Roles of the narJ and narI Gene Products in the Expression of Nitrate Reductase in Escherichia coli*

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Nitrate reductase, released and purified from membrane fractions of Escherichia coli, is composed of three subunits. Formation of the enzyme depends on induction of the nar operon, narGHJI, which is composed of four open reading frames (ORF). Previous studies established that the first two genes in the operon narG and narH encode the α and β subunits, respectively, while formation of the γ subunit, cytochrome b₉₃, depends on expression of the promoter distal genes. The narJ and narI genes were subcloned separately into plasmids where each was under the control of the nar promoter. Expression of these plasmids in a mutant which forms only α and β subunits revealed that expression of the narI gene is sufficient to restore normal levels of cytochrome b₉₃, but expression of both genes is required for assembly of fully active, membrane-bound nitrate reductase. The amino acid composition, the N-terminal sequence, and the sequence of cyanogen bromide fragments derived from the isolated γ subunit corresponds to that expected for a protein produced by the narI ORF. A protein corresponding to the narJ ORF did not appear to be associated with the purified nitrate reductase complex or with the complex immunoprecipitated from Triton X-100-solubilized membrane preparations. We conclude that narI encodes the γ subunit (cytochrome b₉₃) and that while the product of the narI gene is required for assembly of fully active membrane-bound enzyme it is not tightly associated with the active enzyme.

Nitrates reductase is a membrane-bound enzyme which is required for the utilization of nitrate as electron acceptor in anaerobic respiration in Escherichia coli. The purified enzyme is composed of three subunits α, β, γ with approximate molecular masses of 143,000, 60,000, and 20,000 kDa, respectively (1–3).

The formation of nitrate reductase is under the control of the nar operon which is expressed only under anaerobic conditions and is induced to maximal expression by the presence of nitrate. Based on characteristics of mutants generated by transposon insertion, the nar operon was postulated to include the structural genes for the three subunits of nitrate reductase organized in the order promoter - narG (α) - narH - narJ (β) - narI (γ) (4–6). Mutants with insertions in the narI region produced nitrate reductase which was active only with artificial electron donors such as reduced methyl viologen (MVH) and was located primarily in the cytoplasm (4–7). Because this "soluble" nitrate reductase was composed only of the α and β subunits and the mutanta which produced it lacked cytochrome b₉₃ (7, 8) it was concluded that the γ subunit was required for binding of nitrate reductase to the membrane as well as for the transfer of electrons from physiological electron donors involving membrane dehydrogenases such as formate or glyceraldehyde 3-phosphate.

We have recently shown by DNA sequencing that the narI region is composed of two open reading frames, designated narI and narJ, respectively (7). Both open reading frames were translated upon induction of the nar operon, but it was not possible to establish which gene encoded the γ subunit of nitrate reductase or what the role of the additional gene product was in the expression of nitrate reductase activity.

In the studies presented here we establish that the fourth open reading frame of the operon (narJ) encodes the γ subunit and that while the product of narJ is also required for the formation of fully active, membrane-bound nitrate reductase, it does not appear to be associated with the active nitrate reductase complex.

MATERIALS AND METHODS

Strains and Plasmids—The bacterial strains used in this study and plasmids pSL962, pES203, and pMV4 have been described previously (7). Plasmids pES203.1 and pMV5 were constructed from plasmids pES203 and pSL962, respectively, as diagrammed in Fig. 1A resulting in the nar operon structures and fusions shown in Fig. 1B. Although plasmid pES203 produced only an intact narJ gene product, strains transformed with it tended to lyse when grown anaerobically, presumably due to the production of the narI::lacZ fusion protein. Plasmid pES203.1 was constructed by deleting much of the lac operon components as diagrammed in Fig. 1A. After digestion with SstI and SmaI, blunt ending and ligation with T4 ligase, plasmid pES203.1 was isolated from ampicillin-resistant transformants of strain RK5274 and its structure confirmed by restriction mapping. This plasmid permitted normal anaerobic growth of transformed strains.

