JOURNAL OF BACTERIOLOGY, Feb. 1992, p. 867–872
0021-9193/92/030867-06$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 174, No. 3

The *narJ* Gene Product Is Required for Biogenesis of Respiratory Nitrate Reductase in *Escherichia coli*

MICHEL DUBOURDIEU† AND JOHN A. DEMOSS*

Department of Biochemistry and Molecular Biology, The University of Texas Medical School at Houston, Houston, Texas 77030

Received 4 September 1991/Accepted 25 November 1991

Respiratory nitrate reductase purified from the cell membrane of *Escherichia coli* is composed of three subunits, α, β, and γ, which are encoded, respectively, by the *narG*, *narH*, and *narI* genes of the *narGHJI* operon. The product of the *narJ* gene was deduced previously to be a highly charged, acidic protein which was not found to be associated with any of the purified preparations of the enzyme and which, in studies with putative *narJ* mutants, did not appear to be absolutely required for formation of the membrane-bound enzyme. To test this latter hypothesis, the *narJ* gene was disrupted in a plasmid which contained the complete *narGHJI* operon, and the operon was expressed in a *narG*::Tn*10* insertion mutant. The chromosomal copy of the *narJ* gene of a wild-type strain was also replaced by the disrupted *narJ* gene. In both cases, when *nar* operon expression was induced, the α and β subunits accumulated in a form which expressed only very low activity with either reduced methyl viologen (MVH) or formate as electron donors, although an α-β complex separated from the γ subunit is known to catalyze full MVH-linked activity but not the formate-linked activity associated with the membrane-bound complex. The low-activity forms of the α and β subunits also accumulated in the absence of the NarJ protein when the γ subunit (NarI) was provided from a multicopy plasmid, indicating that NarJ is essential for the formation of the active, membrane-bound complex. When both NarJ and NarI were provided from a plasmid in the *narJ* mutant, fully active, membrane-bound activity was formed. When NarJ only was provided from a plasmid in the *narJ* mutant, a cytosolic form of the α and β subunits, which expressed significantly increased levels of the MVH-dependent activity, accumulated, and the α subunit appeared to be protected from proteolytic clipping which occurred in the absence of NarJ. We conclude that NarJ is indispensable for the biogenesis of membrane-bound nitrate reductase and is involved either in the maturation of a soluble, active α-β complex or in facilitating the interaction of the complex with the membrane-bound γ subunit.

Membrane-bound respiratory nitrate reductase is encoded by the *narGHJI* operon in *Escherichia coli* (2, 5, 22, 23). The purified enzyme is composed of three subunits, α, β, and γ, which are encoded, respectively, by the *narG*, *H*, and *I* genes (2, 6, 15, 22). The α and β subunits form a complex which contains molybdenum-pterin cofactor and nonheme iron and can catalyze the reduction of nitrate with reduced methyl viologen (MVH) as an artificial electron donor (1, 12, 14). The α-β complex is normally tightly bound to the membrane-associated γ subunit (cytochrome b_{NR}), which transfers electrons for nitrate reduction from the quinone pool of the cell membrane (6, 7, 13, 17). An active form of the α-β complex can be released from the γ subunit and the membrane by heat treatment (1, 12, 15), and it has been reported to accumulate in an active, cytoplasmic form in some *narI* mutants (13).

The sequence of the *narJ* gene indicates that it encodes a 26.5-kDa hydrophilic protein, and studies with *narJ*::*lacZ* fusions have established that a NarJ protein is expressed when the operon is induced (22). However, a protein corresponding to NarJ has not been found in any of the purified preparations of the enzyme, and its role in the formation or activity of nitrate reductase is unknown.

The results of complementation of a putative *narJ*::Tn*10* insertion mutant with plasmids expressing the *narJ* and *narI* genes suggested that NarJ facilitated, but was not absolutely required for, assembly of fully active, membrane-bound nitrate reductase (21). However, the interpretation of these results was complicated by the imbalance of the *nar* operon gene products resulting from expression of some of the genes from a multicopy plasmid and by the lack of a well-defined *narJ* mutant.

