Purification of Nitrogen Regulator II, the Product of the *glnL* (*ntrB*) Gene of *Escherichia coli*

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We purified the product of *glnL* (*ntrB*), NR_{II}, and the product of a mutant *glnL* allele, NR_{II}2302. In vitro transcription of the nitrogen-regulated promoter *glnAp2* by purified components of *Escherichia coli* required NR_{II} or NR_{II}2302 when the template DNA was linear.

In enteric bacteria, the expression of *glnA*, the structural gene for glutamine synthetase, and of a set of genes and operons known collectively as the Ntr regulon is regulated according to the quality and abundance of the nitrogen source (8). This regulation requires the *rpoN* (*glnF* *ntrA*) gene product σ^{60} and the *glnG* (*ntrC*) gene product NR_I and is affected by the products of three other genes: *glnB* (PII), *glnD* (uridylyltransferase), and *glnL* (NR_{II}) (2, 3-7, 9, 10). Genetic and physiological evidence suggests that PII and uridylyltransferase constitute a system for sensing the availability of ammonia; this information is communicated to NR_{II}, which in turn regulates the activity of NR_I (1, 3, 4).

It is known that σ^{60} and NR_I exert their effect by activating the initiation of transcription from nitrogen-regulated promoters such as *glnAp2* (7, 11). Wild-type *Escherichia coli* is able to adjust very rapidly the level of transcription from *glnAp2* in response to changes in the availability of ammonia. NR_{II} is required for this rapid response. In addition, mutations in *glnL* can result in an altered product, such as NR_{II}2302, which causes the activation of transcription from nitrogen-regulated promoters even under conditions of nitrogen excess (4).

Recent work in our laboratory has included the study of the transcription of *glnA* by use of purified components of *E. coli* (7). It was shown that the initiation of transcription from *glnAp2* carried on a supercoiled template required core RNA polymerase and σ^{60} . This transcription was greatly stimulated by the addition of NR_I and NR_{II}2302 together but not by either protein alone. When a linear DNA template was used, both NR_I and NR_{II}2302 were required for transcription from *glnAp2*. In that study, the NR_{II}2302 preparation used was purified by hydroxylapatite, heparin-agarose, and DEAE-Sephacel chromatography and was judged to be 70 to 80% pure by gel electrophoresis.

We report here the purification of NR_{II} and NR_{II}2302 by a different method. In each case, we used a high-copy-number plasmid containing a fusion of the desired *glnL* allele to a promoter-up mutation at *glnLp* (12) to cause the overproduction of the desired protein. These plasmids are pglN110 (*glnL*⁺) and pTH819 (*glnL*2302) (7). The *glnL* gene product was identified in minicell experiments on one- and two-dimensional gels as a protein with a molecular weight of 36,000 (data not shown). During the purification of NR_{II} and NR_{II}2302, we monitored the presence of the proteins by gel electrophoresis of samples from the various fractions. We

observed that the maximal overproduction of NR_{II} occurred when a strain with a deletion of the *glnALG* operon, YMC21 (pglN110) (1), was grown to saturation in glucose-glutamine-ampicillin medium (3). Under these conditions, which were used for both proteins, NR_{II} was shown to constitute roughly 1% of the total cell protein.

The purification protocol for NR_{II} was as follows. The cells from a saturated 19-liter culture were collected by centrifugation, suspended in saline, sedimented again, and stored as a paste at -20°C. The cells were thawed, suspended in 300 ml of buffer A (50 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7], 1 mM dithiothreitol, 20% [vol/vol] glycerol), and sonicated. The debris was removed by centrifugation, and the supernatant was made to contain 2% (wt/vol) streptomycin sulfate by the addition of the solid salt. The suspension was stirred at 4°C for 30 min, and the precipitate was removed by centrifugation. To the supernatant, solid ammonium sulfate (0.2 g/ml) was added, and the suspension was stirred for 5 h at 4°C, after which the precipitate was collected by centrifugation and suspended in 10 ml of buffer A. This sample was diluted with 40 ml of buffer B (buffer A plus 0.14 g of ammonium

FIG. 1. Sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis of purified NR_{II} and NR_{II}2302. Lanes: 1 and 12, partially purified core RNA polymerase enzyme; 2 to 6, 5, 3, 1, 0.5, and 0.3 mg of NR_{II}, respectively; 7 to 11, 5, 3, 1, 0.5, and 0.3 mg of NR_{II}2302, respectively; 13, protein standards from Bio-Rad Laboratories. The numbers to the right indicate the molecular weights of the standards in thousands. The proteins are stained with Coomassie brilliant blue R-250. Since the sum of the contaminants in lane 2 was less than the amount of NR_{II} in lane 5, we estimated greater than 90% purity for NR_{II}. Comparison of lanes 7 and 8 with lanes 10 and 11 suggested that the NR_{II}2302 preparation was approximately 90% pure.

