Site-Directed Mutagenesis of Conserved Cysteine Residues within the β Subunit of *Escherichia coli* Nitrate Reductase. Physiological, Biochemical, and EPR Characterization of the Mutated Enzymes†

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ABSTRACT: We have used site-directed mutagenesis to alter the ligands to the iron–sulfur centers of *Escherichia coli* nitrate reductase A. The β subunit of this enzyme contains four Cys groups which are thought to accommodate the single [3Fe-4S] center and the three [4Fe-4S] centers involved in the electron-transfer process from quinol to nitrate. The third Cys group (group III) contains a Trp at a site occupied by a Cys residue in typical ferredoxin arrangements or in the DmsB subunit of dimethyl sulfoxide (DMSO) reductase. In an attempt to determine the coordination site of the different iron–sulfur centers in the amino acid sequence, we have changed the Trp of group III to Cys, Ala, Phe, and Tyr and the first Cys residue of groups II–IV to Ala and Ser. Physiological, biochemical, and EPR studies were performed on the mutated enzymes. Substitution of Ala for either Cys_{184}, Cys_{217}, or Cys_{244} results in the full loss of all four iron–sulfur centers present in the wild-type enzyme. These inactive enzymes still possess the α, β, and γ polypeptides associated in a membrane-bound complex. These Cys have important structural roles and are very likely involved in the coordination of the iron–sulfur centers. Substitution of Cys_{184} with a Ser residue produces an enzyme containing the four iron–sulfur centers, but displaying reduced activity. EPR studies suggest that Cys_{184} is a ligand of the [4Fe-4S] center whose midpoint potential is −200 mV in the native enzyme. All substitutions performed in this study on Trp_{220} lead to mutant enzymes harboring the four iron–sulfur centers and a nitrate reductase activity close to that of the wild-type. In spite of the high similarity between the NarH and DmsB subunits, the Trp_{220} → Cys substitution does not allow the conversion of the [3Fe-4S] center of the nitrate reductase into a [4Fe-4S] center. Therefore, Trp_{220} does not seem to play any major role in the β subunit.

During anaerobic growth of *Escherichia coli*, nitrate can be used as an electron acceptor. The enzyme which performs this oxidoreduction reaction, nitrate reductase A, is a membrane-bound complex of three subunits, NarG (α), NarH (β), and NarI (γ). The γ subunit is a *b*-type cytochrome which receives electrons from quinones in the membrane and transfers them to iron–sulfur centers of the β subunit. Finally, the electrons are delivered from the β subunit redox centers to the molybdenum cofactor carried by the α subunit where actual nitrate to nitrite reduction takes place. Previous EPR[1] studies have indicated that nitrate reductase A contains several iron–sulfur centers (Johnson et al., 1985). Recently, Guigliarelli et al. (1992) have demonstrated that the β subunit contains one [3Fe-4S] and three [4Fe-4S] centers and that these fall into two classes according to their redox potentials: the [3Fe-4S] and one of the [4Fe-4S] centers have high redox potentials, +60 and +80 mV, respectively, while the remaining two [4Fe-4S] centers have markedly negative potentials, −200 and −400 mV.

The *narGHJI* operon encoding the various subunits of nitrate reductase A has been completely sequenced (Sodergren & DeMoss, 1988; Blasco et al., 1989). It encodes four polypeptides: NarG, NarH, NarJ, and NarI. The NarG polypeptide (α subunit) contains several amino acid sequences characteristic of molybdoenzymes and is proposed to bind the Mo cofactor (Bilous et al., 1988; Blasco et al., 1989). In the Mo(V) valence state, this redox center gives a pH-dependent characteristic EPR spectrum (Vincent & Bray, 1978). This α subunit does not contain any motif likely to bind heme or non-heme iron. In contrast, four Cys groups (groups I–IV) typical of iron–sulfur centers of ferredoxins are present in the NarH polypeptide (β subunit). These groups are thought to accommodate the four iron–sulfur centers which have been detected in nitrate reductase (Blasco et al., 1989). The NarI polypeptide (γ subunit) is a hydrophobic subunit which possesses characteristics of *b*-type cytochromes. In addition to receiving electrons from quinones, it is thought to be responsible for membrane attachment of the αβ complex: in the absence of the γ subunit, the αβ complex is soluble rather than membrane-bound and is active only with nonphysiological electron donors (Blasco et al., 1992b). The NarJ polypeptide (δ), which is not part of the final enzyme, is implicated in the activation of the αβ complex (Blasco et al., 1992b).

As already stated, the β subunit of nitrate reductase contains four Cys groups which are thought to accommodate the single [3Fe-4S] center and the three [4Fe-4S] centers of the enzyme. The same is true for the B subunit (DmsB) of the dimethyl sulfoxide reductase (Dms) respiratory system, which also contains four Cys groups, although the Dms enzyme contains four [4Fe-4S] centers. Two facts concerning the Cys groups

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[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; EPR, electron paramagnetic resonance; NR, nitrate reductase.

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Table I: Bacterial Strains and Plasmids[a]

strain or plasmid	description/genotype	source
E. coli strains		
MC4100	*ara*D139Δ(*lac*IPOZYA-*arg*F)*rps*L,*thi*	Casadaban (1976)
LCB79	MC4100 with *ϕ79* (*nar-lac*)	Pascal (1982)
TG1	Δ(*lac-pro*) sup E thi hsd D5/F′ tra36 proA$^+$B$^+$*lacI*^{Q}lacZ ΔM15	Amersham
plasmids		
Bluescript KS$^+$ Ap^R		Stratagene
pBS57	Ap^R (narH)$^+$	this study
pJF119EH	*tacP*, rrnB, lacI^Q, Ap^R	Fürste et al. (1986)
pACYC184	Cm^R, Tet^R	Biolabs
pVA7	pACYC 184 Cm^R (*narJ*)$^+$	this study
pVA14	pACYC 184 Cm^R Tet^R (*narJI*)$^+$	this study
pVA50	pJF119 EH Ap^R (*narGH*)$^+$	this study
pVA50-C_{184}A	pJF119 EH Ap^R (*narGH*[C184→A184])$^+$	this study
pVA50-C_{184}S	pJF119 EH Ap^R (*narGH*[C184→S184])$^+$	this study
pVA50-C_{217}A	pJF119 EH Ap^R (*narGH*[C217→A217])$^+$	this study
pVA50-C_{217}S	pJF119 EH Ap^R (*narGH*[C217→S217])$^+$	this study
pVA50-W_{220}C	pJF119 EH Ap^R (*narGH*[W220→C220])$^+$	this study
pVA50-W_{220}A	pJF119 EH Ap^R (*narGH*[W220→A220])$^+$	this study
pVA50-W_{220}Y	pJF119 EH Ap^R (*narGH*[W220→Y220])$^+$	this study
pVA50-W_{220}F	pJF119 EH Ap^R (*narGH*[W220→F220])$^+$	this study
pVA50-C_{244}A	pJF119 EH Ap^R (*narGH*[C244→A244])$^+$	this study
pVA50-C_{244}S	pJF119 EH Ap^R (*narGH*[C244→S244])$^+$	this study

[a] Abbreviations: Ap^R, ampicillin-resistant; Cm^R, chloramphenicol-resistant; Tet^R, tetracycline-resistant; *tacP*, tac promoter.

should be noted. First, in both subunits, three of the four Cys groups (II–IV) are located within short amino acid segments (80 residues in NarH, 79 residues in DmsB) which have 46% identity. Second, group III of NarH, $CX_2WX_2CX_3CP$, contains a Trp at a site where group III of DmsB, CX_2CX_2-CX_3CP, contains a Cys. We wondered whether this substitution was responsible for the presence of a [3Fe-4S] center in NarH.

These observations led us to focus our work on the last three Cys groups of the NarH subunit. Accordingly, we have used the technique of site-directed mutagenesis to change (i) the Trp residue of the third group and (ii) the first Cys residue of each group, since it is thought to be of paramount importance in coordinating iron–sulfur centers. In an attempt to determine the coordination sites of the different iron–sulfur centers in the amino acid sequence, we have analyzed how these substitutions altered the various known biological and physicochemical characteristics of the enzyme. This paper reports the physiological, biochemical, and EPR results obtained with the different mutated nitrate reductases.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. All *E. coli* strains and plasmids used in this study are listed in Table I.