Plasmid pMV5 was constructed as diagrammed in Fig. 1A by deleting all of narH and most of narG and narI, leaving narJ intact and still under the control of the nar promoter. Plasmid pSL962 was digested with BamHI and BglII and the mixture ligated with T4 ligase. Plasmid pMV5 was isolated from ampicillin-resistant transformant of RK5274 produced from the mixture, and its structure was confirmed by restriction endonuclease mapping.

Growth, Extract Preparation, and Assays—Cells were grown in L broth (9) with or without 1% nitrate and supplemented with the appropriate antibiotic in the case of plasmid-bearing strains. Ampicillin was used at 50 µg/ml and tetracycline at 20 µg/ml. Aerobic

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1 The abbreviations used are: MVH, reduced methyliodogen; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography.
cultures were grown on a rotary shaker and anaerobic cultures in stationary, filled bottles at 37 °C.

When the suspensions were prepared by centrifuging cultures for 10 min at 10,000 × g and resuspending at 10 g wet weight/100 ml in 50 mM potassium phosphate, pH 7.0. Crude extracts were prepared by passing the whole cell suspension through the French press two times at 15,000 psi and centrifuging 10 min at 100,000 × g to yield a supernatant fraction and a membrane pellet fraction which was suspended in 50 mM potassium phosphate, pH 7.0.

MNV, and formate-nitrate reductase activities were assayed as previously described (10, 11). The low temperature-reduced minus-oxidized cytochrome spectra were determined on membrane fractions containing approximately 1 mg of protein/ml as previously described (12). The relative content of cytochrome b562 was calculated as the peak height of the α band, i.e., at the 418 nm divided by the mg of protein present in the 1-ml sample. This calculation includes all cytochrome b components which contribute to the α band absorption and tends to minimize the changes specifically in cytochrome b562.

Purification of Nitrate Reductase—MNV-nitrate reductase was released from the membrane pellet by deoxycholate treatment and concentrated by ammonium sulfate precipitation as previously described (13). The purified enzyme was absorbed to a phenyl-Sepharose column. Fractions containing pure α subunit and the column was eluted with 3 volumes of 1 mM Tris-HCl, pH 7.5, followed by a gradient in the same buffer of 0-0.8% Triton X-100. The active fractions which eluted in the gradient were pooled, concentrated by ultrafiltration with a PM-20 filter, and the buffer replaced with 20 mM Tris-acetate, pH 7.4, containing 0.1% Triton X-100. The concentrated fraction was fraction was finally purified by HPLC on a 0.925-cm column of 10 pm Vydac TP silica coated with polyethyleneimine (14) which was eluted with a 0-1 M sodium acetate gradient in 20 mM Tris-acetate, pH 7.4. The major activity peak was composed of underaged α, β, and γ subunits and had a specific activity of 30-50 units/mg of protein when assayed under standard assay conditions (11).

Isolation of Subunits—The α and β subunits were separated from γ subunits as follows. The purified enzyme was heated at 60 °C for 20 min and then, after cooling, centrifuged at top speed in an Eppendorf centrifuge. The pellet was washed two times with 50 mM Tris·HCl, pH 7.5, and 100 mM dithiothreitol, and 100 mM sodium phosphate, pH 6.1. The α subunit was recovered in the supernatant fraction was dissolved in 1% SDS·polyacrylamide gel electrophoresis, were combined and dialyzed against 4 M urea in 200 mM Tris-HCl, pH 7.5, and then 0.2% SDS prior to N-terminal sequencing. To isolate the β subunit, the lyophilized residue of the α-RK5274 was excised and electrophoresed on 10% SDS·polyacrylamide gel electrophoresis, were combined and dialyzed against 4 M urea in 200 mM Tris-HCl, pH 7.5, and then 0.2% SDS prior to N-terminal sequencing.

