Complete Coordination of the Four Fe-S Centers of the β Subunit from *Escherichia coli* Nitrate Reductase. Physiological, Biochemical, and EPR Characterization of Site-Directed Mutants Lacking the Highest or Lowest Potential [4Fe-4S] Clusters

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ABSTRACT: The β subunit of the nitrate reductase A from *Escherichia coli* contains four groups of cysteine residues (I−IV) which are thought to bind the four iron−sulfur centers (1−4) of the enzyme. The fourth Cys residue of each group was replaced by Ala by site-directed mutagenesis, which led to the C26A, C196A, C227A, and C263A mutants. Physiological and biochemical effects of these mutations were investigated on both the membrane-bound and the soluble forms of the enzyme. In addition, detailed redox titrations of the mutants were monitored by EPR spectroscopy. The C196A and C227A mutations resulted in the full loss of the four Fe-S clusters and of the Mo-cofactor, leading to inactive enzymes. In contrast, the C26A and C263A mutants retained significant nitrate reductase activities. The EPR analysis showed that the highest redox potential [4Fe-4S] cluster (center 1) was selectively removed by the C263A mutation and that the C26A replacement likely eliminated the lowest potential [4Fe-4S] cluster (center 4). In both mutants, the three remaining Fe-S clusters kept the same spectral and redox properties as in the wild type enzyme. These results enabled the determination of the Cys ligands of center 1 to be completed and led to a proposed model for the coordination of the four Fe-S centers by the four Cys groups of the β subunit. In this model, the four clusters are organized in two pairs, (center 1, center 4) and (center 2, center 3), which is in good agreement with the magnitude of intercenter magnetic interactions observed by EPR and with the stability of the different mutants. The possible implications on the intramolecular electron transfer pathway are discussed.

In *Escherichia coli*, the nitrate reductase A enzyme is induced when the bacterium is grown anaerobically in the presence of nitrate. This terminal enzyme of the respiratory chain is a membrane-bound complex composed of three subunits, NarH (α, 139 kDa), NarG (β, 58 kDa), and NarI (γ, 25 kDa). A catalytic dimer results from the association of the α and β subunits, which bear, respectively, a molybdenum cofactor and four iron−sulfur centers. The third subunit, γ, is a hydrophobic subunit exhibiting characteristics of a *b*-type cytochrome and is responsible for the attachment of the $\alpha\beta$ complex to the membrane. In addition, the γ subunit receives electrons from physiological membrane-embedded quinones, and in its absence, the $\alpha\beta$ complex is soluble and active only with nonphysiological electron donors such as benzyl viologen. A fourth polypeptide NarJ (δ, 25 kDa), which is not part of the final enzyme, is required to obtain the nitrate reductase enzyme in an active form.

Usually, the binding of iron−sulfur centers in proteins involves the typical motif of cysteine residues Cys-X-X-Cys-X-X-Cys-X-X-X-Cys-Pro (Bruschi & Guerlesquin, 1988). Recent biochemical and EPR studies (Blasco et al., 1989; Guigliarelli et al., 1992) have shown that the β subunit contains four such groups of cysteine residues, numbered I−IV from the N-terminal to the C-terminal part of the polypeptide chain (Figure 1), and carries three [4Fe-4S] clusters and one [3Fe-4S] cluster. According to their markedly different redox potentials, these clusters belong to two classes. One of the [4Fe-4S] clusters, center 1, and the [3Fe-4S] cluster, center 2, are characterized by unusually high redox potentials, +80 and +60 mV, respectively, and seem to be competent for the intramolecular electron transfer between the γ subunit and the Mo catalytic site of the α subunit. The two other [4Fe-4S] clusters, centers 3 and 4, have much lower redox potentials, −200 and −400 mV respectively, and their role in the enzyme is presently unknown.

In an attempt to determine the coordination sites of the different Fe-S centers in the β subunit, we have undertaken a site-directed mutagenesis program to substitute the two first Cys residues of each cysteine groups by Ala or Ser residues (Augier et al., 1993a,b). Except for the replacement of the first Cys of group II by Ser, all the substitutions of the two first Cys of groups II, III, and IV have resulted in the loss of the four Fe-S centers and of the molybdenum cofactor. In contrast, the mutations of the two first Cys of group I have led to the removal of only one Fe-S cluster, center 1, the other centers keeping almost unchanged characteristics. On the basis of redox titrations monitored by EPR[1] and

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[1] Abbreviations: EPR, electron paramagnetic resonance; NR, nitrate reductase; IPTG, isopropyl β-D-thiogalactopyranoside; BV, benzyl viologen; PMSF, phenylmethylsulfonyl fluoride.