Oligonucleotide-Directed Mutagenesis and Expression of the Altered narH Gene. Oligonucleotide-directed mutagenesis was performed using the Amersham in vitro mutagenesis kit based on the method of Taylor et al. (1985). The single-stranded template used for mutagenesis was pBS57 (Figure 1). It is a Bluescript KS$^+$ (Stratagène) derivative containing, in the *Sma*I–*Sst*II sites of the polylinker, the *Hpa*I–*Sst*II fragment caring the wild-type *narH* gene, derived from pSR95 (Rondeau et al., 1984). Oligonucleotides for mutagenesis and sequencing were purchased from the "Centre de Recherche de Biochimie et de Génétique Cellulaires de Toulouse". The oligonucleotides differ from the wild-type by having 1 or 2 nucleotides altered, thus changing $Cys_{184,217,and244}$ to Ala and Ser, and changing Trp_{220} to Cys, Ala, Tyr, or Phe (Table I). These substitutions affect Cys groups II–IV of NarH (β subunit). After mutagenesis, progeny were first screened by appropriate restriction analysis of plasmids isolated from individual clones. Cultures of TG1 harboring the putative

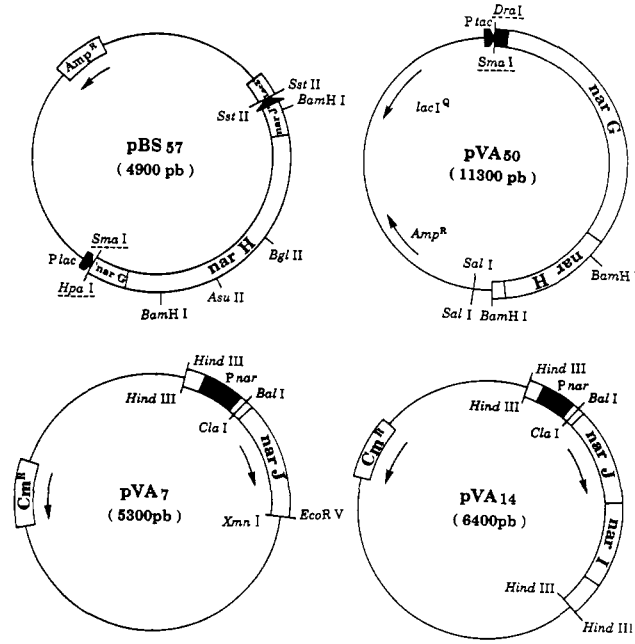

FIGURE 1: Plasmids used in this study. The procedures for constructing plasmids are described in Experimental Procedures. The arrows indicate the direction of transcription of the genes. The promoters are depicted by a black box for the *narGHJI* promoter and by a black arrow for the *tac* promoter or the *lac* promoter.

mutant plasmids were infected with the helper phage R-408 (Stratagène), and single-stranded templates were prepared as described by Russel et al. (1986). The mutations were confirmed by DNA sequence analysis using the dideoxy termination procedure of Sanger et al. (1977) using the Sequenase II system of U.S. Biochemicals (Cleveland, OH). After the whole sequence of the 350 bp *Asu*II–*Bgl*II fragment of pBS57 containing the desired mutation was confirmed, this fragment was cloned back to the wild plasmid pBS57. The plasmids carrying the mutations are then digested by *Bam*HI, and the 1.5 kb *Bam*HI–*Bam*HI fragments carrying the mutations are cloned back into pVA50, a pJF119EH derivative (Fürste et al., 1986) carrying the *narG* and *narH* genes (Figure 1). These successive subcloning steps, depending on the available restriction sites, are the simplest way to avoid

any undesirable mutations that could arise accidentally elsewhere in the plasmids during mutagenesis. The physical structures of the recombinant plasmids were verified according to cleavage of appropriate sites, and the mutations in these constructs were then definitely confirmed by double-stranded DNA sequencing. For this purpose, the plasmids were prepared and purified according to the Geneclean technique (Bio 101).

Expression of nitrate reductase complexes can be obtained from separate transcriptional units (Blasco et al., 1992a). For reasons detailed in Results, pVA50 has been used with pVA7 to express concomitantly the α, β, and δ polypeptides of the nitrate reductase (i.e., without cytochrome *b*) or with pVA14 to express the complete nitrate reductase (α, β, δ, and γ polypeptides).

Plasmid pVA7 (5300 bp) was obtained by ligating the 1056 bp *Hin*dIII–*Xmn*I fragment from pFB74 (Blasco et al., 1992b) containing the *narJ* gene into the *Hin*dIII–*Eco*RV sites of pACYC184 (Biolabs) (Figure 1).

Plasmid pVA14 (6400 bp) was obtained by inserting the 2.2 kb *Hin*dIII–*Hin*dIII fragment from pFB74 carrying the *narJ* and *narI* genes under the nitrate reductase promoter (*Pnar*), into the *Hin*dIII site of pACYC184 (Figure 1).

Manipulation of DNA. The recombinant DNA techniques, i.e., isolation of plasmid DNA, restriction enzyme digestion, polymerase polishing, ligation, transformation, and electrophoresis, were essentially adapted from Maniatis et al. (1982). Unless otherwise indicated, enzymes were purchased from BRL and used with the supplied or recommended buffer.

Culture Conditions. The strains were grown on basal medium as described previously (Giordano et al., 1978). Complex medium was prepared by adding Difco yeast extract and Difco Bactopeptone (2 g L^{-1} of each) to the basal medium. The carbon source was glucose (2 g L^{-1} for anaerobiosis), and 1 μM sodium molybdate was routinely included. For the induction of nitrate reductase, potassium nitrate was added to a final concentration of 1 g L^{-1}.

For anaerobic growth on minimum medium, Difco yeast extract and Difco Bactopeptone were omitted, glucose was replaced by glycerol (2 g L^{-1}), and amino acids required by the strains (all at 10 mg L^{-1}) were added to the medium.

Strains carrying plasmids were grown by adding appropriate antibiotics (ampicillin, 50 μg mL^{-1}; chloramphenicol, 10 μg mL^{-1}) and, when needed, IPTG (0.2 mM) to induce the *tac* promoter (*tacP*).

Preparation of Subcellular Fractions. The cells were harvested during the exponential phase of growth, suspended in 50 mM Tris-HCl (pH 7.6), and disrupted in a French press. The crude extract was centrifuged at 18000*g* for 15 min in order to sediment unbroken cells. The supernatant fraction obtained was further centrifuged at 120000*g* for 90 min, and the soluble and membrane fractions were retained. All procedures were performed at 4 °C.

Enzyme Assays. Nitrate reductase activity was measured spectrophotometrically (Jones & Garland, 1977) by the oxidation of reduced benzyl viologen which leads to the reduction of nitrate to nitrite. One unit of nitrate reductase activity is the amount catalyzing the production of 1 μmol of nitrite min^{-1} (mg of protein)$^{-1}$. Proteins were estimated by the technique of Lowry et al. (1951).

Formate-dependent nitrate reduction was measured on whole cells with formate as electron donor (Pichinoty, 1969). For the determination of nitrite the reaction was stopped by the addition of 2 mL of acetone; after centrifugation, nitrite was determined in an aliquot of the supernatant (Iobbi et al., 1987).

Quantification of Nitrate Reductase. Quantification of nitrate reductase antigen present in nitrate reductase preparations was performed by rocket immunoelectrophoresis analysis (Graham et al., 1980). The reference curves were established with fully purified nitrate reductase A and immunoadsorbed anti-nitrate reductase A serum. The samples (6 μL) were electrophoresed at 2 mA overnight in 4- × 4-cm (1% w/v) agarose plates buffered with 20 mM sodium barbital (pH 8.6) containing (1% w/v) Triton X-100 and 0.05% (w/v) sodium azide. Antiserum (100 μL) was included in the agarose medium.