To circumvent these difficulties, we have determined the effects of NarJ deficiency on nitrate reductase formation by utilizing plasmid constructions which permit balanced subunit production from plasmids at both high and low copy numbers as well as mutants with well-defined chromosomal *narJ* gene disruptions. The results indicate that NarJ is required for the formation or stabilization of the active α-β complex which catalyzes the reduction of nitrate with an artificial electron donor.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Strain RK5274P was constructed by transferring the *pcnB* mutation (10) into strain RK5274 by P1 transduction by using the selection procedure described by Walker and DeMoss (27). To construct *narJ* mutants MD100 and MD101, the *narJ* gene disrupted with a kanamycin resistance cartridge in plasmid pMD31 was transferred into the chromosomal *narGHJI* operon of strain RK4353 by a two-step process. First, the disrupted gene was moved into *recBC* mutant JC7623 by homologous recombination. Kanamycin-resistant deriva-

* Corresponding author.
† Permanent address: Department of Biology, University de los Andes, Mérida 5101, Venezuela.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
RK4353	F[-] *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 ptsF25 deoC1 rbsR gyrA non*	23
RK5265	RK4353 *narG*::Tn*10*	23
RK5274	RK4353 *narI*::Tn*10*	23
MD100	RK4353 *narJ*::Kan[r] cartridge	This work
MD101	RK4353 *narJ*::Kan[r] cartridge	This work
JC7623	*recB21 recC22 sbc15*	G. Weinstock[a]
Plasmids		
pSL962	*narGHJI*	21
pMV4	*narJI*	21
pMV5	*narI*	21
pES203.1	*narJ*	21
pMD31	*narGH narJ*::Kan[r] cartridge	This work
pMD32	*narGHI narJ*::12-bp insert	This work
pMD33	*narGHI* Δ*narJ*	This work
pUC71K	Kan[r] cartridge	24

[a] University of Texas Medical School, Houston, Tex.

tives of JC7623, resulting from transformation with pMD31 linearized at a unique *Afl*II site located at the 5′ end of the *narG* gene, were isolated and shown to lack the transforming plasmid by their sensitivity to ampicillin. The disrupted gene was then moved into the RK4353 background by P1 transduction of the kanamycin resistance marker into strains RK4353 and RK5274, resulting in strains MD100 and MD101, respectively. Strain MD101 was sensitive to tetracycline, demonstrating that incorporation of the region containing the kanamycin resistance marker had replaced the Tn*10* insert in the *narI* region. The location of the fragment bearing the kanamycin cartridge in the *nar* operon of strain MD100 was verified by a Southern blot of *Eco*RI-*Sal*I restriction digests with plasmid pMV4 as a radioactive probe. Only strain MD100 was studied in detail since the two strains appeared to be identical in preliminary studies of their growth characteristics and biochemical properties.

Plasmid pSL962, which carries the complete *narGHJI* operon, has been described previously (9). Plasmid pMD31 was constructed by inserting a fragment containing a kanamycin resistance gene into a *Bam*HI site in the *narJ* gene of pSL962. The 1.4-kb fragment containing the kanamycin resistance gene obtained from digestion of plasmid pUC71K (24) with *Bam*HI was ligated with the 11.3-kb fragment purified from a partial *Bam*HI digest of pSL962, which contains two *Bam*HI sites. The location of the kanamycin cartridge in the *narJ* gene of plasmid pMD31 was verified by restriction analysis.

Plasmid pMD32 was derived from pMD31 by removal of the kanamycin cartridge by using an alternative restriction site in the flanking polylinker region. After digestion of pMD31 with *Sal*I, the mixture was religated and used to transform RK4353 to ampicillin resistance. Plasmid pMD32 was isolated from a kanamycin-sensitive isolate, and the loss of the fragment containing the kanamycin cartridge was verified by restriction analysis. Based on the structure of the polylinker region flanking the kanamycin cartridge (24), this construction is equivalent to plasmid pSL962 with a 12-bp insert at the *Bam*HI site in the *narJ* gene.

Plasmid pMD33 was constructed by deleting a 98-bp fragment between the *Bam*HI and *Sac*II sites within the *narJ* gene of plasmid pSL962. pSL962 was digested overnight with *Sac*II and then partially digested with *Bam*HI. The digestion mixture was treated with Klenow fragment to fill in the overhangs generated by *Bam*HI and then incubated with T4 polymerase to remove the overhangs generated by *Sac*II. The resulting 11.2-kb fragment was purified on an agarose gel and ligated in the presence of an 8-bp *Sal*I linker. A plasmid isolated from an ampicillin-resistant transformant was exhaustively digested with *Sal*I to remove excess linkers, and the religated plasmid was isolated from an ampicillin-resistant transformant. The general structure of the resulting plasmid, pMD33, was verified by restriction analysis. This construction replaced the deleted 98-bp fragment with the 8-bp *Sal*I linker, resulting in the restoration of the *narJ* reading frame after the *Sac*II site.