To isolate the γ subunit, the pellet fraction resulting from heating the purified enzyme at 60 °C was dissolved in a solution containing 1% SDS and 10 mM dithiothreitol and heated 2 min at 100 °C. The cooled fraction was chromatographed on a Bio-Gel A-15m column equilibrated and eluted with 0.1% SDS. The fractions containing pure β subunit, as determined by SDS·polyacrylamide gel electrophoresis, were pooled and used directly for N-terminal sequencing.

To isolate the γ subunit, the pellet fraction resulting from heating the purified enzyme at 60 °C was dissolved in a solution containing 1% SDS and 10 mM dithiothreitol and heated 2 min at 100 °C. The cooled fraction was chromatographed on a Bio-Gel A-15m column equilibrated and run with the same buffer. The fractions containing pure α subunits, as determined by SDS·polyacrylamide gel electrophoresis, were combined and dialyzed against 4 M urea in 200 mM Tris-HCl, pH 7.5, and then 0.2% SDS prior to N-terminal sequencing.

To isolate the α subunit, the supernatant fraction was dissolved in 1% SDS and 10 mM dithiothreitol and heated 2 min at 100 °C. The cooled fraction was chromatographed on a Bio-Gel A-15m column equilibrated and run with the same buffer. The fractions containing pure α subunit, as determined by SDS·polyacrylamide gel electrophoresis, were combined and dialyzed against 4 M urea in 200 mM Tris·HCl, pH 7.5, and then 0.2% SDS prior to N-terminal sequencing.

Analytical Procedures—Hydrolyses of proteins and amino acid analyses were carried out as described by Walker and DeMoss (15). Nitrogen contents were determined by the method of Kjeldahl. The results were expressed as the percentage of nitrogen in the samples. The analysis of amino acid sequences was performed as described by Hackett and Bragg (8) to have an altered cyto-
chrome spectrum, indicating that cytochrome \( b_{NR} \) was not produced under the conditions which induce nitrate reductase formation. Based on these observations it was concluded that RK5274 contained a Tn10 insertion located 3' to the \( narG \) and \( narH \) genes in the \( narl \) gene, which was assumed to be the structural gene for cytochrome \( b_{NR} \) (5). This assumption was supported by the demonstration that plasmid pMV4 (Fig. 1), containing a subcloned fragment of the 3' end of the \( nar \) operon under the control of the \( nar \) promoter, complemented mutant RK5274, restoring normal levels of cytochrome \( b_{NR} \) and promoting the assembly of nitrate reductase on the membrane in a fully functional form (7).

DNA sequencing of the 3' end of the operon, however, established that there are two open reading frames (7), designated \( narJ \) and \( narI \) respectively, in addition to the \( narG \) and \( narH \) genes which are located proximal to the promoter (6, 19, 21). Southern blots (22) revealed that the Tn10 insertion in RK5274 was located downstream from \( narH \), but it was not possible to discern whether the insertion was in the \( narJ \) or the \( narI \) open reading frame. We therefore provisionally designate mutant RK5274 and a \( narJI \) mutant.

In order to determine which of the two functions are required for complementation of RK5274, we subcloned the \( narJ \) and \( narI \) open reading frames separately by constructing plasmids pES203.1 and pMV5 (Fig. 1). In both cases, as well as in plasmid pMV4, the subcloned genes remained under the control of the \( nar \) promoter region so that growth anaerobically in the presence of nitrate would induce cells carrying the plasmids to express the subcloned genes.

As previously shown (7), mutant RK5274 produced reduced levels of MVH-nitrate reductase and 80% of the activity was located in the supernatant fraction (Table I), in contrast to the parental wild type strain, RK4353, where the activity was associated mainly with the membrane fraction. The mutant strain was completely devoid of formate-nitrate reductase activity, reflecting the inability of the cytoplasmic nitrate reductase to accept electrons from membrane-associated dehydrogenases such as formate dehydrogenase.

