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The *Escherichia coli efg* Gene and the *Rhodobacter capsulatus adgA* Gene Code for NH_3-Dependent NAD Synthetase

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The essential gene *efg*, which complements ammonia-dependent growth (*adgA*) mutations in *Rhodobacter capsulatus* and is located at 38.1 min on the *Escherichia coli* chromosome, was found to code for NH_3-dependent NAD synthetase. Crude extracts from a strain which overproduces the *efg* gene product contained up to 400 times more activity than crude extracts from the control strain, and the purified Efg protein possessed NH_3-dependent NAD synthetase activity. Glutamine-dependent NAD synthetase activity was found in crude extracts of *E. coli* but not in the purified enzyme, suggesting that it may be catalyzed by an additional subunit. An *R. capsulatus* strain carrying an *adgA* mutation was found to be deficient in NAD synthetase activity, and activity was restored by complementation with the *E. coli* gene. In accordance with the nomenclature proposed for *Salmonella typhimurium* (K. T. Hughes, B. M. Olivera, and J. R. Roth, J. Bacteriol. 170:2113–2120, 1988), the *efg* and *adgA* genes should now be designated *nadE*.

The essential gene *efg*, located at 38.1 min on the *Escherichia coli* chromosome (12), was originally identified by its ability to complement ammonia-dependent growth (*adgA*) mutations in the photosynthetic bacterium *Rhodobacter capsulatus* (2, 17). The *efg* gene product has been purified and shown to be a homodimer with a subunit M_r of 30,600 and a pI of 5.6 (2). The *R. capsulatus adgA* gene has been cloned and sequenced, and it codes potentially for a polypeptide with an M_r of 59,712 (13). The *efg* gene product and the C-terminal region of the *adgA* gene product have sequence homology, including three highly conserved regions of about 20 amino acids each. One of these regions corresponds to a glycine-rich sequence, which is also found in the GMP synthase from *E. coli* (13). In addition, an essential gene from *Bacillus subtilis*, *outB*, has been shown to code for a protein with high-level sequence identity to the *efg* gene product (1).

The pleiotropic phenotype of *adgA* mutants of *R. capsulatus* originally suggested a regulatory role for this gene ("Ntr-like" phenotype) (2) but is also consistent with an indirect metabolic effect of the mutation (13). It was recently suggested that *adgA* (and, by analogy, *efg*) might code for an enzyme which catalyzes an unidentified amide transfer reaction (13). In wild-type strains, this enzyme would use either NH_3 or glutamine as the amide (N) donor, whereas in Adg^- mutants, it may have lost the ability to use glutamine as the N donor. Several enzymes catalyzing this type of reaction have been described for *E. coli*, but the genes coding for most of these enzymes have been cloned and sequenced, and their map positions are known (see, e.g., references 4 and 10). An exception is NAD synthetase (EC 6.3.5.1), which catalyzes the synthesis of NAD from either NH_3 or glutamine and nicotinic acid adenine dinucleotide (NaAD) (15):

$$\text{NaAD} + \text{ATP} + \text{NH}_3 \rightarrow \text{NAD} + \text{AMP} + \text{PP}_i$$

$$\text{NaAD} + \text{ATP} + \text{glutamine} \rightarrow \text{NAD} + \text{AMP} + \text{PPi} + \text{glutamate}$$

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NAD synthetase has been purified from *E. coli* and characterized enzymatically and was found to use NH_3 in preference to glutamine (11), in contrast to the yeast enzyme, which is able to use the two substrates with similar efficiencies (15). In *Salmonella typhimurium*, the gene for NAD synthetase, designated *nadE*, was found to be located at 27 min on the chromosome (6). However, the cloning of the gene from either *S. typhimurium* or *E. coli* has not been reported. The experiments described below show that the gene previously designated *efg* is in fact the structural gene for NH_3-dependent NAD synthetase and that the *R. capsulatus adgA* gene is probably also the structural gene for this enzyme. We also report the existence, in *E. coli*, of a previously undescribed glutamine-dependent NAD synthetase activity, which may be catalyzed by an additional subunit of the enzyme.