The structure of the altered regions in plasmids pMD32 and pMD33 were confirmed by dideoxy-sequencing (16, 24, 25) of fragments containing the *narJ* region cloned into the phagemid vector pTZ18R (Pharmacia LKB Biotechnology).

Growth conditions and cells. For determination of activities, cultures were grown overnight in L broth (8) supplemented with the appropriate antibiotics on a rollerdrum apparatus and were used as a 1% inoculum for larger volumes of L broth supplemented with the appropriate antibiotics. The inoculated culture was grown on a shaker to a turbidity of 60 Klett units (with a 540 filter) and then shifted to anaerobic conditions with the addition of 1% glucose and 1% potassium nitrate. The cultures were grown to approximately 100 Klett units (log phase), harvested by centrifugation, and resuspended in 50 mM potassium phosphate buffer, pH 7.0.

In some cases, crude extracts were prepared by passing cell suspensions (approximately 20% [wt/vol]) through the French press and removing cell debris by centrifugation for 10 min at 10,000 × *g*. To determine the localization of activities, the crude extracts were centrifuged for 1 h at 100,000 × *g*. The resulting supernatants were assumed to represent the cytosolic fractions, while the pellets, suspended in an equal volume of buffer, were assumed to represent the membrane fractions.

Enzyme activities. Nitrate reductase activity was determined by using reduced MVH or formate as electron donors as described previously (19, 20), except that samples of the reaction mixtures were analyzed at 5-min intervals to establish linear reaction rates. Activities are expressed as units (micromoles of nitrite formed per minute) per milligram of protein.

Cytochrome b_{NR} was determined in either the whole-cell suspensions or the membrane fractions in a double-beam spectrophotometer, as previously described (19). The relative content of cytochrome b_{NR} was determined as the peak (558 nm at room temperature) height in absorbancy units per milligram of protein. For a comparative set of determinations, the spectra were run on samples with identical protein concentrations. Cells grown anaerobically in the absence of nitrate or the mutants blocked in the formation of cytochrome b_{NR} produced negligible amounts of cytochromes and did not interfere with this determination in whole cells or membrane fractions.

Protein was determined by the Lowry reaction (11).

Immunoblot procedure. The immunoblot was run according to a procedure previously described (26) with antibodies purified from an antiserum prepared in rabbits against the purified α-β complex (12). The antibodies were purified by the procedure of Olmsted (18), with α and β subunits purified by gel electrophoresis.

TABLE 2. Effect of plasmid copy number on complementation of mutant RK5274

Strain (plasmid)	Subunits expressed by plasmids	Nitrate reductase (U/mg of protein)	
		MVH-linked	Formate-linked
RK4353[a]		1.82	0.099
RK5274[b]		0.57	<0.001
RK5274*pcnB*		0.62	<0.001
RK5274(pMV4)	NarJ, NarI	1.85	0.050
RK5274*pcnB*(pMV4)		1.40	0.072
RK5274(pMV5)	NarI	1.26	0.066
RK5274*pcnB*(pMV5)		1.54	0.080
RK5274(pES203.1)	NarJ	0.93	<0.001
RK5274*pcnB*(pES203.1)		1.11	<0.001

[a] RK4353, wild-type *narGHJI*.
[b] RK5274, *nar*::Tn*10*.