Table I also shows the effects of transforming mutant RK5274 with plasmids which express the \( narJ \) and \( narI \) genes. Transformation with plasmid pMV4, which expresses both the \( narJ \) and \( narI \) genes, restored both formate and MVH-nitrate reductase activities to approximately 65% the wild type level and more than 80% of the MVH-nitrate reductase was associated with the membrane fraction. Transformation with plasmid pES203.1, which expresses \( narJ \) only, did not significantly change the levels of the nitrate reductase activities or the distribution of MVH-nitrate reductase between the supernatant and membrane fractions, indicating that the \( narJ \) product alone is not sufficient to restore the wild type phenotype. Transformation with plasmid pMV5, which expresses \( narI \) only, resulted in low but significant increases in

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**A. Plasmid Constructions**

![Plasmid Constructions Diagram](image)

**B. nar Operon Structures**

| Plasmid | Structure Diagram |
|---------|------------------|
| pSL962  | ![pSL962 Diagram](image) |
| pMV4   | ![pMV4 Diagram](image) |
| pES203.1 | ![pES203.1 Diagram](image) |
| pMV5   | ![pMV5 Diagram](image) |
**narJ and narI Gene Products**

**TABLE I**

| Strain          | Fraction | Formate-NRase | MVH NRase | % Total | $\epsilon_{max}\ (\alpha)$ | Cytochrome $b_{NR}$ relative content* |
|-----------------|----------|---------------|-----------|---------|----------------------------|---------------------------------------|
| RK4353          | Whole cells | 0.22          | 1.61      | 28      | 556                        | 0.49                                  |
|                 | Supernatant | 0.04          | 0.35      | 72      |                            |                                       |
|                 | Pellet     | 0.23          | 1.90      |         |                            |                                       |
| RK5274          | Whole cells | 0.00          | 0.24      | 81      | 558                        | 0.25                                  |
|                 | Supernatant | 0.00          | 0.59      |         |                            |                                       |
|                 | Pellet     | 0.01          | 0.45      | 19      |                            |                                       |
| RK5274 (pMV4)   | Whole cells | 0.14          | 1.06      | 18      | 556                        | 0.47                                  |
|                 | Supernatant | 0.02          | 0.21      |         |                            |                                       |
|                 | Pellet     | 0.16          | 1.66      | 82      |                            |                                       |
| RK5274 (pES203.1) | Whole cells | 0.01         | 0.27      | 83      | 558                        | 0.27                                  |
|                 | Supernatant | 0.00         | 0.26      |         |                            |                                       |
|                 | Pellet     | 0.01         | 0.16      | 17      |                            |                                       |
| RK5274 (pMV5)   | Whole cells | 0.05          | 0.32      | 51      | 557                        | 0.51                                  |
|                 | Supernatant | 0.00         | 0.31      |         |                            |                                       |
|                 | Pellet     | 0.07         | 0.93      | 49      |                            |                                       |

* Relative contents of cytochrome $b_{NR}$ were calculated from the spectra in Fig. 2 and equal to the peak height of the $\alpha$ band of cytochrome $b$ divided by the $\mu$g of proteins/ml in the sample.

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The reduced versus oxidized spectra of the membrane fractions from the above strains are shown in Fig. 2 and the $\epsilon_{max}$ and relative content values for cytochrome $b$ are summarized in Table I. As previously shown (11, 12), under conditions of full nitrate reductase induction the $\epsilon_{max}$ for the $\alpha$ band of cytochrome $b$ in the wild type strain was 556 nm. In mutant RK5274, the relative content was reduced about 50% and the $\epsilon_{max}$ for the $\alpha$ band was shifted to approximately 558 nm. Transformation of the mutant with either plasmid pMV4 (narJ-narZ) or pMV5 (narJ) restored the $\epsilon_{max}$ for the $\alpha$ band and the relative content of cytochrome $b$ to the wild type values. Transformation with plasmid pES203.1 (narJ) appeared to affect neither the $\epsilon_{max}$ nor the relative content of cytochrome $b$ found in RK5274. These results suggest that the expression of both narJ and narI are required for the assembly of fully active nitrate reductase but that a functional
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The narl gene is sufficient for the formation of the cytochrome b component of nitrate reductase.

