *nadG* genes was determined by mapping the nearby Tn*10* insertions by using the method of Benson and Goldman (1). More accurate mapping was done by testing transductional linkage to known markers in the region identified.

Isolation of *nadF* deletion mutants. Cells (10^8 to 10^9) of a strain carrying a Tn*10*d(Tc) insertion linked to the *nadF*$^+$ gene were spread on Bochner plates (2), which allow growth only of tetracycline-sensitive colonies. Spontaneous mutants which had lost tetracycline resistance were recloned on Bochner plates and were screened for the presence of a deletion that removed both tetracycline resistance and the ability to grow on minimal medium. The isolated auxotrophic deletion mutant (*nadF553*) proved to be deficient in NAD kinase activity.

Protein determination. Protein concentration was determined by the method of Lowry et al. (17) with bovine serum albumin as a standard.

β-Galactosidase assay. Assays of β-galactosidase activity were performed as described by Miller (21), with sodium dodecyl sulfate-chloroform-permeabilized cells. The β-galactosidase activity is reported as nanomoles per minute per optical density unit (650 nm) of cells.

TABLE 2. Phenotypes of NAD kinase mutants

Strain	Relevant genotype[a]	Lac phenotype on minimal medium + X-Gal[b]				Growth on rich medium (NB)[c]			
		Low[d] NA		High NA[e]		−QA		+QA[f]	
		30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
TT10738	Wild type	B	B	W	W	+	+	+	+
TT17510	*nadF548*(Cs)	−	LB	−	W	+	+	−	+
TT17512	*nadF549*(QA^s)	−	−	−	−	+	+	−	−
TT17542	*nadF551*(Ts)	LB	−	W	−	+	+	+	−
TT17540	Δ*nadF553*	−	−	−	−	+	+	−	−
TT17511	*nadG550*(Ts)	LB	−	W	−	+	+	+	+
TT17543	*nadG552*(Ts)	LB	−	W	−	+	+	+	+
TT17545	Δ*nadF553 nadG552*(Ts)	−	−	−	−	+	−	−	−

[a] All strains have the *nadB449*::MudJ (Lac) insertion.
[b] Letters indicate color of colonies growing on medium containing X-Gal. B, dark blue; LB, light blue; W, white. A minus sign indicates no growth.
[c] +, growth; −, no growth.
[d] Low NA (10^{-6} M); *nadB* auxotrophs grow with derepressed *nadB* and *nadA* genes.
[e] High NA (2×10^{-4} M); *nadB* auxotrophs grow with repressed *nadB* and *nadA* genes.
[f] 30 mM.

NAD pool assay. A fresh overnight culture was diluted into minimal medium (10 ml) and was grown to 100 Klett units. Radioactive [carbonyl-^{14}C]Na was added to final concentration (0.01 μCi/5ml). Cells were grown for 30 min, pelleted, washed twice with cold medium, and resuspended in 0.5 ml of 6 N HCl to lyse the cells. The cell lysate was spotted onto DEAE paper and was chromatographed by using 0.25 M ammonium bicarbonate as solvent. Unlabeled NAD and NADP were spotted onto the paper as markers. The chromatogram was cut vertically into strips, one for each lane. Each such strip was cut horizontally into 1.5-cm pieces, and each was counted with a Beckman LS200 liquid scintillation counter. These general assay methods have been described previously (12, 13, 18).

Preparation of cell extracts and assay of NAD kinase. A 500-ml volume of an exponentially growing cell culture was centrifuged, washed, and suspended in 5 ml of 0.02 M Tris buffer (pH 7.5). The cells were lysed with a French press at 20,000 lb/in^2. The extract was centrifuged at 17,000 × *g* for 30 min to remove debris. Crude extracts were supplemented with glycerol to a final concentration of 15% and were divided into small samples (500 μl), which were quickly frozen in dry ice-ethanol and stored at −70°C. Extracts stored in this way retained full activity for more than 1 month.

The NAD kinase assay procedure was that of K. T. Hughes (10a). The reaction mixture contained 10 μmol of Tris-HCl (pH 7.5), 48 nmol of NAD with 0.1 μCi of [carbonyl-^{14}C]NAD, 0.5 μmol of ATP, 0.5 μmol of $MgCl_2$, 0.2 μmol of dithiothreitol, and 35 μl of crude extract in a total volume of 100 μl. The reaction proceeded at 37°C for 30 min and was stopped by boiling for 30 s. Denatured protein was pelleted in a microcentrifuge, and the supernatant was spotted with cold NAD and NADP markers onto DEAE paper. Chromatography was performed as described above. All figures reported are from assays in which the amount of NADP measured fell within the linear dependence of the assay on time and protein.