RESULTS

Complementation of *nar* mutant RK5274. Sodergren et al. (21) previously demonstrated that complementation of a putative *narJ*::Tn*10* mutant, *E. coli* RK5274, with a multicopy plasmid expressing only the *narI* gene partially restored the activity of membrane-bound nitrate reductase, while complementation with a plasmid expressing both the *narJ* and *narI* genes yielded full wild-type levels of activity. Since a mutant with a Tn*10* insertion in the *narJ* gene should produce α and β subunits but neither the *narJ* nor *narI* gene products, these results suggested that the NarJ protein was not absolutely required for the expression of the physiologically active, membrane-bound α-β-γ complex. To explore whether the NarJ protein might be required for expression when the γ subunit was present at levels normally produced from the chromosome, we repeated these complementation studies with a *pcnB* (10) derivative of strain RK5274 to limit the plasmid copy number to two or three per cell (Table 2). Nitrate reductase was determined in cell suspensions with MVH as electron donor to assess the total amount of active enzyme produced and with formate as electron donor to assess the amount of enzyme capable of accepting electrons from the membrane-bound quinone pool. In each case, complementation at high and low plasmid copy numbers yielded similar results, indicating that the imbalance of *nar* gene products produced with multicopy plasmids had a minimal effect, if any, on the biogenesis of nitrate reductase. More informative, however, was the demonstration that complementation with NarI alone (pMV5) restored both MVH- and formate-linked nitrate reductase produced by RK5274 to the levels produced with NarI plus NarJ (pMV4). Complementation with NarJ alone (pES203.1) led to a slight increase in the level of MVH-nitrate reductase produced by RK5274, but no formate-linked activity was detectable in this case. Although not entirely conclusive, the simplest interpretation of these results was that the Tn*10* insertion in RK5274 is in the *narI* gene rather than the *narJ* gene, since the provision of NarI alone restored essentially full levels of both activities. Therefore, whether NarJ is required for the expression of nitrate reductase activity remained an open question.

Effect of plasmid *narJ* disruption. To examine more critically the possible requirement for NarJ in nitrate reductase biogenesis, we constructed specific lesions in the *narJ* gene on a plasmid which carried the entire *narGHJI* operon and expressed the resulting plasmids in a *narG* mutant (RK5265) that produced none of the operon products from the chromosomal locus (Table 3). RK5265 produced undetectable levels of MVH- and formate-linked nitrate reductase activities and cytochrome b_{NR}. RK5265 transformed with plasmid pSL962, which contains the entire *narGHJI* operon, overproduced MVH-linked activity and cytochrome b_{NR} two- to threefold relative to the wild-type strain RK4353, and formate-nitrate reductase activity was restored to the wild-type level. The *narJ* gene of plasmid pSL962 (9) was disrupted in three different ways. In plasmid pMD31, a kanamycin resistance gene cartridge (24) was inserted into a unique *Bam*HI site located within the *narJ* gene approximately 100 bp from the 5′ end of the open reading frame. When expressed in RK5265, this construction produced no detectable cytochrome b_{NR}, but surprisingly, it produced a low but significant level of both MVH- and formate-linked activities (Table 3).

Plasmid pMD32 was constructed by removing the kanamycin cassette from pMD31 by digestion with *Sal*I followed by religation, leaving a 12-bp insert at the *Bam*HI site which restored the original *narJ* reading frame. This construction behaved exactly as did plasmid pSL962 by overproducing cytochrome b_{NR} and MVH-nitrate reductase and restoring formate-nitrate reductase to the wild-type level (Table 3), indicating that insertion of four amino acids at the position in the NarJ protein defined by the *Bam*HI site did not adversely affect the formation of nitrate reductase.

Plasmid pMD33 was constructed by deleting a 96-bp fragment between the blunted *Bam*HI site and a blunted *Sac*II site within the *narJ* gene (21, 22), which resulted in the deletion of a 32-amino-acid fragment from the NarJ protein

TABLE 3. Effects of *narJ* gene disruption on *nar* operon expression from multicopy plasmids

Strain (plasmid)	Structure of *narGHJI* in plasmid	Nitrate reductase (U/mg of protein)		Cytochrome b_{NR} relative content[a]
		MVH-linked	Formate-linked	
RK4353[b]		2.34	0.25	0.003
RK5265[c]		<0.01	<0.01	<0.001
RK5265(pSL962)	Wild type	4.60	0.25	0.010
RK5265(pMD31)	*narJ*::Kan^r cartridge	0.30	0.09	<0.001
RK5265(pMD32)	*narJ* insertion (12 bp)	4.88	0.28	0.011
RK5265(pMD33)	Δ*narJ* (90 bp)	0.14	0.09	0.010

[a] See Materials and Methods.
[b] RK4353, wild-type *narGHJI*.
[c] RK5265, *narG*::Tn*10*.