Limited Proteolysis by Trypsin. Partially purified wild-type or mutated nitrate reductases (50 μg of protein) were incubated with an equal mass of trypsin for 1 h at 37 °C at pH 7.6. Trypsin was inactivated by the addition of disaggregation buffer followed by a heat treatment, and the samples were loaded on an SDS–PAGE (7.5% w/v).

Polyacrylamide Gels. Electrophoresis under denaturing conditions was carried out in polyacrylamide gels (7.5% w/v) at pH 8.8. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970).

Western Blotting Analysis. Western blotting analysis was performed as previously described. In all our experiments, the molecular weights of the native and mutated nitrate reductases subunits were estimated with standards stained by the nonpermanent stain Ponceau S before western blotting (Blasco et al., 1992a).

Partial Purification of Mutated Nitrate Reductases. All procedures were carried out at 4 °C. Wet cells (20 g) were suspended in 80 mL of 50 mM Tris-HCl, pH 8.3. The crude extract (3000 mg of protein) obtained after breakage of the cells was centrifuged twice at 120000*g* for 90 min. The supernatant fraction (2000 mg of protein) was applied to two DEAE-Sepharose CL-6B columns (2.6 cm × 85 cm) equilibrated with 100 mM NaCl. Subsequently, 2 column volumes of equilibrating buffer were passed through the column. Finally, 1 L of a 100–500 mM linear NaCl gradient in the same buffer was applied. Fractions of inactive mutated nitrate reductases were identified by rocket immunoelectrophoresis. All the wild-type and mutated nitrate reductase fractions were eluted in the range 0.35–0.38 M NaCl and then pooled, dialyzed, freeze-dried, and resuspended to a concentration of 150 mg mL^{-1} in 0.1 M Tris-HCl, pH 8.3.

Redox Titration and EPR Spectroscopy. The redox titration and EPR experiments were performed on control and mutated nitrate reductases, as previously described by Guigliarelli et al. (1992).

RESULTS

Part of this study was performed on cells expressing the *narI* gene, producing the NarI (γ) subunit, in which case the nitrate reductase complex was bound to the membrane. For reasons that will be developed later, another part had to be performed in cells devoid of the *narI* gene, in which case the $\alpha\beta$ complex was soluble in the cytoplasm.

Construction of NarH Mutant Proteins by Site-Directed Mutagenesis. Nucleotide substitutions were introduced at different positions in the structural gene for the NarH (β) subunit. In all cases, the DNA fragment subjected to oligonucleotide-directed mutagenesis was totally sequenced to verify the presence of the specified mutation as well as to ensure that no other mutation had been introduced (for details see Experimental Procedures). Ten different mutated β

Table II: Benzyl Viologen Activity, Formate–Nitrate Reductase Activity, and Anaerobic Growth on Glycerol–Nitrate Medium of Strains Transformed with pVA14 and Various pVA50 Plasmids Carrying the Mutated *narH* Gene[a]

strain	benzyl viologen–nitrate reductase activity in crude extracts		formate–nitrate reductase activity		growth on glycerol–nitrate medium
	total activity[b]	sp act. (%)[c]	nmol of nitrite formed min^{-1} (mg dry weight)$^{-1}$	(%)[d]	
MC4100	81	62.3 (99)	55	(100)	+++
LCB79	1	<0.3 (<1)	<0.3	(<1)	no growth
LCB79/pVA14+pVA50	650	63 (100)	54	(100)	+++
LCB79/pVA14+pVA50-$C_{184}A$	2	<0.3 (<1)	<0.3	(<1)	no growth
LCB79/pVA14+pVA50-$C_{184}S$	78	7.1 (11)	17.5	(32)	++
LCB79/pVA14+pVA50-$C_{217}A$	1.5	<0.3 (<1)	<0.3	(<1)	no growth
LCB79/pVA14+pVA50-$C_{217}S$	1	<0.3 (<1)	<0.3	(<1)	no growth
LCB79/pVA14+pVA50-$W_{220}C$	352	24.6 (39)	23	(42)	++
LCB79/pVA14+pVA50-$W_{220}A$	450	41.7 (66)	45	(83)	+++
LCB79/pVA14+pVA50-$W_{220}Y$	951	62.2 (98)	49	(91)	+++
LCB79/pVA14+pVA50-$W_{220}F$	557	50.2 (80)	54	(100)	+++
LCB79/pVA14+pVA50-$C_{224}A$	1.8	<0.3 (<1)	<0.3	(<1)	no growth
LCB79/pVA14+pVA50-$C_{244}S$	1.2	<0.3 (<1)	<0.3	(<1)	no growth

[a] Plasmid pVA14 encodes for *narJ* and *narI* genes. pVA50 encodes for *narG* and *narH* genes (Figure 1). [b] Total benzyl viologen–nitrate reductase activities of crude extracts were expressed in μmol of nitrate reduced min^{-1}. [c] Specific benzyl viologen–nitrate reductase activities were expressed in μmol of nitrate reduced min^{-1} (mg of nitrate reductase)$^{-1}$ and as a percent (%) of the specific activity found in strain LCB79/pVA14+pVA50. [d] Formate nitrate reductase activities, from cells grown anaerobically in the presence of glucose (2 g L^{-1}), were measured as described in Experimental Procedures. Activity is given as nmol of nitrite formed min^{-1} (mg dry weight)$^{-1}$ and as a percent (%) of the activity found in strain LCB79/pVA14+pVA50.

subunits (β^*) were obtained depending on the substituted residue and on the nature of the substitution. Cys_{184}, the first Cys residue of the second Cys group (group II), was substituted with Ala or Ser, leading to the $\beta(C_{184}A)$ or $\beta(C_{184}S)$ mutated subunits. The same was carried out for Cys_{217}, the first Cys residue of the third Cys group (group III), and for Cys_{244}, the first Cys residue of the fourth Cys group (group IV), leading to the $\beta(C_{217}A)$, $\beta(C_{217}S)$, $\beta(C_{244}A)$, and $\beta(C_{244}S)$ mutated subunits, respectively. In the case of Trp_{220} which occupies an atypical position in the group III of NarH, four different substitutions with Cys, Ala, Tyr, and Phe were produced, leading to $\beta(W_{220}C)$, $\beta(W_{220}A)$, $\beta(W_{220}Y)$, and $\beta(W_{220}F)$, respectively.

Benzyl Viologen Activity of the Mutated Nitrate Reductases in Cells Expressing the narI Gene. Biochemical studies of the various mutated enzymes were performed on a bacterial strain devoided of the chromosomal *narGHJI* structural genes but which carried the *narG* and *narH* genes on one plasmid, pVA50, and the *narJ* and *narI* genes on a second plasmid, pVA14 (Figure 1). With wild-type genes carried on plasmids, crude extracts of cells grown anaerobically in the presence of nitrate display a total nitrate reductase activity about 8 times that of the parental strain (MC 4100) expressing the genes from a single chromosomal copy (Table II). This result is consistent with previous studies which demonstrated that the different nitrate reductase subunits, produced concomitantly but independently from separate transcriptional units, can meet efficiently and associate to form active enzymes (Blasco et al., 1992a). When the mutated *narH* genes were introduced in place of the wild-type *narH* gene, different results were obtained (Table II). The presence of the *narH*($C_{184}A$) (group II) or *narH*($C_{217}A$), *narH*($C_{217}S$) (group III), or *narH*($C_{244}A$), *narH*($C_{244}S$) (group IV) mutated genes led to cells completely devoid of benzyl viologen-dependent nitrate reductase activity. In contrast, the presence of the *narH*($C_{184}S$) mutated gene led to cells which had 11% of the specific benzyl viologen-dependent nitrate reductase activity of control strain (LCB79/pVA14+pVA50). The presence of a *narH* gene mutated at codon 220 specifying Trp in the wild-type gene resulted in all cases in cells displaying a specific nitrate reductase activity ranging from 39% of the control for cells carrying the *narH*($W_{220}C$) allele to 98% for those carrying the *narH*($W_{220}Y$) allele (Table II).