RESULTS

Isolation of NAD kinase mutants. The rationale for isolation of NAD kinase mutants was based on the following assumptions. (i) NAD is probably the effector molecule for repression of the *nadB* and *nadA* genes by NadI protein. (ii) A partial defect in NAD kinase might cause an increase in the intracellular NAD pool. (iii) A complete lack of NAD kinase might be lethal. (iv) A temperature-sensitive NAD kinase mutant might fail to grow at the nonpermissive temperature and might have a partial defect in kinase at the permissive temperature. (v) The elevated NAD level caused by a partial defect in NAD kinase at the permissive temperature might lead to superrepression of a *nadB*::*lac* fusion. (vi) Superrepressed mutants might be detected as white colonies if cells carried a *nadB*::*lac* fusion and were plated on X-Gal plates.

In applying these assumptions, a *nadB*::*lac* fusion strain (TT10738) was mutagenized with diethyl sulfate and was plated for single colonies on nutrient broth at 30°C. The mutagenized clones were replica printed to minimal X-Gal medium with 1 μM NA (a concentration that is too low to repress *nadB* expression in normal cells) at 30 and 42°C. On this medium, the parent *nadB*::*lac* strain showed blue colonies. Plates were screened for clones that formed a lighter blue colony at one temperature and failed to grow at the other temperature. Three types of mutants were isolated, and their phenotypes are presented in Table 2. All types grow on NB medium at both the high and the low temperature. Cold-sensitive mutants failed to grow on minimal medium at low temperature; heat-sensitive mutants failed to grow on minimal medium at high temperature. The third type of mutant failed to grow on minimal medium at both the high and the low temperatures (30 and 42°C) but grew on minimal medium at 37°C. All mutants grew on NB at both temperatures (30 and 42°C). Growth of the third mutant type on NB was inhibited by 30 mM quinolinic acid (QA). On the basis of genetic tests to be described later, the three mutant types were found to affect two different chromosomal loci. The *nadF* locus included mutations of all three types; only temperature-sensitive alleles were found at the *nadG* locus. An insertion mutant with Tn*10*d(Tc) or Tn*10*d(Cm) near each locus was isolated. By using these nearby insertions, several additional *nadG* and *nadF* mutants were isolated following localized mutagenesis of the two regions. Properties of several of these mutants are included in Table 2.

Effects of *nadF* and *nadG* mutations on expression of a *nadB*::*lac* fusion. The rationale for isolating *nadF* and *nadG* mutants was to look for conditionally lethal mutants which superrepressed expression of the *nadB* gene when grown at the permissive temperature. We hoped that a partial defect in NAD kinase at the permissive temperature would lead to an increase in the NAD level and secondarily to repression of the *nadB* and *nadA* genes, which are thought to be controlled by this effector. The *nadF* and *nadG* mutants were initially

TABLE 3. Effects of NAD kinase mutations on expression of a *nadB*::*lac* operon fusion

Strain	Relevant genotype[a]	β-Galactosidase activity[b] in cells grown in minimal medium at the indicated temperature					
		30°C		37°C		42°C	
		Low NA	High NA	Low NA	High NA	Low NA	High NA
TT10738	Wild type	220	15	246	8	213	8
TT17510	*nadF548*(Cs)	NG[c]	NG	NG	NG	70	7
TT17512	*nadF549*(QA^s)	NG	NG	85	8	NG	NG
TT17542	*nadF551*(Ts)	100	8	NG	NG	NG	NG
TT17511	*nadG550*(Ts)	150	13	NG	NG	NG	NG
TT17543	*nadG552*(Ts)	164	5	NG	NG	NG	NG
TT15430	$nadF^+$ $nadG^+$ *nadI579*::Tn*10*d(Tc)	250	265	250	235	260	280
TT17560	*nadF548*(Cs) *nadI579*::Tn*10*d(Tc)	NG	NG	NG	NG	250	220
TT17562	*nadF549*(QA^s) *nadI579*::Tn*10*	NG	NG	230	277	NG	NG
TT17561	*nadG550*(Ts) *nadI579*::Tn*10*d(Tc)	240	265	NG	NG	NG	NG

[a] All the strains have the *nadB449*::MudJ mutation.
[b] Strains were grown at the permissive temperature in minimal medium supplemented with 1 μM (low) or 200 μM (high) NA. Activity is expressed in Miller units (27). For values below 20 Miller units, the standard deviation was less than 20%; for all higher values, the standard deviation was less than 6%.
[c] NG, assay was not done; strain does not grow at this temperature on minimal medium.

identified only by their colony color on medium with X-Gal. The putative regulatory phenotype of *nadF* and *nadG* mutations was tested by assaying β-galactosidase activity produced by a *nadB*::*lac* operon fusion (Table 3).

Expression of the *nadB*::*lac* fusion is controlled by the internal level of pyridine nucleotides, presumably NAD (13). Cells with a *nadB*::*lac* fusion but no *nadF* or *nadG* mutation repress their *nadB*::*lac* fusion when they grow on medium with a high level of NA. When these cells are grown with a low level of exogenous NA, the *nadB*::*lac* fusion is derepressed (see first line in Table 3). Addition of a *nadI* (repressor) mutation leads to constitutive high expression of the *nadB* fusion under all growth conditions (see line 7 in Table 3).