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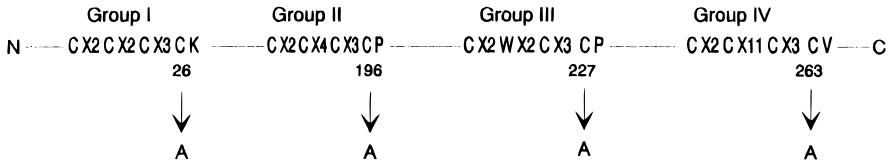

FIGURE 1: Different groups of cysteine residues in the β subunit of *E. coli* nitrate reductase A.

Table 1: Bacterial Strains and Plasmids[a]

strains or plasmids	description/genotype	source
E. coli strains		
MC4100	*ara*D139 Δ(*lacl*POZYA-*arg*F) *rsp*L *thi*	Casadaban (1976)
LCB79	MC4100 ϕ (*nar-lac*)	Pascal (1982)
plasmids		
pJF119EH	*tacP*, *rrn*B, *lacl*^{q}, Ap^r	Fürste et al. (1986)
pBS57	Bluescript:KS* (*narH*) Ap^r	Augier et al. (1993a)
pVA50	Bluescript KS$^+$ (*narGH*) Ap^r	Augier et al. (1993a)
pVA7	pACYC184 (*narJ*) Cm^r	Augier et al. (1993a)
pVA700	pJF119EH (*nar GHJ*I) Ap^r	this study
pVA700-C26A	pJF119EH (*nar GH*[C26A] *JI*) Ap^r	this study
pVA700-C196A	pJF119EH (*nar GH*[C196A] *JI*) Ap^r	this study
pVA700-C227A	pJF119EH (*nar GH*[C227A] *JI*) Ap^r	this study
pVA700-C263A	pJF119EH (*nar GH*[C263A] *JI*) Ap^r	this study
pVA50-C26A	pJF119EH (*narGH*[C26A]) Ap^r	this study
pVA50-C196A	pJF119EH (*narGH*[C196A]) Ap^r	this study
pVA50-C227A	pJF119EH (*narGH*[C227A]) Ap^r	this study
pVA50-C263A	pJF119EH (*narGH*[C263A]) Ap^r	this study

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

carried out on the wild type enzyme and site-directed mutants, a correspondence between the first Cys of groups I, II, III, and IV and Fe-S clusters 1, 3, 2, and 4, respectively, has been proposed (Augier et al., 1993b).

In a typical [4Fe-4S] center coordination, the first three Cys of one group are ligand of one cluster, whereas the fourth binds another cluster (Bruschi & Guerlesquin, 1988; Bilou et al., 1988; Manodori et al., 1992; Yano et al., 1994). Thus, each Cys group binds two Fe-S clusters and maintains them in close proximity, leading to EPR detectable magnetic interactions in their reduced state (Mathews et al., 1974; Guigliarelli et al., 1993). Inversely, each Fe-S center connects two Cys groups and then two different parts of the polypeptide chain, playing then a structural role in the folding of the protein and in its stability. The understanding of the cluster coordination within the β subunit is then of fundamental importance since it could provide information about the spatial arrangement of the four Fe-S centers and help to understand the intramolecular electron transfer mechanism. In order to solve this question, we have then undertaken site-directed mutagenesis experiments to change the last Cys residue of each Cys group of β into Ala (Figure 1), and we have performed biological, biochemical, and biophysical studies on the mutated enzymes. This work allows us to identify the fourth Cys ligand of centers 1 and 4 and to propose a model for the organization of the four Fe-S centers in the β subunit of nitrate reductase.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. All *E. coli* strains and plasmids used in this study are listed in Table 1. Plasmid pVA700 (12.8 kbp) was obtained by inserting the 8 kbp *Dra*I−*Ssp*I fragment carrying the nar*GHJI* operon into the *Sma*I site of pJF119EH. In this construction, the expression of the nitrate reductase was induced by the addition of 0.2 mM IPTG.