**Identification of the γ Subunit Structural Gene**—To determine directly which open reading frame encodes the γ subunit, we resolved this subunit from purified nitrate reductase (Fig. 3, lane 1) by the procedure described under “Materials and Methods.” As described by Chaudhry and MacGregor (3), this subunit is extremely hydrophobic and tends to aggregate irreversibly when heated. We therefore resolved the γ subunit on a SDS-polyacrylamide gel after treatment at 37°C with buffer containing 0.2% SDS (Fig. 3, lane 2) and isolated the subunit by elution from the gel (Fig. 3, lane 3).

In Table II the amino acid composition of the purified γ subunit is compared to the amino acid compositions deduced for the theoretical products of the narJ and narl open reading frames. The composition of the γ subunit appears to correspond closely to that deduced for the narl product and to be significantly different from that deduced for the narJ product (see “Discussion”).

Initial attempts to determine the N-terminal sequence for the isolated γ subunit established that the subunit contained a blocked N terminus. Therefore, in order to derive sequence information the isolated subunit was subjected to cyanogen bromide cleavage as described under “Materials and Methods.” The products of cyanogen bromide cleavage were extremely hydrophobic, as was the isolated subunit, and it was not possible to resolve individual peptides by either gel electrophoresis or HPLC. For that reason, the entire cyanogen bromide-cleaved mixture was subjected to sequential Edman degradation to derive sequence information.

In Table III the amino acid residues identified after each round of Edman degradation of the mixture resulting from cyanogen bromide treatment of the γ subunit are compared to the residues expected for the cyanogen bromide fragments predicted from the narJ and narl sequences. There is excellent agreement between the theoretical and experimental data for the narl gene product and the γ subunit. When fragments shorter than 5 residues were ignored, essentially all the residues expected for cyanogen bromide fragments derived from the NarI protein were present in each of the first 15 cycles of Edman degradation of the γ subunit. The exceptions were residues which are poorly recovered (such as W, C, and T) and residues which are close to the carboxyl end of the fragments. In contrast, most of the residues expected for cyanogen bromide fragments from the NarJ protein were not found in the mixtures from each cycle of Edman degradation of the γ subunit. Significantly, essentially every residue found in each cycle could be rationalized by the theoretical composition of the NarI protein while only a limited number of residues (12 of a total of 66) found corresponded to those expected from the theoretical composition of the NarJ protein.

Finally, it was possible to establish an N-terminal sequence for the γ subunit after treatment of the isolated preparation with 30% trifluoroacetic acid, a procedure which has been shown to remove the formyl group from N-terminal blocked peptides (15). Although this hydrolytic procedure results in a high background at each Edman degradation cycle of the treated protein, the major amino acid residues released at each cycle could be clearly discerned. The sequence observed corresponded to that expected for the NarI protein as deduced from the nucleotide sequence of the narl open reading frame.

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**Table II**

| Amino acid | NarJ (theoretical) | Narl (theoretical) | γ Subunit (experimental) |
|------------|-------------------|-------------------|-------------------------|
| D          | 9                 | 6                 | 12                      |
| N          | 4                 | 3                 | 8                       |
| T          | 8                 | 9                 | 8                       |
| S          | 11                | 13                | 9*                      |
| E          | 19                | 4                 | 17                      |
| Q          | 18                | 9                 |                         |
| P          | 9                 | 7                 | 8                       |
| G          | 11                | 21                | 23                      |
| A          | 31                | 17                | 19                      |
| C          | 1                 | 2                 |                         |
| V          | 10                | 15                | 18                      |
| M          | 4                 | 12                | 16*                     |
| I          | 10                | 15                | 14                      |
| L          | 35                | 33                | 32                      |
| Y          | 7                 | 8                 | 7                       |
| F          | 8                 | 18                | 18                      |
| K          | 8                 | 6                 | 6                       |
| H          | 8                 | 9                 | 8                       |
| R          | 13                | 12                | 12                      |
| W          | 2                 | 6                 |                         |
| **Total**  | 236              | 225              | 225*                    |

* Based on the nucleotide sequences of narJ and narl open reading frames (7).