The *E. coli efg* gene is the structural gene for NH_3-dependent NAD synthetase. The *efg* gene was previously overexpressed in *E. coli* by cloning of the gene downstream from the *tac* promoter in the expression vector pKK223-3, to give plasmid pAP112, followed by induction of gene expression in strain JM105(pAP112) with IPTG (isopropyl-β-D-thiogalactopyranoside) (2). We have subsequently found that the *efg* gene is constitutively overexpressed in *E. coli* HB101 containing pAP112, apparently with no detrimental effect on cell growth. Crude cell extracts were prepared as follows. Cells were grown to stationary phase in Luria-Bertani medium containing 50 μg of ampicillin per ml, harvested by centrifugation, and washed by centrifugation and resuspension in 50 mM Tris-HCl (pH 7.6)–1 mM dithiothreitol–1 mM EDTA. After resuspension in 1/20 of the original culture volume of the same buffer, the cells were broken by sonication as described previously (2) and centrifuged for 5 min at 20,000 × *g* to remove cell debris. The crude extract was then dialyzed for 3 h at 4°C against 50 mM Tris-HCl (pH 7.6)–0.1 M NaCl. Protein in the cell extract was assayed by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.).

When dialyzed crude extracts of strain HB101(pAP112) were assayed spectrophotometrically for NAD synthetase, as described previously (11), the specific NH_3-dependent activity was found to be 400 times greater than that in extracts of the

TABLE 1. NAD synthetase activity in dialyzed crude extracts of *E. coli* strains

N donor	NAD synthetase activity[a] (nmol/min/mg of protein)	
	HB101(pAP112)[b]	HB101(pKK223-3)
None	<1.0	<1.0
$(NH_4)_2SO_4$ (1 mM)	8,330	20.7
Glutamine (2 mM)	10.3	10.8

[a] Rate of NAD formation corrected for nonspecific activity in the absence of substrate (NaAD). The values shown are averages of results from two separate experiments, with the assays performed in duplicate.

[b] The plasmid pAP112 contains the *E. coli efg* gene cloned behind the *tac* promoter in the expression vector pKK223-3 (2).

control strain, HB101(pKK223-3) (Table 1), whereas the glutamine-dependent NAD synthetase activity was similar in extracts of both strains. In undialyzed crude extracts, the NAD synthetase activity in the presence of glutamine was only slightly greater than that in the absence of added N donor (data not shown). In a previous study, the specificity of NAD synthetase in crude extracts was not examined (11).

The *efg* gene product was purified from undialyzed cell extracts of strain HB101(pAP112) by ion-exchange chromatography and gel filtration, as described previously (2). At each stage of the purification, NH_3-dependent NAD synthetase activity coeluted with the major protein fraction, and the final protein fraction was >95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The specific activity of the purified protein was 7.0 μmol of NAD synthesized per min per mg of protein, which is almost identical to the value of 6.9 μmol/min/mg of protein reported by Spencer and Preiss (11). It should be noted, however, that the specific activity in extracts of strain HB101(pAP112) was greater than that of the purified enzyme, suggesting that some loss of activity occurs during purification (Table 1).

In the presence of 2 mM glutamine, the purified NAD synthetase showed an apparent glutamine-dependent activity which was approximately 200-fold lower than the NH_3-dependent activity. The rate of NAD synthesis increased with increasing enzyme concentration but reached a saturation value of approximately 0.08 nmol/min/ml of reaction medium. This value is similar to the rate of spontaneous hydrolysis of glutamine to NH_3, which was determined to be 0.27%/h at pH 8.5 and 37°C, equivalent to a rate of 0.086 nmol/min/ml at a glutamine concentration of 2 mM. The apparent glutamine-dependent activity of the purified enzyme can therefore be attributed to the spontaneous hydrolysis of glutamine to NH_3. In contrast, the rate of glutamine-dependent NAD synthesis in crude extracts was 100 times greater than the spontaneous rate of glutamine hydrolysis (Table 1). *E. coli* therefore contains a glutamine-dependent NAD synthetase activity, which does not copurify with the NH_3-dependent enzyme. The glutamine-dependent activity is probably catalyzed by an additional subunit rather than by a distinct enzyme, since the NH_3-dependent NAD synthetase is essential for growth (6, 12).