TABLE 4. Effects of *narJ* gene disruption on chromosomal *nar* operon expression

Strain (plasmid)	MVH-nitrate reductase		Cytochrome b_{NR}[a] relative content[b]
	U/mg of protein[c]	% Membrane bound[d]	
RK4353	1.28	85	0.011
MD100	0.06	84	<0.002
MD100(pMV4)	1.08	88	0.011
MD100(pMV5)	0.09	86	0.012
MD100(pES203.1)	0.40	47	<0.002

[a] Determined in the pellet fractions after centrifugation of the crude extract.
[b] See Materials and Methods.
[c] Determined in crude extracts.
[d] Crude extracts were centrifuged at 100,000 × g for 60 min, and the pellet was suspended to a volume equal to that of the original sample. The percentage of membrane-bound enzyme was based on the total units of activity in the supernatant plus pellet fractions.

but the restoration of the *narJ* reading frame thereafter. This construction, like pMD32, overproduced cytochrome b_{NR}, but it produced only a very low level of MVH- and formate-nitrate reductase activities in RK5265 (Table 3). These results demonstrated that when the α and β subunits were expressed in the absence of NarJ (with either pMD31 or pMD33), very low but significant levels of MVH- and formate-nitrate reductase activities were formed. Furthermore, whether cytochrome b_{NR} was provided (with pMD33 but not with pMD31) had little or no effect on the level of either activity produced in the absence of NarJ. We conclude that in the absence of the NarJ protein, the balanced overproduction of subunits α, β, and γ from a multicopy plasmid does not result in the formation of either fully active α-β or α-β-γ complex.

Effect of chromosomal *narJ* disruption. To test the effects of disruption of the *narJ* gene in the chromosomal *narGHJI* operon, the disrupted *narJ* gene containing the kanamycin resistance cartridge in plasmid pMD31 was transferred into the RK4353 background by the procedure described in Materials and Methods. The resulting strain, MD100, expressed less than 5% of the wild-type level of MVH-nitrate reductase activity (Table 4) and, as expected for a *narJ* gene disruption, undetectable amounts of NarI (cytochrome b_{NR}). Transformation of MD100 with plasmid pMV4 (NarJ$^+$ NarI$^+$) resulted in essentially full restoration of the wild-type level of MVH-nitrate reductase activity, indicating that normal levels of the α and β subunits were expressed by MD100. Transformation of MD100 with pMV5 (NarI$^+$ only) resulted in no significant increase in the level of MVH-nitrate reductase activity, although wild-type levels of cytochrome b_{NR} were produced. In contrast, transformation with pES203.1 (NarJ$^+$ only) resulted in the formation of more than 30% of the wild-type level of MVH-nitrate reductase activity. The relative levels of MVH-nitrate reductase in these strains were the same when determined in whole-cell suspensions and in crude extracts prepared in the French press, eliminating any possible differences in enzyme accessibility due to differential localization. However, when the crude extracts were fractionated into membrane and cytosolic fractions by centrifugation, the activity produced by strain MD100(pES203.1) was distributed more or less equally between the cytosolic and membrane fractions, while that produced by the other strains, including the low level in MD100, was localized mainly in the membrane fraction as in the wild type (Table 4).

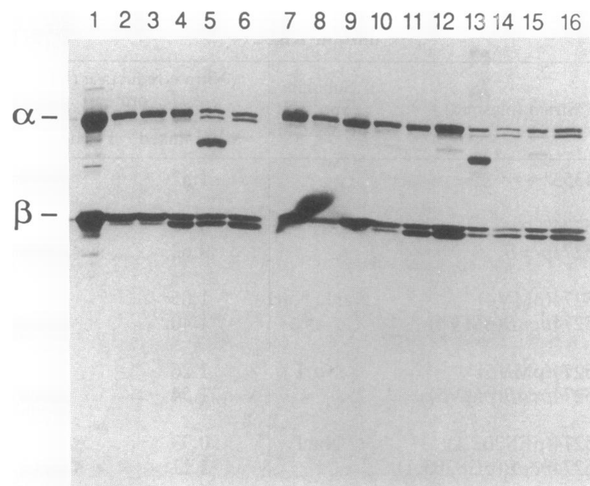

FIG. 1. Immunoblot determination of the distribution and properties of α and β subunits accumulated in *narJ* mutant derivatives. Lanes: 1, purified nitrate reductase; 2 to 6, 20 μg (each lane) of protein of whole-cell suspensions; 2, RK4353; 3, MD100(pMV4); 4, MD100(pES203.1); 5, MD100(pMV5); 6, MD100; 7 to 16, equal volumes of either the membrane or cytosolic fractions, prepared as described in Materials and Methods; 7, RK4353 membrane; 9, MD100(pMV4) membrane; 11, MD100(pES203.1) membrane; 13, MD100(pMV5) membrane; 15, MD100 membrane; 8, 10, 12, 14, and 16, cytosolic fractions.