Oligonucleotide Site-Directed Mutagenesis. Oligonucleotide site-directed mutagenesis, based on the method of Taylor et al. (1985), was performed using the Amersham *in vitro* mutagenesis kit Sculptor. The plasmid template used was pBS57. Oligonucleotides for mutagenesis were purchased from Genset. 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.B. (Cleveland, OH). The mutated fragments were then subcloned into the pVA700 or pVA50 plasmids.

Growth of Bacteria. Strains carrying plasmid pVA700 were grown anaerobically at 37 °C on 2TY medium supplemented with glucose (2 g/L). Strains carrying plasmids pVA50 and pVA7 were grown as described previously by Augier et al. (1993a). When needed, IPTG was added to a final concentration of 0.2 mM. Ampicillin and chloramphenicol were used, respectively, at 50 and 10 μg/mL.

Preparation of Subcellular Fractions. The cells were harvested during the exponential phase of growth, suspended in 100 mM Tris-HCl, 1 mM $MgCl_2$ (pH 8.3), and disrupted in a French press. The buffer used during the French press step contained 0.2 mM PMSF. The crude extract was centrifuged at 18 000*g* for 15 min in order to sediment unbroken cells. The supernatant obtained was further centrifuged at 120 000*g* for 90 min, and the soluble and membrane fractions were retained.

Enzyme Assays. Nitrate reductase activity was measured spectrophotometrically (Jones & Garland, 1977) by the oxidation of reduced benzyl viologen (BV) 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}.

Quinol-nitrate reductase activities were measured spectrophotometrically according to Unden and Kröger (1986) by the extinction changes caused by the oxidation of ubiquinol analog tetramethyl-*p*-benzoquinone (duroquinol,

$E'_0 = +35$ mV) (Aldrich) and menaquinol analog 2-methyl-1,4-naphthoquinone (menadiol, $E'_0 = -1$ mV) (Aldrich) by nitrate. Quinone analogs were directly reduced in the cuvette by a freshly prepared solution of KBH_4 (5 g/L of H_2O). Assays were initiated by addition of nitrate (37.5 mM) to the anaerobic assay buffer containing the subcellular fractions (1.1 μg of NR/mL) and the electron donors (130 μM menadiol or 150 μM duroquinol). The difference of millimolar extinction coefficients (red-ox) used for menadiol and duroquinol are $\epsilon_{260} = 17.18$ and $\epsilon_{270} = 21.26$, respectively. One unit of quinol-nitrate reductase activity is the amount of nitrate reductase catalyzing the production of 1 μmol of quinone min^{-1}.

Formate-dependent nitrate reduction was measured on whole cells with formate as electron donor (Iobbi et al., 1987).

Quantification of Nitrate Reductase. Quantification of nitrate reductase antigen present in nitrate reductase preparations was performed by rocket immunoelectrophoresis analysis as previously described by Augier et al. (1993a).

Partial Purification of Soluble Mutated Nitrate Reductases. All procedures were carried out at 4 °C as previously described (Augier et al., 1993a).

Redox Titrations and EPR Spectroscopy. The redox titrations were performed anaerobically at 21 °C, in 0.1 M Tris-HCl, pH 8.3 buffer, under argon atmosphere. Potentials were adjusted with small additions of 20 mM sodium dithionite and measured with an Ag/AgCl/KCl (3M) electrode in the presence of the same mediators as those previously used for the titration of the wild type enzyme (Guigliarelli et al., 1992). All quoted potentials are given with respect to the standard hydrogen electrode.

Photochemical reductions were carried out by adding 5-deazaflavin (10 μM) and EDTA (10 mM) to dithionite-reduced EPR samples and by anaerobically illuminating the EPR tubes with a 150 W slide projector for 5−10 min.

EPR spectra were recorded on a Bruker ESP300 spectrometer equipped with an Air-Products Helitran gas flow system. Spin intensity measurements were performed in nonsaturating conditions for Fe-S signals (1 mW microwave power at 12 K).

RESULTS

The last Cys residue of each of the four Cys groups present in the NarH (or β) subunit of nitrate reductase (Figure 1) was replaced by an Ala residue by site-directed mutagenesis, which led to the C26A, C196A, C227A, and C263A mutated proteins (Figure 1). Each *narH* mutated allele (*narH**) was then introduced into the plasmid pVA700 in which the *nar* genes are under control of the strong *tacP* promoter, and the resulting construct was used to transform the strain LCB79 devoid of the chromosomal *narGHJI* operon. The overexpression of *narGH**JI genes carried by this plasmid allowed all studies on the mutated enzymes to be performed in good experimental conditions.