Assay of NAD synthetase activity in *R. capsulatus* wild-type strain B10. NAD synthetase activity in crude extracts of *R. capsulatus* B10 was found to be approximately 50-fold lower than that in the *E. coli* control strain (Table 2) and could be assayed only fluorimetrically. Cultures were grown photosynthetically to mid-exponential phase in minimal RCV medium (5) as described previously (2). Crude extracts were prepared by sonication as described above, except that cells were washed and resuspended in buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 0.1 mM EDTA. To eliminate the high level of background activity observed in the absence of added N donor (NH_4^+ or glutamine), the crude extracts were dialyzed against 50 mM Tris-HCl, pH 7.5. The assay medium was similar to that used for *E. coli*, except that the concentration of NaAD was decreased from 1 mM to 100 μM (it was verified that the apparent K_m for NaAD was <10 μM in crude extracts). The reaction medium (1 ml), containing 100 to 120 μg protein, was incubated at 30°C for 30 min, and then the reaction was quenched by the addition of 100 μl of 3 N $HClO_4$. The amount of NAD formed was then determined with alcohol dehydrogenase (7), using a Perkin-Elmer MPF-2A fluorescence spectrophotometer with an excitation wavelength of 360 ± 14 nm and an emission wavelength of 460 ± 5 nm. The fluorescence changes were calibrated by the addition of 125 pmol of NADH to the 1-ml reaction cuvette. The rate of NAD synthesis was observed to be constant up to 30 min, but it decreased rapidly after this time. In contrast to the case with the *E. coli* enzyme, which has a pH optimum (with NH_4^+ as the substrate) of 8.5, the pH optimum of the *R. capsulatus* enzyme in crude extracts was found to be 10.4. This high pH optimum would be consistent with NH_3, rather than NH_4^+, being the active form.

With glutamine as the added N donor, the *R. capsulatus* enzyme showed a much broader pH profile, although the pH optimum also appeared to be greater than 10 (Table 2). The rate of NAD synthesis in the presence of glutamine was lower than the rate of spontaneous hydrolysis to NH_3. No conclusion

TABLE 2. NAD synthetase activity in dialyzed crude extracts of *R. capsulatus* strains

Strain[a]	Relevant genotype	NAD synthetase activity[b] (nmol/min/mg of protein)			
		NH_3 dependent		"Glutamine dependent"[c]	
		pH 8.5	pH 10.4	pH 8.5	pH 10.4
B10(pRK290)	*adgA*$^+$	0.28 ± 0.15	0.41 ± 0.13	0.08 ± 0.03	0.06 ± 0.02
B10(pAP100)	*adgA*$^+$/*efg*$^+$	0.56 ± 0.12	0.70 ± 0.09	0.22 ± 0.05	0.30 ± 0.05
B10(pAP127)	*adgA*$^+$/*adgA*$^+$	0.95 ± 0.22	1.95 ± 0.06	0.54 ± 0.16	0.74 ± 0.23
RC34(pRK290)	*adgA34*	0.16 ± 0.05	0.09 ± 0.05	0.02 ± 0.01	0.02 ± 0.02
RC34(pAP100)	*adgA34*/*efg*$^+$	0.75 ± 0.28	0.72 ± 0.06	0.27 ± 0.04	0.27 ± 0.03
RC34(pAP127)	*adgA34*/*adgA*$^+$	0.63 ± 0.07	0.98 ± 0.17	0.25 ± 0.02	0.25 ± 0.03

[a] Plasmid pAP100 contains the *E. coli efg* gene (2), and pAP127 contains the *R. capsulatus adgA* gene (17); both were cloned in the broad-host-range vector pRK290 (3).