The new *nadF* and *nadG* mutations caused a reduction in *nadB* gene expression when cells were grown on low NA. This repression is presumably caused by an abnormally high level of NAD which accumulates because of a NAD kinase deficiency. As can be seen in Table 3, a high level of exogenous NA caused full repression in all strains, but repression is eliminated in all mutants and on all media by a *nadI* mutation.

Effects of *nadF* and *nadG* mutations on NAD pools. The effects of *nadF* and *nadG* mutations on intracellular NAD and NADP pools were assayed directly (Table 4). Both *nadF* and *nadG* mutations cause an increase in the NAD/NADP ratio. The effect of a conditional *nadF* mutation is greater at the nonpermissive temperature, but the effect of temperature on pools in the *nadG550*(Ts) mutant is only slightly greater at the nonpermissive temperature.

Genetic characterization of the *nadF* locus. To locate the *nadF* gene on the genetic map, we mapped a Tn*10* element (*zbc-3767*) which is 70% cotransducible with a *nadF* mutation. The Tn*10* insertion was mapped by the method of Benson and Goldman (1), which involves transducing the Tn*10*(Tc^r) insertion mutant to tetracycline sensitivity by using a series of donor lysates each prepared by inducing a well-mapped Mud P22 lysogen. Each such donor lysate transduces a characteristic region of the chromosome with high frequency. By this method, we mapped the insertion *zbc*-3767::Tn*10*d(Tc) between min 12 and 13 on the *Salmonella* chromosome.

Deletion *nadF553* (described above) is presumed to remove the parent Tn*10* insertion and extend into the nearby *nadF* gene. To test this, the deletion mutant was transduced with phage grown on the parent Tn*10* insertion mutant with or without a nearby *nadF*(Cs) point mutation. Selection was made for tetracycline-resistant transductants which were then scored for coinheritance of the donor *nadF*(Cs) mutation. In a standard ($nadF^+$) recipient, this Tn*10* element shows about 75% cotransduction with the *nadF* point mutations. If the deletion crosses the site of the Tn*10* insertion, all transductants should repair the deletion and acquire either a cold-sensitive or a wild-type phenotype. If the new deletion mutation removes the site of the Tn*10* insertion and extends into the *nadF* gene, it should cause an increase in the linkage between the donor Tn*10* insertion and a *nadF* point mutation. If the deletion removes both the site of the Tn*10* insertion and the site of the *nadF*(Cs) mutation, all Tc^r transductants should show the donor Cs phenotype. Both *nadF548*(Cs) and *nadG551*(Ts) mutations show 70 to 75% linkage with the

TABLE 4. NAD pools of NAD kinase mutants[a]

Strain	Relevant genotype	NAD pool/NADP pool ratio[b] at:		
		30°C	37°C	42°C
TT10738	*nadB449*::MudJ	2,001/424 = 4.7	3,730/764 = 4.8	4,504/921 = 4.8
TT17510	*nadB449*::MudJ *nadF548*(Cs)	6,594/344 = 19.2		6,980/694 = 10.1
TT17512	*nadB449*::MudJ *nadF549*(QA^s)		5,104/640 = 12.8	
TT17511	*nadB449*::MudJ *nadG550*(Ts)	3,261/423 = 7.7		6,188/664 = 9.3

[a] Cells were grown in 10 ml of minimal medium to 100 Klett units at permissive temperature, and the culture was split; one half (5 ml) was shifted to the nonpermissive temperature, and the other half was left at the permissive temperature. [carbonyl-^{14}C]NA was added to both halves. Cells were grown for 30 min to label pools. Cells were washed, pelleted, and lysed with HCl, and the entire supernatant was chromatographed. The NAD and NADP spots were excised as described in Materials and Methods.
[b] The ratio of NAD/NADP was calculated as the ratio of radioactivity in the NAD and NADP spots on the DEAE chromatograph. The counts are reported as (cpm)/5 × 10^9 cells. The standard deviation was less than 10%.

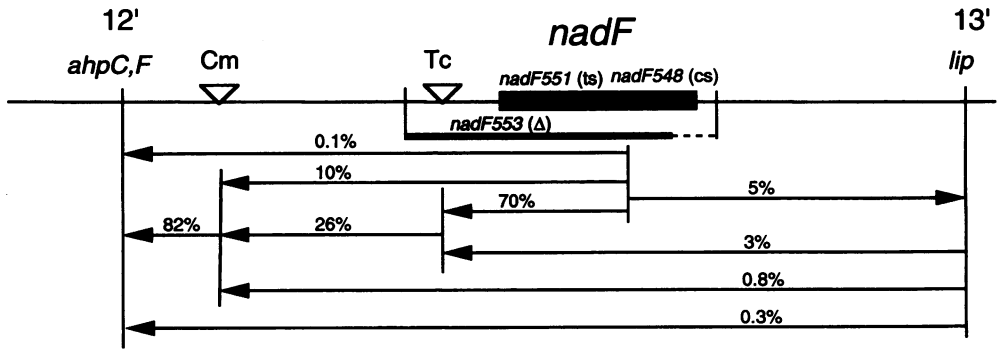

FIG. 2. Map position of the *nadF* gene in the *S. typhimurium* chromosome. Cm, insertion *zbc-3776*::Tn*10*d(Cm); Tc, insertion *zbc-3767*::Tn*10*d(Tc). The extent of the *nadF553* deletion is indicated just below the map. All the linkages were determined by P22 transduction crosses. For each cross, the phenotype of 120 transductants was scored. The arrowheads point to the donor marker selected in the cross.

nearby Tn*10* element when crossed into a wild-type recipient strain. The same two *nadF* mutations are 100% (250 of 250) linked to the Tn*10* insertion when they are crossed with a recipient carrying the *nadF553* deletion. Therefore, it is likely that the deletion removes both the site of the Tn*10* insertion and the site of the two *nadF* point mutations tested.