Physiological Activity of the Mutated Nitrate Reductases in Cells Expressing the narI Gene. Quinones, which are reduced during the oxidation of formate by formate dehydrogenase, are the physiological electron donors for the reduction of nitrate. They provide electrons to the γ subunit which transfers them to the α subunit via the iron–sulfur centers of the β subunit. As seen in Table II, no formate-dependent nitrate reductase activity was detected in cells carrying the *narH*($C_{217}A$), *narH*($C_{217}S$) (group III), *narH*($C_{244}A$), *narH*($C_{244}S$) (group IV), or *narH*($C_{184}A$) (group II) mutated genes, whereas the presence of the *narH*($C_{184}S$) mutated gene led to cells with 32% of the formate-dependent nitrate reductase activity of control strain (LCB79/pVA14+pVA50). Cells carrying a *narH* gene mutated at codon specifying Trp_{220} in the wild-type gene displayed a formate-dependent nitrate reductase activity ranging from 42% of the control in cells carrying the *narH*($W_{220}C$) allele to 100% in those carrying the *narH*($W_{220}F$) allele (Table II).

A direct way to check the physiological functioning of the mutated enzymes was to test their capacity to sustain growth of the cells in conditions requiring the anaerobic respiration of nitrate. Accordingly, the cells were incubated under anaerobic conditions in nitrate minimal medium supplemented with glycerol, a nonfermentable carbon source. The results of these growth experiments correlated well with the formate-dependent nitrate reductase activities observed for the different mutant strains (Table II); no growth was observed when no activity was detected, and conversely. Moreover, it should be noted that cells carrying the *narH*($C_{184}S$) or *narH*($W_{220}C$) alleles, which had shown the lowest formate-dependent activities, also showed the slowest growth capacities, whereas cells carrying the *narH*($W_{220}A$, Y, or F) alleles showed almost normal growth abilities.

Cellular Localization of the Mutated Nitrate Reductases in Cells Expressing the narI Gene. The absence of activity in cells carrying the *narH*($C_{217}A$ or S), *narH*($C_{244}A$ or S), or *narH*($C_{184}A$) mutated genes might be due to the absence of αβ complexes, resulting from a pronounced instability of the β subunit and/or of the complex formed between the different

Table III: Nitrate Reductase Activities and Amounts of Immunoprecipitated Nitrate Reductase in Soluble and Membrane Fractions of Strains Transformed with pVA14 and Various pVA50 Plasmids Carrying the Mutated *narH* Gene

strain	fraction	benzyl viologen–nitrate reductase activity			immunoprecipitated nitrate reductase	
		total activity	(%)[a]	sp act.[b]	mg of protein	(%)[c]
MC4100	M	54	(73)	60	0.9	(68)
	S	20	(27)	47.5	0.42	(32)
LCB79/pVA14+pVA50	M	404	(64)	59	6.8	(66)
	S	227	(36)	65	3.5	(34)
LCB79/pVA14+pVA50-$C_{184}A$	M	<0.1		<0.3	8.4	(65)
	S	<0.1		<0.3	4.6	(35)
LCB79/pVA14+pVA50-$C_{184}S$	M	54	(75)	7	8	(72)
	S	18	(25)	6	3	(28)
LCB79/pVA14+pVA50-$C_{217}A$	M	<0.1		<0.3	6.5	(66)
	S	<0.1		<0.3	3.4	(34)
LCB79/pVA14+pVA50-$C_{217}S$	M	<0.1		<0.3	6	(50)
	S	<0.1		<0.3	6	(50)
LCB79/pVA14+pVA50-$W_{220}C$	M	182	(56)	25	7.3	(51)
	S	144	(44)	20.5	7	(49)
LCB79/pVA14+pVA50-$W_{220}A$	M	220	(54)	40	5.5	(51)
	S	190	(46)	36	5.3	(49)
LCB79/pVA14+pVA50-$W_{220}Y$	M	464	(52)	58	8	(52)
	S	425	(48)	58	7.3	(48)
LCB79/pVA14+pVA50-$W_{220}F$	M	302	(60)	48	6.3	(57)
	S	200	(40)	42	4.8	(43)
LCB79/pVA14+pVA50-$C_{244}A$	M	<0.1		<0.3	7.4	(66)
	S	<0.1		<0.3	4.8	(34)
LCB79/pVA14+pVA50-$C_{244}S$	M	<0.1		<0.3	6.1	(60)
	S	<0.1		<0.3	4	(40)

[a] Total benzyl viologen nitrate reductase activities of membrane (M) and soluble (S) fractions were expressed in μmol of nitrate reduced min^{-1} or as a percent (%) of the sum of the activities present in the soluble and membrane fractions. [b] Specific benzyl viologen-nitrate reductase activities were expressed in μmol of nitrate reduced min^{-1} (mg of nitrate reductase)$^{-1}$. [c] The amount of total immunoprecipitated nitrate reductase in membrane and soluble fractions was expressed in mg of protein estimated by rocket immunoelectrophoresis as described in Experimental Procedures and as a percent (%) of the sum of the immunoprecipitated nitrate reductase present in the soluble and membrane fractions.

subunits. To check this point, the technique of rocket immunoelectrophoresis was used to quantify the amount of material cross-reacting with antiserum raised against purified native nitrate reductase as well as to analyze the distribution of this material between the soluble and membrane fractions. As indicated in Table III, at least 50% of the immunoprecipitated inactive enzymes could be found in the membrane. Furthermore, the total amount of immunoprecipitated inactive nitrate reductases (soluble plus membrane-bound) was never less than 90% of the control cells (LCB79/pVA14+pVA50). It is obvious that, in all cases, (i) cross-reacting material was overproduced and (ii) this material was capable of binding to membranes.

As physiological nitrate reduction can only occur when nitrate reductase is membrane-bound, it was expected that the mutated nitrate reductases which were still active would be, at least in part, bound to the membrane. Table III confirmed that such was the case: 52–75% of the total benzyl viologen-dependent activity of cells carrying the *narH*($C_{184}S$) or *narH*($W_{220}C$, A, Y, or F) alleles was measured in the membrane. Furthermore, the quantification of the enzyme by the rocket technique indicated that the partition of the enzyme between soluble and membrane fractions was in good agreement with the partition of the benzyl viologen-dependent activity (Table III).

Benzyl Viologen Activity of the Mutated Nitrate Reductases in Cells Genetically Devoid of the narI Gene. We have recently performed a detailed analysis of the EPR signals of nitrate reductase in all characteristic redox states of the enzyme (Guigliarelli et al., 1992). In this study, signals arising from nitrosylated forms of the *b*-type cytochrome (γ subunit) were found to interfere strongly with those given by the iron–sulfur centers, which complicated their interpretation. To avoid this problem, the αβ complex was partially purified free of the γ subunit using a technique based on a heat treatment (60 °C, 30 min) of membrane fractions (Lund & DeMoss, 1976). Unfortunately, when the same treatment was applied to the membrane fractions of cells expressing the mutated enzymes, the yield of recovered material was dramatically low (2% of the control); furthermore, the homogeneity of such a preparation was questionable. This fact rendered almost impossible the preparation of each individual mutated enzyme in sufficient amount for EPR studies to be performed. Therefore, we changed the starting material: plasmid pVA14 (*narJI*) was replaced by plasmid pVA7 (*narJ*), since we had previously demonstrated that (i) the *narJ* gene product is required for the activation of nitrate reductase and (ii) the absence of the *narI* gene product leads to the accumulation in the cytoplasm of an active soluble αβ complex (Blasco et al., 1992b). Table IV indicates that, in these conditions, the active mutated enzymes were located almost exclusively in the soluble cellular fraction as is the control enzyme produced by the strain LCB79/pVA7+pVA50. Note that, in this strain, the total nitrate reductase activity of the soluble fraction was 30 times that found in the soluble fraction of parental strain MC4100 expressing the chromosomal genes and that the specific activity was unchanged. In the case of the active mutated enzymes, the specific activity was also unchanged whether the strain was expressing the γ subunit or not (Tables III and IV). In cells carrying a mutated *narH* gene which did not lead to active nitrate reductase, the enzyme was also localized in the soluble fraction (Table IV), and we have noted that the amount of this material is close to that detected in the control cells irrespective of the presence of the γ subunit (data not shown).