Subunit accumulation in the *narJ* mutant. The accumulation of the α and β subunits in the *narJ* mutant and its transformed derivatives was determined directly with immunoblots with antibodies against the purified α-β complex (Fig. 1). Although the antibody preparation stained the β subunits relatively more intensely than the α subunits, the immunoblot of whole-cell extracts (lanes 2 to 6) showed that both subunits were partially converted to presumedly degraded, faster-migrating forms in mutant MD100 (lane 6) compared with those in the parental strain RK4353 (lane 2), in which they appeared not to be degraded. In strain MD100 transformed with pMV4, which expressed essentially full wild-type activity, the α and β subunits appeared similar to those in the wild type (lane 3), while in strain MD100 (pMV5), which produced cytochrome b_{NR} but not NarJ, both subunits were degraded to the same extent as those in MD100 (lane 5). Strain MD100(pMV5) also accumulated large amounts of a NarG-NarJ fusion protein, as expected from the construction of pMV5 (21), in which the 3′ end of *narG*, all of *narH*, and the 5′ half of the *narJ* gene were deleted. In strain MD100(pES203.1), which produced NarJ as well as the α and β subunits, the α subunit appeared to be completely protected from degradation (lane 4), while the β subunit seemed to be degraded similarly to that in the mutant (lane 6). Figure 1 also shows the results of immunoblots for equal amounts of the membrane and cytosolic fractions separated from the crude extract of each strain by differential centrifugation (lanes 7 to 16). In the strains which produced fully active enzyme, RK4353 (lanes 7 and 8) and MD100(pMV4) (lanes 9 and 10), the α and β subunits were localized predominantly in the membrane fractions (lanes 7 and 9) in which the activity was located (Table 4). In *narJ* mutant MD100 (lanes 15 and 16) and MD100(pMV5) (lanes 13 and 14), the undegraded forms of the subunits were equally distributed between the membrane (lanes 15 and 13) and supernatant (lanes 16 and 14) fractions as was the

degraded form of β, but the degraded form of α remained mainly in the supernatant. Thus, in the absence of NarJ, both the α and β subunits accumulated in an inactive form, which appears to be susceptible to proteolytic degradation and to tend to remain in the cytosol even when membrane-bound cytochrome b_{NR} is produced. Furthermore, the portion of the α-β complex which was associated with the membrane fraction appeared to represent the specific form of the complex which included the undegraded α subunit.

In contrast to the other cases, the α and β subunits which accumulated in the presence of NarJ in MD100(pES203.1) were localized more in the supernatant fraction (lane 12) than in the membrane fraction (lane 11). Together with the restoration of activity observed with this strain (Table 4), these results suggest that NarJ protects a partially active, cytoplasmic form of the complex from proteolytic degradation and retards its binding to the membrane in the absence of cytochrome b_{NR}.

DISCUSSION

Contrary to the conclusions reached in our previous study (21), we demonstrate here that there is an absolute requirement for the NarJ protein in the assembly of physiologically active, membrane-bound nitrate reductase. Expression of the *narGHJI* operon containing a disrupted *narJ* gene either from a multicopy plasmid in a *narG*::Tn*10* insertion mutant or from a single chromosomal copy resulted in severely reduced levels of both MVH-linked and formate-linked nitrate reductase activities. The two activities are not restored in the mutant with a disrupted *narJ* gene when NarI is provided from a plasmid but only when both NarJ and NarI are provided. Thus, although NarJ does not appear to be associated with membrane-bound nitrate reductase, it is required for the assembly of the active complex.