Expression and Cellular Location of the Mutated Enzymes. The amount of enzyme present in each subcellular fraction and the subunit composition of each mutated protein were determined by rocket immunoelectrophoresis and immunoblot techniques. In all cases, the molecular weight of the C26A, C196A, C227A, and C263A mutated enzymes was identical to that of the control enzyme, the amount of overexpressed mutated enzymes was ten times that of the parental strain MC4100, and about 90% of the enzyme was associated to the membrane (Table 2). In a previous work (Augier et al., 1993a), we have shown that only 50%−75% of the enzyme was associated to the membrane when it was obtained by expressing the *narGH** and *narJI* genes from two different plasmids which did not express the genes stoichiometrically.

Table 2: Benzyl Viologen−Nitrate Reductase Activities and Amounts of Immunoprecipitated Nitrate Reductase in Soluble and Membrane Fractions of Strains Transformed with Various pVA700 Plasmids Carrying the Mutated *narH* Gene[a]

	immunoprecipitated nitrate reductase		benzyl viologen−nitrate reductase activity		
strains	mg of protein	(%)	total activity[b]	(%)[c]	sp activity[d]
MC4100					
M	6	92	598	94	100
S	0.5	8	37	6	74
LCB79/pVA700					
M	62	93	6110	95	100
S	5	7	322	5	65
LCB79/pVA700 C26A					
M	60	92	5385	93	90
S	5	8	405	7	84
LCB79/pVA700 C196A					
M	57	90	<0.1	/	<0.3
S	6	10	<0.1	/	<0.3
LCB79/pVA700 C227A					
M	58	88	<0.1	/	<0.3
S	8	12	<0.1	/	<0.3
LCB79/pVA700 C263A					
M	59	87	362	87	6
S	9	13	54	13	6

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

As far as the benzyl viologen−nitrate reductase activity of the enzymes was concerned, three different cases were observed (Table 2). The C196A (last Cys of group II) and the C227A (last Cys of group III) mutants were totally devoid of activity. The C263A (last Cys of group IV) mutated enzyme displayed a weak but significant activity of about 6 units/mg of proteins, similar to that observed with the enzymes mutated on the first or second Cys residue of group I (Augier et al., 1993b). Finally, the activity of the C26A mutant was comparable to that of the control wild type enzymes from strains MC4100 and LCB79/pVA700, *i.e.*, 90% of the maximum activity. Such a high BV−nitrate reductase activity has never been obtained before by substituting a Cys residue presumably engaged in the coordination of an Fe-S cluster. It is worth noting that in the case of the C26A and C263A mutants, the partition of the enzyme between the membrane and supernatant fractions estimated from BV−nitrate reductase activity agreed well with that estimated by rocket immunoelectrophoresis. Taken together these results indicated that if none of the substitutions of Ala for Cys reported above modified the cellular location of the cognate mutated enzymes, some of them affected electron transfers in the corresponding proteins.

Physiological Activity of Mutated Enzymes. Various approaches were used to gain some information on the

Table 3: Nitrate Reductase Activities and Anaerobic Growth on Glycerol−Nitrate Medium[a]

strain	duroquinol−nitrate reductase activity		menaquinol−nitrate reductase activity		formate−nitrate reductase activity	growth on glycerol−nitrate medium
	sp activity	%	sp activity	%		
MC4100	5.5	100	14	100	59	+++
LCB79/pVA700	5.4	100	14	100	55	+++
LCB79/pVA700 C26A	1.2	22	3.7	26	25	++
LCB79/pVA700 C196A	0	0	0	0	0	no growth
LCB79/pVA700 C227A	0	0	0	0	0	no growth
LCB79/pVA700 C263A	3.9	72	0	0	18	++

[a] Quinol−nitrate reductase activities from membrane extracts were measured as described in experimental procedures and given as μmol of quinone formed min^{-1} (mg of nitrate reductase)$^{-1}$ and as a percent (%) of the activity found in strain LCB79/pVA700. Formate−nitrate reductase activity was measured on whole cells as described in Experimental Procedures and given as nmol of nitrite formed min^{-1} (mg dry weight)$^{-1}$.

physiological electron transfer in the mutated NR complexes. First, the nitrate reductase activity was measured with formate as electron donor. This method gives an estimation of the efficiency of the whole electron transfer system between the physiological donor and acceptor *via* the physiological membrane-embedded quinones. As expected, the lack of BV−nitrate reductase activity of C196A and C227A enzymes was accompanied by the absence of formate−nitrate reductase activity. In contrast, the C26A and C263A mutants displayed activities of 25 and 18 units, respectively, versus 55−59 units for the reference enzyme, an indication that some electron transfer, at least, occured *via* the pool of physiological quinones (Table 3).