Subunit Composition of the Mutated Enzymes in Cells Genetically Devoid of the narI Gene. To allow better detailed

Table IV: Nitrate Reductase Activities and Amounts of Immunoprecipitate Nitrate Reductase in Soluble Fractions of Strains Transformed with pVA7 (Lacking *narI* Gene) and Various pVA50 Plasmids Carrying the Mutated *narH* Gene

strain	benzyl viologen–nitrate reductase activity in soluble fraction			immunoprecipitated nitrate reductase in soluble fraction (%)[c]
	total activity[a]	sp act.	(%)[b]	
MC4100	20	60	(97)	32
LCB79/pVA7+pVA50	600	62	(100)	98
LCB79/pVA7+pVA50-C_{184}A	1.8	<0.3	(<1)	96
LCB79/pVA7+pVA50-C_{184}S	70	5	(8)	99
LCB79/pVA7+pVA50-C_{217}A	1.4	<0.3	(<1)	98
LCB79/pVA7+pVA50-C_{217}S	2	<0.3	(<1)	96
LCB79/pVA7+pVA50-W_{220}C	304	21.7	(35)	97
LCB79/pVA7+pVA50-W_{220}A	528	48	(77)	95
LCB79/pVA7+pVA50-W_{220}Y	975	65	(105)	99
LCB79/pVA7+pVA50-W_{220}F	590	59	(95)	94
LCB79/pVA7+pVA50-C_{244}A	1.5	<0.3	(<1)	96
LCB79/pVA7+pVA50-C_{244}S	1	<0.3	(<1)	95

[a] Total benzyl viologen–nitrate reductase activities of the soluble fraction were expressed in μmol of nitrate reduced min^{-1}. [b] Specific benzyl viologen–nitrate reductase activities were expressed in μmol of nitrate reduced min^{-1} (mg of nitrate reductase)$^{-1}$ and as a percent (%) of the specific activity found in strain LCB79/pVA7+pVA50. [c] The amount of immunoprecipitated nitrate reductase in the soluble fraction of each strain was expressed as a percent (%) of the immunoprecipitated nitrate reductase present in the corresponding crude extract.

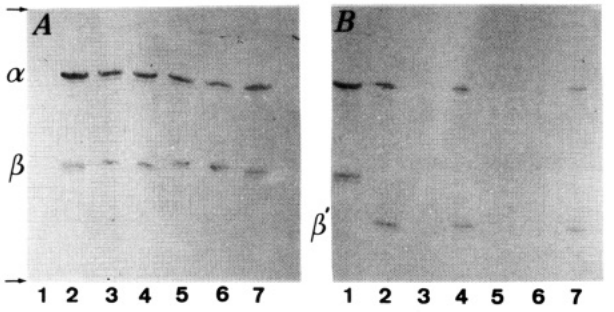

FIGURE 2: Analysis by immunoblotting of the subunit composition of various mutated nitrate reductases: effect of trypsin digestion. Trypsination, electrophoresis, and western blot of partially purified nitrate reductase were performed as described in Experimental Procedures. (Panel A) Lane 1: 2 μg of Triton X-100-solubilized crude extract of strain LCB79. Lane 2: 1 μg of partially purified nitrate reductase of strain LCB79/pVA7+pVA50. Lane 3: 1 μg of partially purified nitrate reductase of strain LCB79/pVA7+pVA50-C_{184}A. Lane 4: 1 μg of partially purified nitrate reductase of strain LCB79/pVA7+pVA50-C_{184}S. Lane 5: 1 μg of partially purified nitrate reductase of strain LCB79/pVA7+pVA50-C_{217}A. Lane 6: 1 μg of partially purified nitrate reductase of strain LCB79/pVA7+pVA50-C_{217}S. Lane 7: 1 μg of partially purified nitrate reductase of strain LCB79/pVA7+pVA50-W_{220}C. (Panel B) Lane 1: 1 μg of partially purified nitrate reductase of strain LCB79/pVA7+pVA50. Lanes 2–7: Samples as in lanes 2–7 of panel A but trypsinated. The arrows indicate the beginning and the end of the migration.

EPR studies, the mutated and control enzymes had to be partially purified. This was done as described in the Experimental Procedures. It is interesting to note that, (i) in all cases, the NaCl concentration for elution of the nitrate reductases from a DEAE-Sepharose CL-6B was between 0.35 and 0.38 M and (ii) the estimated relative molecular mass of the mutated active or inactive enzyme was about 200 000 Da (data not shown). This last result strongly suggested that the enzyme contained associated α and mutated β* subunits. This was confirmed by determining by immunological techniques the subunit composition of the active and inactive enzymes. In all cases, two polypeptides with relative molecular masses of 150 000 and 60 000 Da were observed, i.e., the same molecular masses respectively as the α and β wild-type subunits isolated from native enzyme (Figure 2A).

Trypsin Treatment of Mutated Nitrate Reductases Isolated from Cells Genetically Devoid of the narI Gene. It is known that trypsin treatment of native nitrate reductase results in cleavage of the β subunit into a β′ form with relative molecular mass 43 000 Da whereas the α subunit remains unaffected (DeMoss, 1977). An identical result was obtained with the control enzyme partially purified from strains which did not express the *narI* gene (LCB79/pVA7+pVA50) (Figure 2B). However, as also indicated in Figure 2B, all inactive enzymes present a different behavior since both the α and the β* subunits of these enzymes were totally degraded by the same trypsin treatment. On the contrary, active enzymes such as the αβ-(C_{184}S), αβ(W_{220}C) (Figure 2B), or αβ(W_{220}A, Y, or F) (data not shown) showed the same behavior as control enzyme.

Molybdenum Cofactor in the Inactive Nitrate Reductases Isolated from Cells Genetically Devoid of the narI Gene. When released from its enzymatic environment, the molybdenum cofactor is a very labile compound which can be degraded into stable compounds with characteristic fluorescence spectra (Johnson et al., 1984). We had previously demonstrated that these stable compounds can be recovered from purified molybdoenzymes by a 5-min treatment at 100 °C (Giordano et al., 1990). When the various inactive nitrate reductases were submitted to the same treatment, no material with the characteristic fluorescence spectra of the cofactor was released, indicating that these mutated enzymes did not contain any molybdenum cofactor. As expected, the same treatment applied to the active mutated enzymes led to the identification of molybdenum cofactor derivatives (data not shown).

EPR Study of the Mutated Nitrate Reductases Isolated from Cells Genetically Devoid of the narI Gene. In recent work, we have demonstrated that native nitrate reductase purified from the membrane fraction contains four iron–sulfur centers which can be divided into two groups depending on their redox potentials (Guigliarelli et al., 1992). For reasons explained earlier, the EPR studies were to be done on mutated enzymes partially purified from material already soluble in the cytoplasm. Therefore, the control enzyme partially purified from material which was also already soluble (from strain LCB79/pVA7+pVA50) was first analyzed in all its characteristic redox states between +200 and −500 mV. As shown in Figure 3, the EPR spectra given by this control enzyme were identical to those observed in the native protein in the whole range of potentials. At high potential, the signal

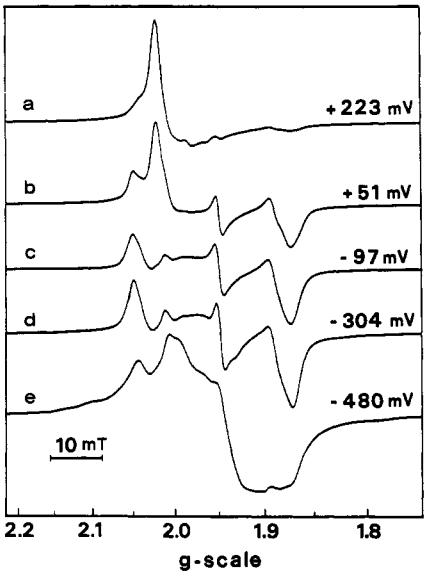

FIGURE 3: Representative EPR spectra obtained during the redox titration of the wild-type nitrate reductase A partially purified from soluble fraction. Experimental conditions: temperature, 15 K; microwave frequence, 9.305 GHz; microwave power, 100 mV; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT.