Although the role of NarJ in the assembly of nitrate reductase has not been defined by these studies, the properties of the α and β subunits which accumulate in the absence or presence of NarJ provide some interesting clues. When the α-β complex is purified after release from the active α-β-γ complex (1, 12, 14, 17), it retains full (or even increased) MVH-nitrate reductase activity along with the Mo-pterin cofactor and the complement of nonheme iron present in the mature complex (1), but it will not accept electrons from quinols which are effective electron donors for the α-β-γ complex (17). The activity of the purified α-β complex is relatively stable, except that the C-terminal end of the β subunit is hypersensitive to the action of endogenous protease action and tends to be converted to a form approximately 2 kDa shorter than the complete subunit without loss of enzyme activity (4). Based on activity and immunoblot analyses, the α and β subunits which accumulated when the chromosomal *narJ* gene was disrupted were quite susceptible to proteolytic clipping. Both the α and β subunits appeared to be partially converted to slightly smaller forms, and only a very low level of MVH-nitrate reductase activity was expressed. When the γ subunit (cytochrome b_{NR}) alone was provided from a plasmid, the activity and subunit characteristics of α and β were unchanged and no active complex was formed. When NarJ alone was provided from a plasmid, the α subunit was completely protected from proteolytic clipping and MVH-nitrate reductase activity was expressed by a form of the resulting α-β complex which tended to partition with the cytosolic fraction. Apparently, NarJ either directly protects the α subunit from proteolysis and the loss of MVH-linked activity or converts the subunit to a more active, protease-resistant form. The latter process could involve the addition or modification of the Mo-pterin cofactor or a facilitated association with the β subunit. In any case, it will be of some interest to purify and compare the composition of the forms of the α and β subunits which accumulate in the presence and absence of NarJ.

When the *narJ* gene was disrupted within the *narGHJI* operon located on plasmid pSL962, low but significant levels of both MVH-linked and formate-linked activities were produced in a *narG*::Tn*10* insertion mutant. The low level of these activities was not due to nitrate reductase produced from the cryptic *narZ* operon described by Bonnefoy et al. (3), since no detectable activity was expressed under the same conditions in the absence of the plasmid (Table 3). Apparently, the α and β subunits which are overproduced from the multicopy plasmid accumulate in the absence of NarJ as a complex which expresses at least low levels of the MVH-linked activity. The fact that some formate-linked activity is also expressed under these conditions suggests that this incomplete complex can accept electrons from the quinone pool of the membrane at a low but finite rate, even in the absence of cytochrome b_{NR}. As shown for mutant MD100 (Table 4), these activities are primarily associated with the membrane fraction and, based on the immunoblot study, the active fraction includes only that portion of the complex with undergraded α subunits. Either association of the α subunit (or an α-β complex) with the membrane protects it from proteolytic clipping and loss of MVH-activity or only the complex with the unclipped form of α can bind to the membrane. In either case, the association with the membrane appears to permit this low-activity form of the complex to accept electrons from the quinol pool (represented by formate-linked activity) in the absence of cytochrome b_{NR} and indicates that the α-β complex may associate with the membrane in a specific manner in the absence of either cytochrome b_{NR} or NarJ. In the presence of NarJ, the α-β complex expresses a significantly higher level of MVH-linked activity, which tends to remain in the cytosol in the absence of cytochrome b_{NR}. Although these results demonstrate that NarJ is required for assembly of the active nitrate reductase, they raise additional questions concerning the sequence of events involved in the addition of cofactors and the assembly of subunits leading to the biogenesis of the physiologically active, membrane-associated α-β-γ complex. On the basis of the available evidence, NarJ may be involved at any of these stages of nitrate reductase biogenesis.

Our conclusion here that the Tn*10* insertion mutant RK5274, previously thought to be a *narJ* mutant, is a *narI* mutant is supported by the characteristics of the mutant with a disrupted *narJ* gene. RK5274 accumulates a form of the α-β complex with significant MVH-nitrate reductase activity (30% of the wild-type level) in the cytosolic fraction (21) (Table 2). This contrasts with the extremely low level of activity expressed by *narJ* mutant MD100 but is similar to the characteristics of MD100 transformed with pES203.1 (Table 4), which forms NarJ in addition to the α and β subunits. The fact that transformation of RK5274 with pES203.1 led to an increased level of MVH-linked activity suggests that the increased level of NarJ stabilizes or produces increased amounts of the active, cytosolic α-β complex, which can accumulate in the absence of cytochrome b_{NR}.

ACKNOWLEDGMENTS

We are indebted to Pei-Young Hsu for effective technical assistance and to Margaret Walker and Violaine Bonnefoy for valuable discussions and criticisms.

This work was supported by Public Health Service grant GM 19511 from the National Institute of General Medical Sciences.