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FIG. 2. In vitro transcription assays with purified components of *E. coli*. In vitro transcription assays were performed and the products were analyzed on acrylamide-urea gels autoradiographed as described previously (7). NR_I , when present, was at 240 nM, core RNA polymerase was at 50 nM, and σ^{60} , when present, was at 270 nM. (A) Transcription assays with a linear template. Lanes: 1, core RNA polymerase alone (E); 2, E + σ^{60} ; 3, E + σ^{60} + NR_I ; 4, E + σ^{60} + NR_I + NR_{II} (40 nM); 5, E + σ^{60} + NR_I + NR_{II} 2302 (20 nM); 6, as in lane 4, but NR_{II} was heated to 90°C for 5 min prior to use; 7, as in lane 5, but NR_{II} 2302 was heated to 90°C for 5 min prior to use. The transcript initiated from *glnAp2* is indicated by the letter b. (B) Transcription assays with a supercoiled template. Lanes: 1, E + σ^{60} ; 2, E + σ^{60} + NR_I ; 3, E + σ^{60} + NR_I + NR_{II} 2302 (20 nM); 4, as in lane 3, but NR_{II} 2302 was heated to 90°C for 5 min prior to use; 5, E + σ^{60} + NR_I + NR_{II} (20 nM); 6, E + σ^{60} + NR_I + NR_{II} (100 nM); 7, as in lane 6, but NR_{II} was heated to 90°C for 5 min prior to use; 8, E + σ^{60} + NR_I + NR_{II} 2302 (40 nM); 9, E + σ^{60} + NR_{II} 2302 (40 nM). The transcript initiated from *glnAp1* is indicated by the letter a (11); that initiated from *glnAp2* is indicated by the letter b.

sulfate per ml) and loaded onto a 50-ml agarose-ethane column (Pharmacia Fine Chemicals) equilibrated in buffer B. The column was washed with 200 ml of buffer B and eluted with a 200-ml linear gradient of buffer B to buffer A. (NR_{II} comes off this column very late in the gradient, $[\bar{c}]$, ~8 to 8.5 mS] and is approximately 80% pure at this point.) The NR_{II} peak fractions were pooled, dialyzed against buffer A, diluted with an equal volume of buffer A, and applied to a 20-ml DEAE-Sephacel column equilibrated in buffer A. The column was washed with 1 liter of buffer A and eluted with a 100-ml linear gradient of 0 to 400 mM NaCl in buffer A. The NR_{II} peak fractions (eluting at ~175 mM NaCl) were collected, glycerol was added to 50% (vol/vol), and the solution was stored at -70°C. The yield was about 5 mg.

A portion of the purified NR_{II} was chromatographed on a 185-ml AcA44 gel filtration column (LKB Instruments, Inc.); NR_{II} eluted at a position corresponding to an M_r of 68,000, identifying the native protein as a dimer (data not shown).

The purification of NR_{II} 2302 from a 4-liter culture was similar through the agarose-ethane step. The pooled NR_{II} 2302 agarose-ethane peak fractions were mixed with an equal volume of saturated ammonium sulfate, and the precipitated NR_{II} 2302 was collected by centrifugation and dissolved in 3 ml of buffer C (50 mM Tris [pH 7.8], 1 mM dithiothreitol, 20% glycerol). The sample was then applied to a 185-ml AcA44 gel filtration column equilibrated in buffer D (buffer C but with 100 mM NaCl and only 10% glycerol). This column was eluted with buffer D, and the pooled NR_{II} 2302 peak (corresponding to an M_r of 68,000) was diluted with an equal volume of buffer C, applied to a 20-ml DEAE-Sephacel column equilibrated in buffer C, and eluted

with a 100-ml linear gradient of 0 to 400 mM NaCl in buffer C. Glycerol was added to the pooled peak fractions to a final concentration of 50% (vol/vol), and the solution was frozen at -70°C. The yield from a 4-liter culture was about 1 mg.

Purified NR_{II} and NR_{II} 2302 were examined by electrophoresis on a sodium dodecyl sulfate-polyacrylamide (10%) gel with a 3% stacking gel (Fig. 1). We estimated that the purity of each protein was about 90%.

We examined the ability of purified NR_{II} and NR_{II} 2302 to stimulate transcription from *glnAp2* in vitro in the presence of σ^{60} , NR_I , and core RNA polymerase. The protocol for these experiments was as previously described (7), except that NR_I , when present, was at 240 nM, core RNA polymerase was at 50 nM, and σ^{60} , when present, was at 270 nM. When the template DNA was linear, transcription from *glnAp2* required NR_{II} or NR_{II} 2302 (Fig. 2A). When the template DNA was supercoiled (Fig. 2B), NR_{II} or NR_{II} 2302 caused much stimulation of transcription from *glnAp2*. These results are entirely in agreement with those of the previous report with NR_{II} 2302 purified in a different way (7). We have extended the previous findings by demonstrating that the wild-type protein, NR_{II} , has similar activity.

We have recently shown that NR_{II} is a protein kinase and a protein-phosphate phosphatase that regulates the activity of NR_I by the addition and removal of phosphate (8a).

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