[b] Rate of NAD formation corrected for nonspecific activity observed in the absence of added substrate (N donor, NaAD, or ATP). The values shown are the averages ± standard errors from three or four separate experiments.

[c] In all cases, the rate of NAD formation in the presence of glutamine was lower than the rate of spontaneous hydrolysis of glutamine to NH_3.

can therefore be drawn as to the presence or absence of glutamine-dependent NAD synthetase in *R. capsulatus*.

Assay of NAD synthetase activity in the Adg$^-$ mutant RC34 and in complemented strains. The specific activity of NAD synthetase in dialyzed crude extracts of strain RC34 was lower than that in extracts of strain B10. Activity was lower both with NH_4^+ and with glutamine as the added N donor, although the activity in the presence of glutamine appeared to be more strongly affected (Table 2). The pH dependence of the enzyme also appeared to be affected, since the NH_3-dependent activity in the mutant was greater at pH 8.5 than at pH 10.4 (Table 2). In addition, the enzyme appeared to be less stable in crude extracts of strain RC34 than in crude extracts of the wild-type strain; NH_3-dependent activity was undetectable in extracts of the mutant after storage for 10 days at −70°C or overnight at 4°C, whereas extracts of the wild-type strain retained more than 50% of their activity under these conditions. These results suggest that in the mutant, both the catalytic activity and the stability of the enzyme are affected, i.e., that the *adgA34* mutation is located within the structural gene for NAD synthetase.

Both the *E. coli efg* gene and the wild-type *adgA* gene restored NAD synthetase activities to levels greater than those found in the wild-type strain (Table 2). In the wild-type strain B10, the presence of a plasmid-borne *efg* gene increased NH_3-dependent NAD synthetase activity 2-fold, and the presence of a plasmid-borne *adgA* gene increased it 4.8-fold. Strain B10(pAP127) could therefore be used as a basis for purification of the *R. capsulatus* enzyme. Specific enzyme activities in strains carrying the *efg* gene were still far below those found in *E. coli*. This is consistent with previous results from Western blotting (immunoblotting) experiments, which indicated that the *efg* gene is weakly expressed in *R. capsulatus* (2). Given the observed amino acid sequence homology between the *efg* and *adgA* gene products (13) together with the evidence provided above that the *adgA34* mutation affects both the catalytic activity and stability of the enzyme, it seems likely that both genes code for the same protein, i.e., NH_3-dependent NAD synthetase. These genes should therefore be redesignated *nadE*, according to the nomenclature proposed for *S. typhimurium* (6).

The *adgA* gene product is predicted to be larger than the Efg protein (13), and the N-terminal region might be expected to correspond to a glutamine amido-transfer domain. However, it does not contain a recognizable glutamine amido-transfer sequence motif (16), and it remains to be determined whether the enzyme does indeed have glutamine-dependent NAD synthetase activity. Although the NH_3-dependent growth phenotype of *adgA* mutants could be explained by a loss of glutamine-dependent activity, it could also be explained by an increase in the K_m for NH_3. The fact that the Efg protein, which lacks glutamine-dependent activity, is able to restore NH_3-independent growth to the *adgA* mutants tends to support the latter hypothesis.

The assignment of an enzymatic function to the *efg* and *adgA* gene products raises the question of a possible regulatory role of NAD synthetase. In this context, it is interesting that a computer search for transmitter and receiver domains in two-component regulatory systems revealed the presence of an atypical receiver domain in the putative AdgA protein (9). Visual inspection of the Efg protein sequence, using its alignment with the AdgA protein sequence, shows that the Efg protein also contains a receiver domain (14). This suggests that the NAD synthetase may be regulated by phosphorylation, and in fact it has been shown that the *B. subtilis outB* gene product, which presumably also codes for NAD synthetase, is phosphorylated when extracts of stationary-phase cells are incubated with [γ-^{32}P]ATP (8).

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