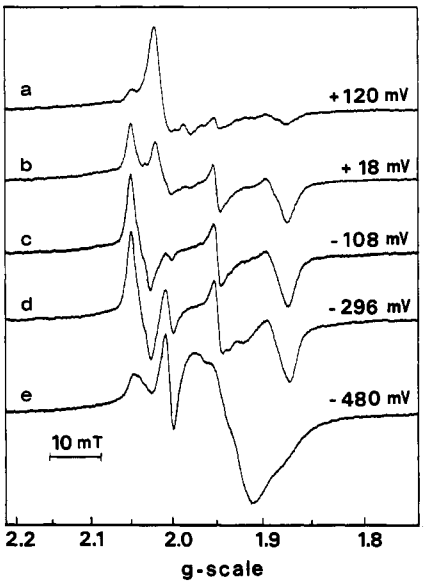

FIGURE 4: Representative EPR spectra obtained during the redox titration of the $\alpha\beta(C_{184}S)$ nitrate reductase. Experimental conditions were the same as for Figure 3.

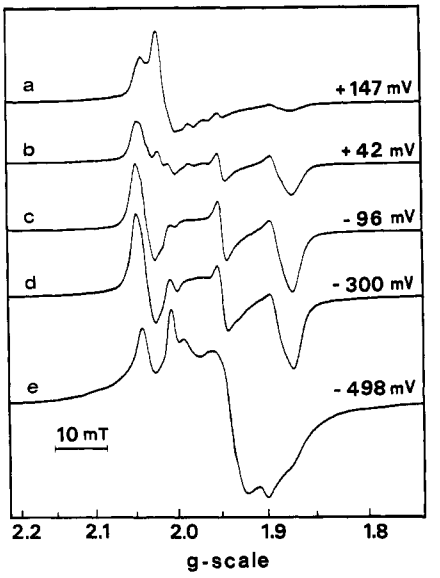

FIGURE 5: Representative EPR spectra obtained during the redox titration of the $\alpha\beta(W_{220}C)$ nitrate reductase. Experimental conditions were the same as for Figure 3.

of the $[3Fe\text{-}4S]^{1+}$ center was observed around $g = 2.01$, carrying on its high-field wing a Mo(V) signal which was strongly saturated at 100 mW (Figure 3a). By lowering the redox potential, the reduction of the high-potential [4Fe-4S] center led to a composite signal with characteristic peaks at $g = 2.04$, $g = 2.003$, $g = 1.95$, and $g = 1.87$. This signal reached its maximum amplitude around −100 mV (Figure 3b,c) with the concomitant disappearance of the signal due to the $[3Fe\text{-}4S]^{1+}$ center. As already reported for the native enzyme, the composite aspect of this spectrum reflects the existence of two different conformations of the enzyme (Guigliarelli et al., 1992). Below −200 mV, reduction of the two low-potential [4Fe-4S] centers led to a general broadening of the EPR spectrum with some modifications in the $g = 1.92$ region (Figure 3d). At very low redox potential, the very broad and almost structureless spectrum was indicative of magnetic interactions between the $[4Fe\text{-}4S]^{1+}$ centers (Figure 3e). The integrated intensities of the EPR spectra recorded in the fully reduced and in the fully oxidized states were in a 3:1 ratio within 10%. This confirmed the presence of three [4Fe-4S] centers and one [3Fe-4S] center in the enzyme. An additional slowly relaxing EPR signal, with a main peak at $g = 2.035$, was present in the whole range of studied redox potentials. This signal is typical of nitrosyl complexes found in proteins containing heme or non-heme iron (Woolum et al., 1968). Its intensity varied depending on the preparation, and it was also observed in the control strain genetically devoid of nitrate reductase, indicating that it belonged to an adventitious species. Note that the control strain lacking nitrate reductase did not present any of the signals characteristic of the above-mentioned iron–sulfur centers.

EPR studies of the partially purified $\alpha\beta(C_{217}A$ or S) enzymes (group III) and of the $\alpha\beta(C_{244}A$ or S) enzymes (group IV) were carried out for redox potentials ranging from +200 to −500 mV. None of these inactive enzymes displayed any signal characteristic either of iron–sulfur centers or of molybdenum. The same observation was made on the $\alpha\beta$-$(C_{184}A)$ enzyme (group II), which is also inactive. On the contrary, the EPR spectra recorded for partially purified $\alpha\beta$-$(C_{184}S)$ enzyme (Figure 4), $\alpha\beta(W_{220}C)$ enzyme (Figure 5), and $\alpha\beta(W_{220}A, Y,$ or F) enzymes (group III) (data not shown) were very similar to those of the active control enzyme (Figure 3). At high redox potential, the $[3Fe\text{-}4S]^{1+}$ signal was found in all five active mutated enzymes. The same was true for the composite signal due to the high-potential $[4Fe\text{-}4S]^{1+}$ center which showed characteristic g values unaffected by the mutation (Figures 4c and 5c). The evolution of the spectra when the redox potential was lowered was reminiscent of the behavior of the native enzyme with an increasing broadening leading to an almost featureless spectrum, the form of which was found to vary slightly from one mutant enzyme to another (Figures 4e and 5e). For each mutant protein, the ratio between the integrated spectral intensities recorded for the fully reduced and the fully oxidized states was 3:1 within 20%. These results indicated that the active mutated enzymes, like the native one, possess three [4Fe-4S] centers and one [3Fe-4S] center.

EPR spectra recorded at lower power (1 mW) showed that molybdenum was present in all these mutated enzymes. As for the native enzyme, at the pH conditions used for the EPR