Transduction crosses were performed to test the linkage of *nadF* mutations to nearby mutations known to lie in this region of the chromosome. The results, summarized in Fig. 2, place the *nadF* locus between the *lip* and *ent* loci.

Genetic characterization of the *nadG* locus. The approximate position of the *nadG* gene was determined by using the method of Benson and Goldman (1) to map a Tn*10* insertion (*zhd-3769*) which is 71% cotransducible with a *nadG* point mutation. The *nadG* locus was located between min 72 and 73 of the *Salmonella* chromosome.

To locate the *nadG* gene more exactly, we determined the transductional linkage between *nadG* mutations, insertions linked to *nadG*, and mutations in genes known to map in this region of the chromosome. The results, summarized in Fig. 3, place the *nadG* locus between the *rpsL* and *argD* loci.

Construction of *nadF nadG* double mutants. Initial assays of NAD kinase activity in single *nadF* or *nadG* mutants indicated that each single mutation eliminated only part of the total activity seen in wild-type cells. Strains were constructed that carried both *nadF* and *nadG* mutations.

A Tn*10* insertion linked to the *nadG*(Ts) mutation in strain TT17543 was transduced into the *nadF* deletion mutant (TT17541); tetracycline-resistant transductants were selected and screened for coinheritance of the donor *nadG* phenotype (temperature-sensitive growth on NB). The presence of the *nadG* mutation in the constructed *nadG nadF* double mutant was demonstrated by transducing Tc^r from the putative double mutant to a wild-type recipient strain (TT10738). As expected, 70% of the tetracycline resistance transductants inherited the temperature-sensitive auxotrophy characteristic of the original *nadG*(Ts) mutation.

The phenotype of the *nadG*(Ts) Δ*nadF* double mutant is shown at the bottom of Table 2. On rich medium, growth of the double mutant is inhibited at high temperature; growth at 30°C on NB is inhibited by 30 mM QA. The double mutant (like the simple *nadF* deletion mutant) does not grow on minimal medium at any temperature.

Effects of temperature on the growth of NAD kinase mutants. Fresh overnight cultures grown in NB were diluted (1:50) into minimal medium, grown at 30°C for 3 h, and then shifted to 42°C. In minimal medium, growth of *nadF* and *nadG* single mutants stopped within about 1 h following the temperature shift; the wild-type control strain showed no change in growth rate (Fig. 4A). In NB, only the *nadF nadG*(Ts) double mutant showed a growth defect after the temperature shift. The growth rate of single mutants in NB did not decrease at the nonpermissive temperature (Fig. 4B).

Effect of QA on the growth of *nadF* and *nadG* mutants. As described above, all *nadF* mutants were able to grow in NB at high and low temperatures. This included nonconditional mutants such as mutant *nadF549* (Tables 2 and 3) and the deletion mutant (*nadF553*). Growth of these *nadF* mutants on NB depended on the possession of a functional *nadG* allele. In the course of determining the growth phenotype of *nadF* and

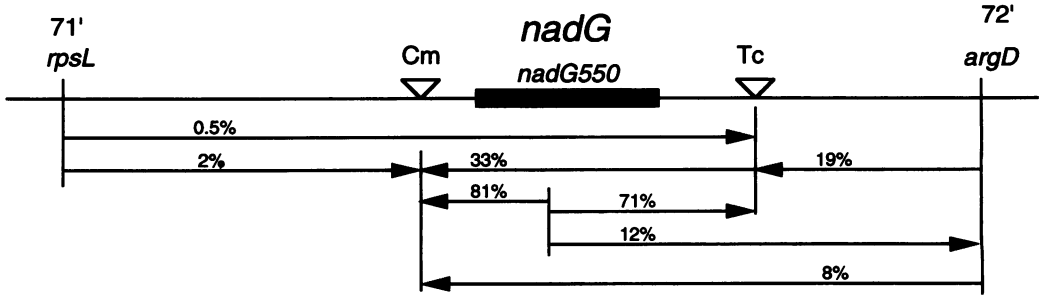

FIG. 3. Map position of the *nadG* gene in the *S. typhimurium* chromosome. Cm, insertion *zhd-3775*::Tn*10*d(Cm); Tc, insertion *zhd-3769*::Tn*10*d(Tc). All the linkages are P22-mediated cotransduction frequencies. For each cross, the phenotype of 120 transductants was scored. The arrowheads point to the donor marker selected in the cross.