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**Table III**

| Edman cycle | NarJ (theoretical) | Narl (theoretical) | γ Subunit (experimental) |
|-------------|-------------------|-------------------|-------------------------|
| 1           | D, F, I, C        | D, N, T, A, Y, K, F, L | N, T, Y, K, F, L        |
| 2           | E, T, P           | D, E, T, G, A, F, L | D, E, T, G, A, F, L    |
| 3           | A, L              | S, G, A, V, F, L   | G, A, V, F, L          |
| 4           | D, N, V, I        | D, E, N, S, G, H, W, K, L | D, S, G, K, L      |
| 5           | A, P, I           | N, G, A, W, I, L   | N, G, A, I, L          |
| 6           | Q, A, V           | S, A, Y, P, F, L   | S, A, Y, P, F, L       |
| 7           | Q, T, K, R        | Q, G, P, F, I      | Q, G, P, F, I          |
| 8           | N, A, Y, L        | E, S, H, Y, V, F   | E, S, Y, V, F          |
| 9           | S, V, I, L        | S, V, I, L         |                         |
| 10          | S, A, L           | G, A, V, K, R, C   | A, V, K                |
| 11          | E, P, R           | Q, T, G, L         | Q, G, L                |
| 12          | Y, K, L, R        | A, K, F, I, L      | A, F, I                |
| 13          | E, P, W, F        | G, V               |                         |
| 14          | D, W              | G, F, I            | G, F, I                |
| 15          | T, A, L, R        | G, V, W, L         | G, V, L                |

* Theoretical mixtures are derived from the nucleotide sequence of the narJ and narl genes (7).
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### Table IV

**N-terminal sequences of nitrate reductase components**

| Component                        | Cycle of Edman degradation |
|----------------------------------|---------------------------|
| M, S                             | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| α Subunit                        | K | F | J | L | R | D | S | Q | F | Y |
| β Subunit                        | D | S | R | Q | F | Y | R | G | K | M |
| γ Subunit (acid-treated)         | M | Q | F | L | N | — | F | F | F | D |
| NarJ protein (theoretical)       | M | I | E | L | N | M | F | F | F | D |
| NarI protein (theoretical)       | M | Q | F | L | N | M | F | F | F | D |

*a* No significant residue detected.

Fig. 4. Immunoprecipitation of nitrate reductase subunits from Triton X-100-treated membrane fractions. The wild type strain, RK4055 was grown and labeled with [35S]methionine, membrane fractions prepared and solubilized with Triton X-100 and nitrate reductase precipitated with purified α subunit-specific antiserum as described under "Materials and Methods." The antigen-antibody complexes were absorbed to protein A-Sepharose. The washed Sepharose pellets were suspended in 2% SDS and heated at 100°C for 3 (lane 2), 3 (lane 3), and 10 (lane 4) min. Lane 1 was similar to lane 3 except antiserum was omitted.

(Table IV) and was distinctly different from that expected for the NarJ protein.

Attempts to Identify the NarJ Protein—We conclude from the above data that the narI gene encodes the γ subunit of nitrate reductase. However, it was still unresolved where the NarJ protein is localized and what its role is in the expression of nitrate reductase activity.