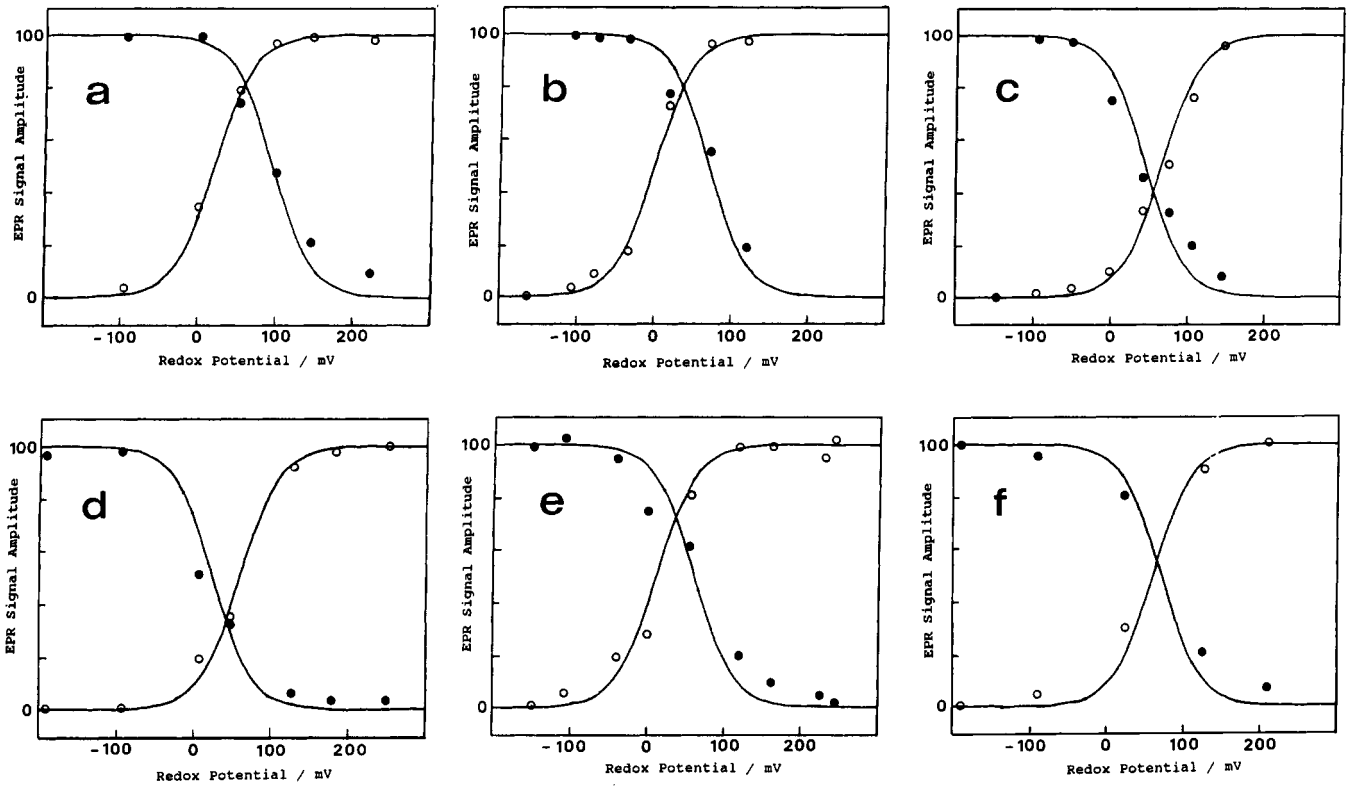

FIGURE 6: Redox behavior of the high-potential iron–sulfur centers in the wild-type and mutated nitrate reductases. (○) Normalized amplitude of the [3Fe-4S]$^{1+}$ EPR signal measured at $g = 2.01$. (●) Normalized amplitude of the [4Fe-4S]$^{1+}$ EPR signal measured at $g = 1.87$. Conditions for EPR spectroscopy were the same as for Figure 3. (a) Wild-type enzyme; (b) $\alpha\beta(C_{184}S)$ mutant; (c) $\alpha\beta(W_{220}C)$ mutant; (d) $\alpha\beta(W_{220}A)$ mutant; (e) $\alpha\beta(W_{220}Y)$ mutant; (f) $\alpha\beta(W_{220}F)$ mutant. Solid lines result from Nernst plots with the midpoint potentials listed in Table V.

studies (pH 8.3), the Mo center gives a high pH type Mo(V) signal. The maximum amplitude of this signal varied depending on the mutant enzyme and on the preparations, suggesting that the Mo cofactor was, somehow, more labile in the mutated protein than in the native one (data not shown). Unlike the native enzyme, only the disappearance of the Mo-(V) signal due to the reduction of Mo(V) into Mo(IV) was observed below +200 mV. Furthermore, for a part of these mutated enzymes, the redox behavior of this Mo(V) signal showed a marked deviation from a Nernstian curve, thus preventing a detailed comparison with the native enzyme. For all these reasons, we did not attempt to further characterize the redox properties of the Mo center.

In order to compare the redox properties of the high-potential iron–sulfur centers in the mutant and control enzymes, the amplitude of the signal given by the [3Fe-4S]$^{1+}$ center, corrected for the Fe–NO signal contribution, and that of the [4Fe-4S]$^{1+}$ center measured at $g = 1.87$ were plotted against the redox potential (Figure 6). We had previously shown that, in the native enzyme, the redox behavior of the high-potential redox centers did not exactly follow a Nernstian curve and could best be described by assuming that the two redox species were coupled by an anticooperative redox interaction of about −50 mV (Guigliarelli et al., 1992). The same redox behavior was observed with the mutated enzymes. Since such a detailed analysis could not be performed because of the large quantities of purified enzymes necessary, we have determined the midpoint potentials of the Nernstian curve which best fitted the experimental data (Figure 6; Table V). For all mutated enzymes, the potentials of the [4Fe-4S] and [3Fe-4S] centers remained close to one another and to the values estimated for the same centers in the native enzyme. Nevertheless, the $\alpha\beta(W_{220}C)$ and $\alpha\beta(W_{220}A)$ mutated enzymes show an inversion of the redox potentials of the [4Fe-4S] and [3Fe-4S] centers with respect to the native enzyme. The possible implications of such a difference on the catalytic properties of these enzymes will be discussed later.

Table V: Midpoint Potentials of the High-Potential Iron–Sulfur Centers in the Wild-Type and Mutated Nitrate Reductases from *E. coli*

strain	midpoint potentials (mV) for [3Fe-4S]	[4Fe-4S]
MC4100	+20[a]	+60[a]
LCB79/pVA7+pVA50	+20	+90
LCB79/pVA7+pVA50-$C_{184}S$	0	+70
LCB79/pVA7+pVA50-$W_{220}C$	+65	+45
LCB79/pVA7+pVA50-$W_{220}A$	+60	+20
LCB79/pVA7+pVA50-$W_{220}Y$	+10	+60
LCB79/pVA7+pVA50-$W_{220}F$	+60	+70

[a] Deduced from the data shown in Guigliarelli et al. (1992).

DISCUSSION

We have focused this study on the first Cys residue of Cys groups II–IV of the NarH (β) subunit.

The EPR signal of the NarI (γ) subunit (a *b*-type cytochrome) in the native membrane-bound enzyme strongly interferes with signals from the iron–sulfur centers of the β subunit. We have overcome this problem by eliminating the γ subunit. In such a strain, the $\alpha\beta$ complex is active with benzyl viologen but no longer with quinones. After partial purification of this soluble $\alpha\beta$ complex, we have demonstrated that the EPR signals of the redox centers are identical to those of the enzyme released from the membrane. This result is important in this study, since it indicates that EPR data recorded from already soluble $\alpha\beta$* mutated complexes would likely be identical to those of the membrane-bound mutated

enzymes and, therefore, can be compared to those of the wild-type enzyme.

Substitution of Cys_{217} (Group III) or Cys_{244} (Group IV). Four different constructs have been made and the cognate mutated proteins, as stated above, will be referred to as β-($C_{217}A$), $\beta(C_{217}S)$, $\beta(C_{244}A)$, and $\beta(C_{244}S)$. It is important to note that the lack of activity in cells expressing these mutated β^* subunits was not due to the absence of the $\alpha\beta^*$ complex since, in all cases, the $\alpha\beta$ complex was found to be as abundant as the wild-type $\alpha\beta$ complex and to be bound to the membrane when the γ subunit was present. Moreover, none of the EPR signals of the iron–sulfur centers of the native β subunit could be detected in these mutated enzymes. It is surprising that this lack of iron–sulfate centers could not prevent the association of the α and β subunits. However, this might be interpreted in supposing that the insertion of the redox centers into the β subunit only takes place once this subunit is associated with the α subunit. This interpretation is supported by the fact that the α subunit of the $\alpha\beta^*$ mutated complexes also lacks its own redox center, the molybdenum cofactor.

According to a second hypothesis, the $\alpha\beta^*$ complexes could result from an abnormal association between the α subunit and an unstructured β^* polypeptide. That this association, however, did not lead to a normal structure was demonstrated by the fact that the $\alpha\beta^*$ mutated complexes are much more sensitive to trypsin proteolysis and, as already pointed out, to heat denaturation than the native one. Whether this association is an artifact due to binding to an abnormal β subunit or reflects the normal course of events, i.e., association between the subunits, followed by the insertion of the Mo cofactor into the α subunit and of the iron–sulfur centers into the β subunit, remains to be tested.

Although only two substitutions have been performed, it can be concluded that the Cys_{217} and Cys_{244} have important structural roles within the β subunit, whether they coordinate iron–sulfur centers or not. The one main conclusion from these substitutions is that all iron–sulfur centers are lost by a single residue change. The loss and/or substitution of Cys_{217} (or Cys_{244} residue, by similar reasoning) must place a constraint on the folding of the β subunit such that the resulting structure is no longer compatible with the insertion of any of the four iron–sulfur centers of the normal subunit. Note that similar results have been reported for some mutants of *E. coli* fumarate reductase (Manodori et al., 1992), *Klebsiella pneumoniae* (Kent et al., 1989), and *Azotobacter vinelandii* (May et al., 1991) MoFe nitrogenases. In this last case, substitution of either Cys_{70} or Cys_{95} residues with Ala or Ser abolishes the MoFe protein activity and the EPR signals.

Substitution of Cys_{184} (Group II). When Cys_{184} was mutated to Ala or Ser, different effects were observed depending on the nature of the substituting residue. The β-($C_{184}A$) mutated subunit had the same general properties as reported above for the other β^* subunits ($C_{217}A$, $C_{217}S$; $C_{244}A$, $C_{244}S$). Consequently, the same comments as above can be made, i.e., a strong structural role for Cys_{184} within the subunit such that its loss leads to a structure in which none of the iron–sulfur centers can be integrated.