REFERENCES

1. **Adams, M. W. W., and L. E. Mortenson.** 1982. The effect of cyanide and ferricyanide on the activity of the dissimilatory nitrate reductase of *Escherichia coli*. J. Biol. Chem. **257:**1791–1799.
2. **Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy.** 1989. Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the α and β subunits in iron binding and electron transfer. Mol. Gen. Genet. **218:**249–256.
3. **Bonnefoy, V. J., F. Burini, G. Giordano, M. C. Pascan, and M. Chippaux.** 1987. Presence in the "silent" terminus region of the *Escherichia coli* K-12 chromosome of cryptic gene(s) encoding a new nitrate reductase. Mol. Microbiol. **1:**143–150.
4. **DeMoss, J. A., T. Y. Fan, and R. H. Scott.** 1981. Characterization of subunit structural alterations which occur during purification of nitrate reductase from *Escherichia coli*. Arch. Biochem. Biophys. **206:**54–64.
5. **Edwards, E. S., S. S. Rondeau, and J. A. DeMoss.** 1983. *chlC* (*nar*) operon of *Escherichia coli* includes structural genes for α and β subunits of nitrate reductase. J. Bacteriol. **153:**1513–1520.
6. **Enoch, H. G., and R. L. Lester.** 1974. The role of a novel cytochrome b-containing nitrate reductase and quinone in the *in vitro* reconstruction of formate-nitrate reductase activity of *E. coli*. Biochem. Biophys. Res. Commun. **61:**1234–1241.
7. **Enoch, H. G., and R. L. Lester.** 1975. The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. J. Biol. Chem. **250:**6693–6705.
8. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P_1. Virology **1:**109–206.
9. **Li, S. F., T. Rabi, and J. A. DeMoss.** 1985. Delineation of two distinct regulatory domains in the 5' region of the *nar* operon of *Escherichia coli*. J. Bacteriol. **164:**25–32.
10. **Liu, J., and J. S. Parkinson.** 1989. Genetics and sequence analysis of the *pcnB* locus, an *Escherichia coli* gene involved in plasmid copy number control. J. Bacteriol. **171:**1254–1261.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
12. **Lund, K., and J. A. DeMoss.** 1976. Association-dissociation behavior and subunit structure of heat-released nitrate reductase from *Escherichia coli*. J. Biol. Chem. **251:**2207–2216.
13. **MacGregor, C. H.** 1976. Biosynthesis of membrane-bound nitrate reductase in *Escherichia coli*: evidence for a soluble precursor. J. Bacteriol. **126:**122–131.
14. **MacGregor, C. H., C. A. Schnaitman, D. E. Normansell, and M. G. Hodgins.** 1974. Purification and properties of nitrate reductase from *Escherichia coli* K-12. J. Biol. Chem. **249:**5321–5327.
15. **McPherson, M. J., A. J. Baron, D. J. C. Pappin, and J. C. Wootton.** 1984. Respiratory nitrate reductase of *Escherichia coli*: sequence identification of the large subunit gene. FEBS Lett. **177:**260–264.
16. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9:**309–321.
17. **Morpeth, F. F., and D. H. Boxer.** 1985. Kinetic analysis of respiratory nitrate reductase from *Escherichia coli* K12. Biochemistry **24:**40–46.
18. **Olmsted, J. B.** 1986. Analysis of cytoskeletal structures using blot-purified monospecific antibodies. Methods Enzymol. **134:** 467–472.
19. **Ruiz-Herrera, J., M. K. Showe, and J. A. DeMoss.** 1969. Nitrate reductase complex of *Escherichia coli* K-12: isolation and characterization of mutants unable to reduce nitrate. J. Bacteriol. **97:**1291–1297.
20. **Showe, M. K., and J. A. DeMoss.** 1968. Localization and regulation of nitrate reductase in *Escherichia coli*. J. Bacteriol. **95:**1305–1313.
21. **Sodergren, E. J., P.-Y. Hsu, and J. A. DeMoss.** 1988. Roles of the *narJ* and *narI* gene products in the expression of nitrate reductase in *Escherichia coli*. J. Biol. Chem. **263:**16156–16162.
22. **Sodergren, E. J., and J. A. DeMoss.** 1988. *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. J. Bacteriol. **170:**1721–1729.
23. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151:**788–799.
24. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertional mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
25. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
26. **Walker, M. S., and J. A. DeMoss.** 1990. Deletion analysis of domain independence in the *TRP1* gene product of *Neurospora crassa*. Mol. Gen. Genet. **223:**49–57.
27. **Walker, M. S., and J. A. DeMoss.** 1991. Promoter sequence requirements for Fnr-dependent activation of the *narGHJI* operon. Mol. Microbiol. **5:**353–360.