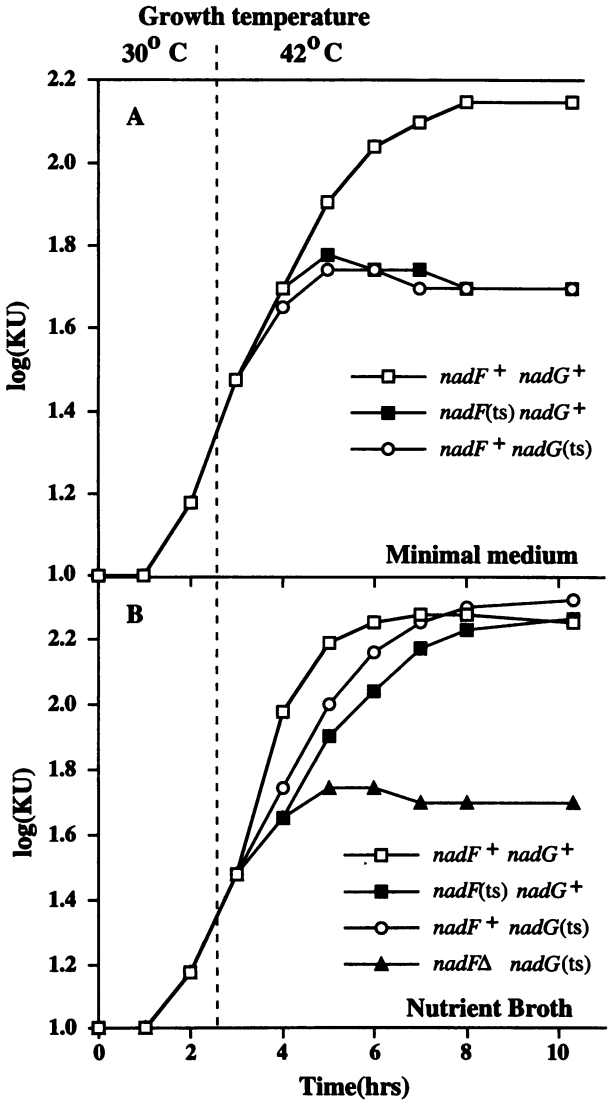

FIG. 4. Effects of temperature on the growth of NAD kinase temperature-sensitive mutants in minimal medium (A) and in NB (B). Fresh overnight cultures were diluted (1:50) in minimal medium (A) or NB medium (B), grown at 30°C for 3 h, and then shifted to 42°C. Strains used were TT10738 *nadF*$^+$ *nadG*$^+$, TT17511 *nadF*$^+$ *nadG*(Ts), TT17542 *nadF551*(Ts) *nadG*$^+$, TT17543 *nadF*$^+$ *nadG552*(Ts), and TT17544 Δ*nadF553 nadG550*(Ts).

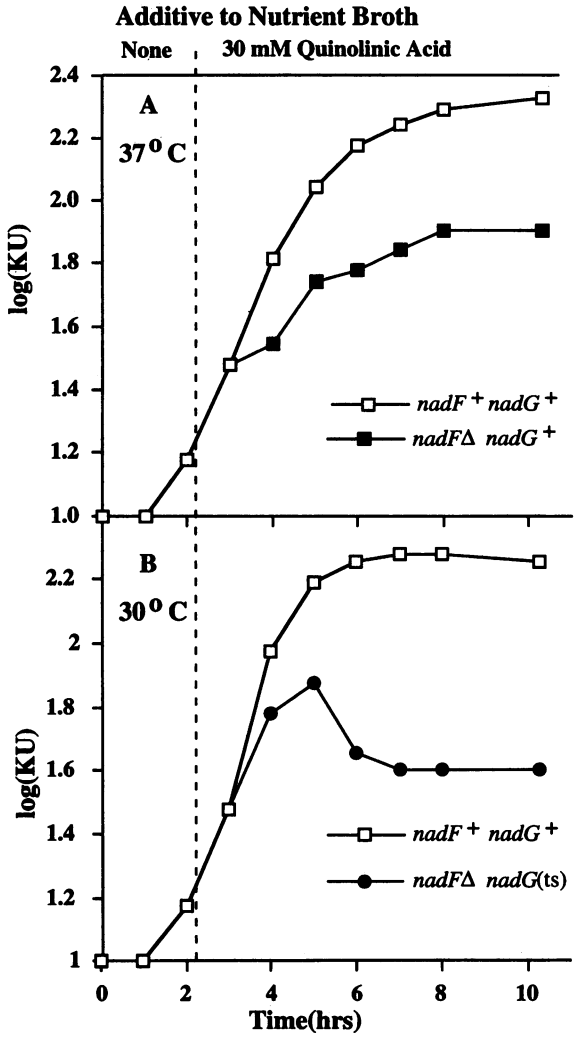

FIG. 5. Effects of QA on growth of *nadF* and *nadG* mutants. A fresh overnight culture was diluted in NB and was grown for 3 h at 37°C (A) or 30°C (B), and then QA was added to a final concentration of 30 mM. Strains used were TT10738 *nadF*$^+$ *nadG*$^+$, TT17540 Δ*nadF553 nadG*$^+$, and TT17544 Δ*nadF553 nadG550*(Ts).

nadG mutants, we tested the biosynthetic intermediate QA, the second intermediate in the pathway (Fig. 1). While this compound did not support growth of either *nadF* or *nadG* mutants on minimal medium, we were surprised to see that it strongly inhibited growth of a *nadF* mutant on NB. Since growth of this mutant on NB depends on the NadG enzyme, we inferred that the QA might act by inhibiting the NadG enzyme.