The effects changed, however, when Cys_{184} was mutated to Ser. Our results indicated that electrons are transferred from the redox centers of the γ subunit to those of the α subunit, and EPR spectroscopy confirmed the presence of the four iron–sulfur centers in the $\beta(C_{184}S)$ subunit. We should have expected important modification of the g-tensor and of the redox properties of one iron–sulfur center to be induced by the substitution of a terminal sulfur ligand with an oxygen ligand as reported for the [2Fe-2S] center of fumarate reductase (Werth et al., 1990). Several explanations can be proposed to rationalize our observations. Cys_{184} might not be involved in the coordination of an iron–sulfur center. This seems very unlikely, if one considers the identity of Cys group II with typical Cys arrangements in ferredoxins and the disappearance of the iron–sulfur signals when Cys_{184} is replaced by Ala. As previously proposed for the Cys_{65} of fumarate reductase (Werth et al., 1990), Cys_{184} of the β subunit could coordinate one iron–sulfur center indirectly through a hydrogen-bonded water molecule. However, such a coordination scheme has not yet been proven in proteins or in model compounds. In fact, an alternative explanation is that Cys_{184} is a ligand for one of the low-potential [4Fe-4S] centers. As mentioned above, only the spectroscopic and redox properties of the high-potential iron–sulfur centers could be accurately studied. Reduction of the low-potential centers does not give well-resolved characteristic EPR features and simply leads to a broadening of the spectrum arising from magnetic interactions. Thus, the $Cys_{184} \rightarrow$ Ser mutation could induce some modifications of one of the low-potential centers' g-tensors which would result in minor changes on the low-potential EPR spectrum as experimentally observed (Figure 3e). Moreover, from the results reported for fumarate reductase (Werth et al., 1990), the Cys → Ser substitution is expected to decrease the midpoint potential of one iron–sulfur center by about 100 mV. At −500 mV, all [4Fe-4S] centers of the $\beta(C_{184}S)$ subunit are fully reduced, and so it is very likely that the [4Fe-4S] center with the lower midpoint potential (center 4: −400 mV in the wild-type enzyme) is not affected by the mutation. On the other hand, the EPR spectra observed at low potential are not inconsistent with a 100-mV decrease of the midpoint potential of center 3 (numbering refering to decreasing redox potential). Taken together, our results suggest that Cys_{184} could ligate the [4Fe-4S] center whose midpoint potential is −200 mV in the native enzyme (center 3 of the β subunit), but further studies are required to confirm this proposal.

Substitution of Trp_{220} (Group III). Four different constructs have been made in which the Trp_{220} residue was substituted with either Ala, Cys, Tyr, or Phe. Cells harboring these β^* subunits showed growth and nitrate reductase and physiological activities close to that of the wild-type, with the exception of those harboring the $\beta(W_{220}C)$ mutated subunit for which the growth was slightly lowered. Moreover, in these constructs, the EPR signals of the iron–sulfur centers did not show any difference with those of the control $\alpha\beta$ complex. In particular, the $Trp_{220} \rightarrow$ Cys substitution, which transforms the Cys group of the β subunit into a Cys arrangement similar to that found in the DmsB subunit, does not allow the binding of an additional [4Fe-4S] center: the [3F-4S] center is still present, and the low-potential spectra due to magnetic interactions between the [4Fe-4S] centers are not changed with respect to the control enzyme. Thus, it appears that the Cys replacing the Trp_{220} residue is not involved in the binding of an iron–sulfur center and does not compete with other Cys residues of the β subunit. This situation is reminiscent of that observed in ferredoxin I (FdI) of *A. vinelandii* which carries one [3Fe-4S] and one [4Fe-4S] center. The primary sequence of this protein exhibits two typical Cys groups; crystallographic studies have shown that the second Cys residue of the first group, Cys_{11}, not only does not participate in liganding the [3Fe-4S] center but is excluded from it (Stout 1988). On the basis of sequence alignments with the first FdI Cys group, and assuming that Cys group III of the β subunit is involved

in the coordination of the [3Fe-4S] center, Brasseur has constructed a model for this Cys group (personal communication). His model predicts that Trp_{220} is excluded from the iron–sulfur center as Cys_{11} in FdI. Thus it can be proposed that the constraints brought about by the amino acid sequence around position 220 are such that, even if the residue at this position is a Cys, it will not be used as a ligand for the iron–sulfur center and will not change dramatically the folding of the protein. It is therefore tempting to propose that the residue at position 220 does not play any major role in the β subunit. It should be noted that the substitution of Trp_{220} by aromatic residues (Phe or Tyr) is less penalizing for the activities than the other substitutions.

Recently, Rothery and Weiner (1991) have proposed that the group III of the NarH subunit is involved in liganding the [3Fe-4S] center of this protein. Our results do not provide any direct argument in favor of or against this proposal. Nevertheless, the permissivity of position 220, consistent with the model of tertiary structure proposed for the Cys group III of the β subunit as ligand of a [3Fe-4S] center, supports this idea.

It should be pointed out that another example of iron–sulfur center conversion has been recently reported in the literature. A site-directed mutant of *E. coli* fumarate reductase in which Val_{207} of B subunit (FrdB) was changed to Cys has been obtain by Manodori et al. (1992). EPR studies of this mutated fumarate reductase have indicated that the indigenous [3Fe-4S] center ($E_m = -70$ mV) has been replaced by a much lower potential [4Fe-4S] center ($E_m = -350$ mV). Moreover, this mutation resulted in a functional membrane-bound enzyme that was able to support growth under anaerobic and aerobic conditions.

The substitutions of Trp_{220} with other amino acids induce moderate shifts of midpoint potential for both the [3Fe-4S] and high-potential [4Fe-4S] centers, which are less than 70 mV in all cases. These concomitant shifts are not surprising when one considers that a given Cys group in the sequence should be involved in the coordination of two iron–sulfur centers. It is interesting to note that, for the two mutants $\beta(W_{220}A)$ and $\beta(W_{220}C)$, the midpoint potential of the high-potential [4Fe-4S] center becomes lower than that of the [3Fe-4S] center. This reverse ordering of potentials could hinder an efficient electron transfer between the γ and α subunits, leading to the lower nitrate reductase activities observed experimentally. However, replacement of Trp_{220} by other aromatic residues gives essentially the same activities as in the wild-type enzyme, although the midpoint potentials of the high-potential centers are somewhat different. Conversely, for the $\beta(C_{184}S)$ mutant, the redox potentials of high-potential centers are close to the values found in the native enzyme, but the activity is strongly reduced. This indicates that the catalytic activity of nitrate reductase is not only dependent on the redox potentials of the high-potential centers, but probably also dependent on other factors like the redox properties of the Mo cofactor or on local conformational changes which could affect the electron-transfer kinetics.

CONCLUSION

The main conclusion emerging from this study is that the Cys_{184}, Cys_{217}, or Cys_{244}, the first Cys residue of the second, third, and fourth Cys groups of the β subunit respectively, are very likely involved in the coordination of iron–sulfur centers. These centers are so structurally interdependent that the loss of any one of them precludes the insertion or the stability of all others. However, the β^* subunits lacking the iron–sulfur centers still bind the α and γ subunits and remain associated with the membrane.

It would be valuable to compare the behavior of the NarH and DmsB subunits by further site-directed mutations. For example, it would be of interest to know if a Cys to Ala substitution of the first Cys residue of any one of the last three Cys groups of DmsB subunit would lead to the same drastic loss of iron–sulfur centers as in NarH, and if the mutated DmsB subunit would retain its capacity to bind the DmsA subunit.

At present we do not know what would be the effects of an Ala substitution for Cys_{16}, the first Cys residue of the NarH first Cys group. We only know that the iron–sulfur center supposed to be coordinated by the first three Cys residues of that group is also lost upon substitution of Cys_{184}, Cys_{217}, or Cys_{244} with Ala. It would, therefore, be very interesting to know whether or not this particular center can be lost without affecting the stabilization of the other three centers. Experiments are in progress to answer this question.

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