To examine the possibility that the NarJ protein is part of the nitrate reductase complex but that it had not been recognized previously because of its similarity in size (26.5 kDa) to that of the NarI protein, the γ subunit (25.5 kDa) (7), we determined N-terminal sequences for isolated α and β subunits and for the purified nitrate reductase complex (Table IV). Both the isolated α and β subunits readily yielded N-terminal sequences with the α sequence corresponding to the first open reading frame of the nar operon (narG) minus the N-terminal Met (20, 21). The isolated γ subunit contained a blocked N terminus and yielded no amino acid derivatives in multiple rounds of Edman degradation. The purified nitrate reductase complex (Fig. 3, lane 1) yielded a mixture of residues at each cycle which corresponded to an equal mixture of α and β subunits and no other N-terminal sequences corresponding to either the narJ or narI sequence were detectable (Table IV). Therefore, any peptides present in purified nitrate reductase other than the α and β subunits must contain blocked N termini. We previously demonstrated that a narJ-lacZ fusion produced a hybrid protein with an N-terminal sequence expected for the NarJ protein, indicating that this open reading frame produced a protein with an unblocked N terminus when the nar operon was induced (7). Since the narJ-lacZ fusion protein had an unblocked N terminus we assume that the NarJ protein would be similarly unblocked and therefore is not present in purified nitrate reductase.

It was also possible that the NarJ protein is part of the assembled nitrate reductase complex but is lost during the lengthy purification procedure. To examine this possibility, antibodies specific for the α subunit were purified by the procedure described under "Materials and Methods" and utilized to precipitate the nitrate reductase complex directly from Triton X-100-treated membrane fractions from 35S-labeled cells (Fig. 4). The antigen-antibody complexes were absorbed to protein A-Sepharose, and the labeled peptides were analyzed on a SDS-polyacrylamide gel. The labeled proteins were released by heating the protein A-Sepharose pellet in 2% SDS for 3, 10, and 10 min at 100°C. In each case bands corresponding to the α, β, and γ subunits in purified nitrate reductase were released. In the case of the unheated sample (lane 2, Fig. 4), an additional band was present on the gels midway between the α and β bands, which we have shown to be unaltered, active αβ complex (data not shown). As reported by Chaudhry and MacGregor (3), the apparent levels of γ subunit were diminished by boiling in SDS, presumably due to its aggregation by heat and removal during centrifugation with the protein A-Sepharose pellet. In each case there is no indication of an additional band in the γ subunit region; the radioactive bands in this region precipitated from the crude fraction corresponded precisely with the stained γ subunit band in the purified complex.

**DISCUSSION**

It was previously established that the α and β subunits of nitrate reductase are encoded by the first two genes in the nar operon, the narG and narH genes, respectively (4-6, 20, 21), and it was proposed from the phenotype of nar mutants lacking cytochrome b_{nor} that a third gene, narL located 3' to the narG and narH genes, encoded the cytochrome b_{nor} or γ subunit of nitrate reductase (4, 5). Nucleotide sequencing,
however, established that in addition to the \textit{narG} and \textit{narH} genes there are two open reading frames at the 3' end of the operon which were designated \textit{narJ} and \textit{narI}, respectively (7). We conclude from the data presented here that the last open reading frame in the operon, \textit{narI}, is the structural gene for the \(\gamma\) subunit. This conclusion is based on the correspondence of the structure and properties of the isolated \(\gamma\) subunit with those expected for a protein with the sequence and composition encoded by the \textit{narI} reading frame. Based on theoretical amino acid compositions the \(\text{NarJ}\) protein would be a distinctly hydrophilic protein with an acidic isoelectric point of greater than 5.5 while the \(\text{NarI}\) protein would be distinctly hydrophobic with a basic isoelectric point of less than 5.5. However, the complementation results with the cloned genes argues that the position of the transposon insertion is in the \textit{narJ} gene and the \(\gamma\) subunit is associated with the nitrate reductase complex. As shown here it is present in either the purified enzyme or in immune precipitates of the crude enzyme which had been solubilized by detergent treatment of membranes. Furthermore, it is not associated with immune precipitates of the soluble complex from extracts of \textit{RK}5247 containing the plasmid expressing only the \textit{narJ} gene.\footnote{J. A. DeMoss and P.-Y. Hsu, unpublished data.} We cannot rule out the possibility, however, that it is loosely associated with nitrate reductase but lost during purification of during washing of the immune precipitates. It is also possible that the \text{NarJ} protein plays some other role in regulation or the uptake of nitrate, but further study will require that the protein be identified and its localization in the cell established.

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