Growth inhibition by 30 mM QA was seen for a simple *nadF* deletion mutant, tested at 37°C (Fig. 5A), and for a *nadF* deletion with a *nadG*(Ts) mutation, tested at the permissive temperature (30°C). Growth inhibition of the Δ*nadF nadG*(Ts) double mutant was somewhat stronger than that of *nadF* single mutants (Fig. 5B). This suggests that the activity of the NadG enzyme (required for growth on NB) is inhibited by QA. The stronger growth inhibition seen in a strain with a partially defective NadG enzyme is consistent with this interpretation, if one assumes that the mutant NadG enzyme has less activity than does the wild-type enzyme but has normal sensitivity to inhibition of QA. Inhibition of the NadG enzyme by QA will be described below.

Assay of NAD kinase from *nadF* and *nadG* mutants. To test the idea that the phenotype of *nadF* and *nadG* mutants is due to a defect in NAD kinase, this enzyme was assayed in *nadF* and *nadG* single and double mutants (Fig. 6). All cells were grown in NB for 3 h, and then the cultures were split; one half was grown at 30°C, and the other half was grown at 42°C. After 3 h, extracts were made from each culture and the NAD kinase activity was assayed at 37°C as described in Materials and Methods. As seen in Fig. 6, NAD kinase activity at the non-permissive temperature is reduced 30% by a *nadG*(Ts) mutation (TT17543) and is decreased 70% by a *nadF*(Ts) mutation. The NAD kinase activity in the *nadF* deletion strain (TT17541) was 70% lower than that in the wild type at both temperatures.

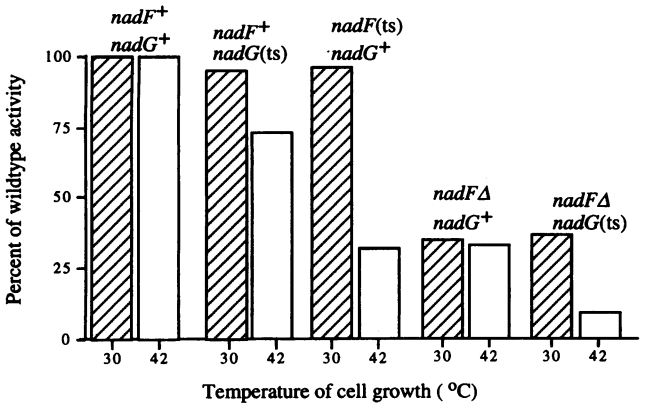

FIG. 6. Assay of NAD kinase of *nadF* and *nadG* mutants. All cells were grown in NB at 30°C for 3 h, and then the culture was split; one half was grown to full density at 30°C and the other was grown at 42°C. Extract procedure and assay conditions are described in Materials and Methods. All the assays were run at 37°C for 30 min. The NAD kinase activity from a wild-type strain (TT10738) (580 pmol/min/mg of protein) was set at 100; all the mutant NAD kinase activity levels are expressed as a percentage of the wild-type level. The strains assayed were TT10738 *nadF*$^+$ *nadG*$^+$, TT17543 *nadF*$^+$ *nadG552*(Ts), TT17542 *nadF551*(Ts) *nadG*$^+$, TT17540 Δ*nadF553 nadG*$^+$, TT17544 Δ*nadF553 nadG550*(Ts).

In a Δ*nadF nadG*(Ts) double mutant (TT17544), the NAD kinase activity was 30% of that of the wild type at 30°C but decreased to nearly zero at 42°C (Fig. 6). The combined data suggest that *nadF* and *nadG* are structural genes that encode two distinct NAD kinases. The *nadF* gene encodes about 70% of wild-type NAD kinase activity, while the *nadG* gene encodes about 30%.

Heat inactivation of NadF and NadG enzymes from temperature-sensitive mutants. To confirm that the temperature-sensitive growth phenotype and reduced NAD kinase activity seen in *nadF* and *nadG* mutants were due to a qualitative change in the NadF and NadG enzymes, the heat inactivation of kinase activity was tested in vitro. Extracts were made from cells grown at 30°C in NB. The extracts were incubated at 65°C; samples were withdrawn and added directly to the reaction mixture to measure the NAD kinase activity. We presume that, in strains with a *nadF* deletion mutation, all kinase is produced by the *nadG* gene. In the Δ*nadF nadG*(Ts) strain, the NAD kinase activity was more rapidly inactivated than in a Δ*nadF nadG*$^+$ strain, indicating that the nadG(Ts) mutation causes a qualitative change in NAD kinase (Fig. 7A).