Second, the growth of cells carrying any one of the four mutated enzymes was estimated in minimal medium supplemented with glycerol and nitrate under anaerobic conditions. In these conditions, only those cells able to perform a physiological electron transfer between formate and nitrate can grow. As expected, cells carrying the C196A or C227A mutants were unable to grow, while those carrying the C26A or C263A enzymes were able to develop. The growth was, however, much slower than that of the parental strain MC4100, indicating that the gain in energy corresponding to the reduced electron transfer observed *via* the quinones, if able to support the cell growth, was not of the same magnitude as that of the control cells.

Third, these observations prompted us to verify whether the active mutated complexes were able to use the same quinones as the native enzyme. Studies performed with *menA*, *ubiA*, and *menA-ubiA* mutants had proven that, unlike most enzymes engaged in anaerobic respiration, NR was able to use ubiquinone, even if the electron transfer occurs preferentially with the menaquinones (Wissenbach et al., 1990; Enoch et al., 1974; Wallace et al., 1977a,b). We have then used the duroquinol ($E'_0 = +35$ mV) and the menadiol ($E'_0 = -1$ mV) as electron donors to measure the nitrate reductase activity. No activity was detected with the C196A and C227A mutants. The other mutated enzymes, C26A and C263A, presented 22% and 72% of wild type NR activity, respectively, when assayed with duroquinol. The C26A mutant was also capable of a reduced but significant electron transfer between the menaquinol and the active site of the enzyme, whereas the C263A enzyme could not (Table 3). Therefore, it seems likely that the C263A mutation, unlike the C26A one, has led to the loss of the physiological ability to use electrons provided by the menaquinols, while saving that provided by ubiquinols.

In order to check this hypothesis, the ability of these donors to reduce the *b*-type cytochrome of nitrate reductase was assayed for the control and the mutated enzymes. In all cases the cytochrome could be reduced by both quinol analogs (data not shown), indicating that none of the studied mutations interfered with the ability of the cytochrome to accept electrons from these donors. As expected, the *b*-type cytochrome of the native enzyme could be totally reoxidized upon addition of nitrate, while that of the C196A and C227A enzymes could not. In all cases the cytochrome could be fully reoxidized by ferricyanide, but the physiological reoxidations of the cytochrome from the C26A and C263A mutated enzymes were different: while the cytochrome of both enzymes previously reduced by duroquinol could be readily reoxidized by nitrate, only that of the C26A mutant reduced by menaquinol could be reoxidized by nitrate. This indicates that the C26A substitution interferes with, but does not eliminate, the electron transfer between the menaquinol-reduced *b*-type cytochrome and the molybdenum cofactor, whereas the C263A mutation interrupts it totally.

EPR Study of Mutated Nitrate Reductases in Cells Devoid of the γ Polypeptide. Previous studies had revealed that membrane-bound NR displayed EPR spectra strongly perturbed by signals arising from heme nitrosyl complexes (Vincent & Bray, 1978). This problem can be avoided by using soluble NR produced in the absence of the γ subunit, and we have shown that the Fe-S centers have the same characteristics in the soluble and membrane-bound forms of the enzyme (Guigliarelli et al., 1992; Augier et al., 1993a). The mutated *narH** genes corresponding to the C26A, C196A, C227A, and C263A mutated enzymes were then subcloned individually into plasmid pVA50 (*narGH*) and then introduced into strain LCB79 already bearing plasmid pVA7 (*narJ*) since the δ (NarJ) subunit is required for activating the $\alpha\beta$ (NarGNarH) complex (Blasco et al., 1992; Dubourdieu et al., 1992). After purification, we have checked that the molecular weights of the α and β subunits were unchanged and that the soluble mutated enzymes presented the same catalytic activities than the membrane-bound forms (Table 4). Only in the case of the C26A mutation was the specific BV-nitrate reductase activity of the soluble enzyme half that of the cognate membrane-bound enzyme.