Because no null alleles of the *nadG* gene were available, the effect of a *nadF*(Ts) mutation (70% of total activity) was tested in strains with a wild-type allele of the *nadG* gene (30% of total activity). The *nadF*(Ts) mutation caused a significant change in the temperature stability of NAD kinase (Fig. 7B). The kinetics of this inactivation are peculiar; an investigation of this will be delayed until purified enzyme is available. We conclude that both *nadF* and *nadG* mutations change the quality of NAD kinase activity and are likely to affect the structural genes for distinct proteins with this activity.

Effects of QA on the *nadG* NAD kinase activity. Inhibition of growth of *nadF* mutant strains by QA (Fig. 8) suggested that this pyridine biosynthetic intermediate might act as an inhibitor of the NadG protein. This was tested by examining the inhibition of NAD kinase activity in vitro. Extracts were made from cells grown at 30°C in NB, and assays were performed with QA added to the reaction mixture (Fig. 8). In strains with

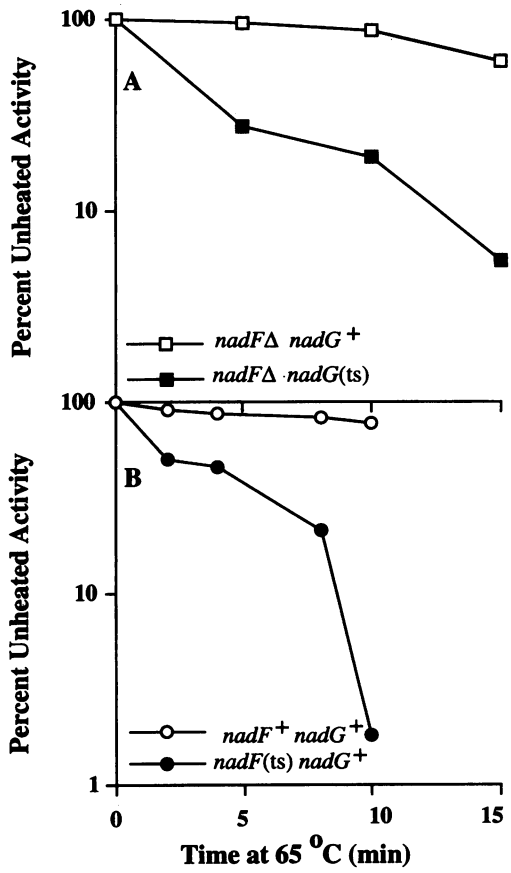

FIG. 7. Heat inactivation of NAD kinase. Extracts were made of cells grown at 30°C in NB. The extracts were incubated at 65°C for the times indicated; samples were withdrawn and added directly to the reaction mixture to measure the NAD kinase activity. Assays were run at 37°C. Initial wild-type activity and mutant activity were set at 100, and later points were expressed as a percentage of the initial activity. Strains used were TT10738 *nadF*$^+$ *nadG*$^+$, TT17542 *nadF551*(Ts) *nadG*$^+$, TT17540 Δ*nadF553 nadG*$^+$, and TT17544 Δ*nadF553 nadG 550*(Ts).

a wild-type *nadF* allele (TT10738 and TT17543), 1 mM QA reduced total kinase activity by 30%. However, in *nadF* deletion strains (TT17540 and TT17544), NAD kinase activity was completely inhibited by 1 mM QA. These data suggested that the NAD kinase activity encoded by the *nadG* gene was sensitive to QA. Since 30 mM QA was required to inhibit growth of the *nadF* mutant in NB, we suspect that QA is poorly transported. Pyridine auxotrophs (*nadA* or *nadB* in Fig. 1) are fed by QA but require 10 mM QA to satisfy their growth requirements (14a).

DISCUSSION

A search for mutants defective in NAD kinase yielded two classes of mutations which mapped at widely separated positions (*nadF* at 13 min and *nadG* at 73 min). No *nad* genes have been previously mapped to these positions.

Our results might be explained in several ways. (i) There could be two independent kinases. (ii) One gene might encode a kinase and the other gene might encode a transcriptional regulator of that kinase. (iii) Each gene might encode a subunit of a single kinase with dissimilar subunits. We believe the data