In order to determine the changes induced by selective amino acid replacements on the four Fe-S centers of nitrate reductase, each mutated $\alpha\beta$ complex was titrated on a wide potential range, from about +200 to −500 mV, and samples poised at different redox potentials were studied by EPR spectroscopy at 12 K. As expected from previous studies on mutated NR (Augier et al., 1993a,b), the Cys→Ala mutation of the fourth Cys residue of each Cys group in the

Table 4: Immunoprecipitated Nitrate Reductase in Soluble Fractions and Nitrate Reductase Activities

strain	immunoprecipitated nitrate reductase (%)[a]	BV−nitrate reductase activity	
		total[b]	specific[c]
LCB79/pVA7+pVA50	9	288	88
LCB79/pVA7+pVA50 C26A	7.5	162	43
LCB79/pVA7+pVA50 C196A	8	<0.1	<0.3
LCB79/pVA7+pVA50 C227A	7.6	<0.1	<0.3
LCB79/pVA7+pVA50 C263A	10	30	5

[a] The amount of immunoprecipitated nitrate reductase in the soluble fraction of each strain was expressed as a percent (%) of the total protein in the soluble fraction. [b] In μmol of nitrate reduced min^{-1}. [c] In μmol of nitrate reduced min^{-1} (mg of nitrate reductase)$^{-1}$.

β subunit had very different effects on the metal center stability. For the $\alpha\beta$(C196A) and $\alpha\beta$(C227A) mutants, neither the EPR signals of Fe-S centers nor that of the molybdenum cofactor were detected in the studied potential range. Thus, these Cys replacements induced the loss of all metal centers of NR, a result consistent with the full inactivation of the corresponding mutated enzymes (Tables 3 and 4). In contrast, for the $\alpha\beta$(C26A) and $\alpha\beta$(C263A) mutant proteins that exhibited significant nitrate reductase activities (Tables 3 and 4), EPR signals similar to those given by the wild type NR (Guigliarelli et al., 1992) were observed. In their oxidized state, both mutants gave a fast relaxing [3Fe-4S]$^{1+}$ signal centered at $g = 2.01$, identical to that of the wild type enzyme (Figures 2a and 3a). In both mutants, an axial Mo(V) signal at $g = 1.98$ and 1.96, which was strongly saturated at 100 mW, was superimposed on the [3Fe-4S]$^{1+}$ signal. In some preparations, an additional signal which likely arose from a weak amount of iron−nitrosyl complex (Guigliarelli et al., 1992) was visible at $g = 2.035$. This signal was strongly saturated at 100 mW and did not interfere with the analysis of the Fe-S spectra.

Upon progressive reduction with sodium dithionite, these two mutants showed distinct spectral behavior. For the $\alpha\beta$-(C26A) protein, the [3Fe-4S]$^{1+}$ signal decreased progressively with a midpoint potential of −40 mV (Figure 4a). This decrease was concomitant with the appearance of a rhombic signal at $g = 2.04$, 1.94, and 1.87 (Figure 2b), which was very similar to that given by the highest potential [4Fe-4S] cluster of the wild type NR, namely, center 1. For potentials more negative than −150 mV, the rhombic signal broadened progressively and a feature increased at $g = 1.92$, suggesting the reduction of other Fe-S clusters (Figure 2c,d). Below −300 mV, this broadening led to a slight decrease of the line amplitude. No significant change of the EPR spectrum was observed below −350 mV. Intensity measurements performed after subtraction of the Fe−NO signal

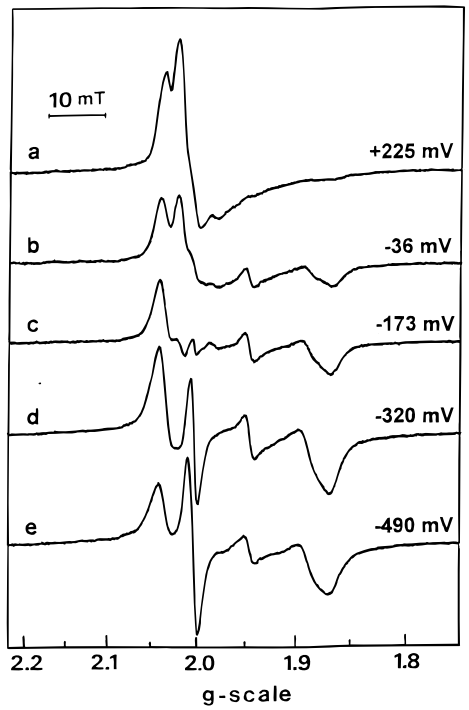

FIGURE 2: Representative EPR spectra obtained during the redox titration of the $\alpha\beta$(C26A) nitrate reductase. Experimental conditions: temperature, 12 K; microwave frequency, 9.305 GHz; microwave power, 100 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT.