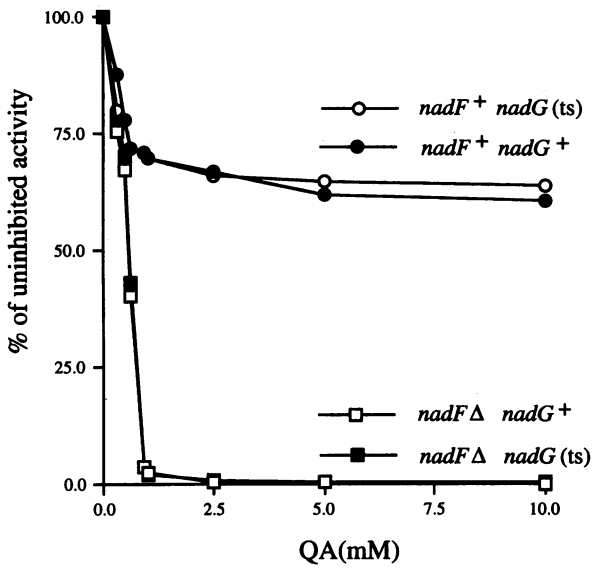

FIG. 8. Effect of QA on NAD kinase activity. All extracts were made from cells grown at 30°C in NB. The assay was done under standard conditions (37°C) as described in Materials and Methods. Wild-type NAD kinase activity without QA was set at 100. All other activities are expressed as a percentage of the uninhibited wild-type NAD kinase activity. Strains assayed were TT10738 *nadF*$^+$ *nadG*$^+$, TT17543 *nadF*$^+$ *nadG552*(Ts), TT17540 Δ*nadF553* *nadG*$^+$, TT17544 Δ*nadF553* *nadG550*(Ts).

best support the existence of two kinases. The idea of a transcriptional regulator is made unlikely by the fact that both *nadF* and *nadG* mutants produce qualitatively distinct enzymes with an increased temperature lability. Furthermore, the residual activity in a *nadF* mutant is inhibited by QA, while the residual activity in a *nadG* mutant is resistant to inhibition. The possibility of a multisubunit protein is harder to eliminate. Since the *nadF* deletion mutant makes an active enzyme, we suppose that the *nadG* protein has some independent kinase activity. While we have not yet found a *nadG* deletion mutant, the existence of temperature-sensitive mutations which retain 30% activity at the high temperature suggests that the NadF protein also has independent activity. It is noteworthy that the *nadF* and *nadG* mutations have additive effects on the total kinase activity assayed, consistent with the existence of two independent kinases. The widely separated map positions of the *nadF* and *nadG* genes are an indirect argument against a multimeric protein, since most such proteins are encoded by adjacent genes. Thus, while we cannot yet absolutely eliminate the possibility of a protein with two dissimilar subunits, we feel the evidence best supports the existence of two independent NAD kinases.

The strategy used to identify these mutants was based on several unproved assumptions. The success of the strategy lends support to the validity of those assumptions. We assumed that NAD kinase is an essential enzyme and that a partial defect might cause an elevation in the cellular NAD level. These are reasonable assumptions and therefore not very informative when supported. More important is the assumption that the internal NAD level serves to regulate NadI-mediated repression of the *nadA* and *nadB* genes. Evidence for this is consistent with the observation of Olivera et al., who showed that starvation of an *nad* auxotroph causes a progressive drop in NAD pools as growth rate and cell size decrease, while NADP pools remain quite constant and large (18). Maintenance of the NADP pool during starvation may reflect the importance of this pool for synthetic metabolism and allows cells to quickly resume growth following pyridine limitation, while the other pool can be sacrificed until growth resumes. The drop in NAD levels is presumed to be responsible for the regulatory effects on transcription of *nadA* and *nadB* genes. It seems likely that a more detailed study of the NAD kinases will reveal intricate controls that regulate the size of the NAD and NADP pools in response to a limitation of pyridine nucleotide production.

Strains carrying a single *nadF* or *nadG* mutation fail to grow on minimal medium but grow on NB. The growth defect could not be corrected by any simple nutrient supplement tested, including amino acids, purines, pyrimidines, and vitamins. We have described these mutants as general auxotrophs, with the idea that a shortage of NADP leads to a pleiotropic defect in many synthetic processes that cannot be corrected by any particular small molecule but can be spared by a complex array of compounds. While this interpretation may be correct, the situation could be more complex. Many temperature-sensitive mutants are able to grow on rich medium at the nonpermissive temperature. This medium could simply reduce synthetic stress on an impaired cell and allow some growth. For a specific example, temperature-sensitive *nadD* mutants (blocked before both NAD and NADP; Fig. 1) can also grow at the nonpermissive temperature on NB (12).

Results presented here suggested that one of the NAD kinases (NadG) is inhibited by QA. While this inhibition may reflect some pathway control, we suspect it is actually part of a response to O_2 stress. It has been suggested that NAD levels may be reduced in response to H_2O_2 so as to minimize production of hydroxyl radicals by the nonenzymatic Fenton reaction (14). This reaction requires a reducing agent (NADH works, but not NADPH) and Fe^{2+}. One might expect cells to defend against this reaction by breaking down NAD during exposure to high O_2 and Fe^{2+}. If this mechanism involved NAD kinase, one might expect kinase to be stimulated by O_2 and/or Fe^{2+}. Since QA chelates iron, it may inhibit *nadG* by removing the activator Fe^{2+}. Other iron chelators also inhibit growth of *nadF* mutants. We know that inhibition of NAD kinase by QA is prevented by the addition of Fe^{2+} in vitro. These results suggest that the NadG enzyme is activated by Fe^{2+} and can be inhibited when the activator is chelated by QA.

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