contribution gave an intensity ratio of 1:2 between the fully oxidized and the fully dithionite-reduced forms of the mutated enzyme. If one considers that in the oxidized state the [3Fe-4S]$^{1+}$ signal corresponds to one spin per molecule, this indicates that only two [4Fe-4S] centers per molecule are reduced at −500 mV. This is in agreement with the amplitude variations of the $g = 1.87$ peak which are well fitted by two Nernstian curves centered at +30 and −220 mV, respectively (Figure 4a). By comparison with the wild type enzyme where the −500 mV EPR spectrum arises from three reduced [4Fe-4S] per molecule, these results suggest that the Cys26→Ala mutation induced either a significant downshift of the midpoint potential of one [4Fe-4S] cluster below −500 mV, or the removal of the cluster. In an attempt to detect an hypothetical very low potential Fe-S center, the mutant protein was photochemically reduced by light-activated 5-deazaflavin, which is known to generate electrons at very low potential, around −650 mV (Massey & Hemmerich, 1978). The photoreduction led to an EPR spectrum identical to that shown in Figure 1e, indicating that no Fe-S center characterized by a midpoint potential more negative than −500 mV is present in the $\alpha\beta$(C26A) mutant.

Table 5: Midpoint Potentials of the Iron−Sulfur Centers in the Wild-Type and Mutant Nitrate Reductase from *E. coli*

strains	midpoint potentials (mV)[a]				refs
	center 1 [4Fe-4S]	center 2 [3Fe-4S]	center 3 [4Fe-4S]	center 4 [4Fe-4S]	
MC4100	+60 (+80)[b]	+20 (+60)	−200	−400	Guigliarelli et al. (1992) Augier et al. (1993b)
LCB79/pVA7 + pVA50	+90	+20	ND	ND	Augier et al. (1993b)
LCB79/pVA7 + pVA50-C26A	+30	−40	−220	/	this work
LCB79/pVA7 + pVA50-C263A	/	−20	−150	−390	this work

[a] These midpoint potentials are those of the Nernstian curves which give the best fit with the experimental data. [b] The high quality of the experimental data obtained in the native enzyme allowed us to show that centers 1 and 2 are likely coupled by an anticooperative redox interaction. These values correspond to the microscopic redox potentials deduced from this analysis (Guigliarelli et al., 1992).

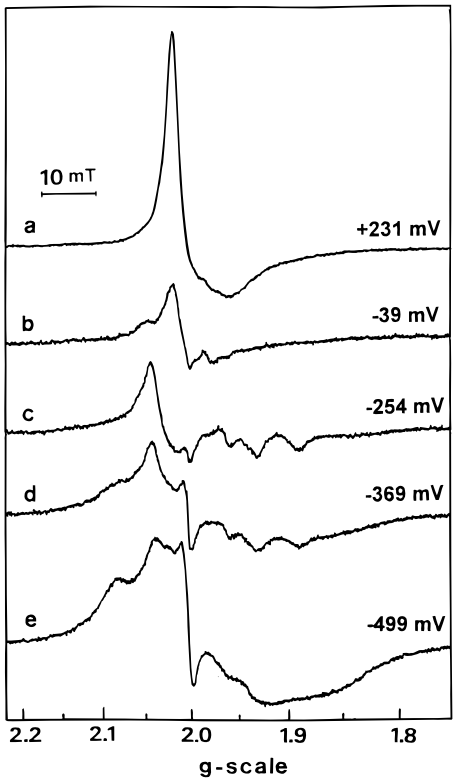

FIGURE 3: Representative EPR spectra obtained during the redox titration of the $\alpha\beta$(C263A) nitrate reductase. Experimental conditions were the same as for Figure 2.

For the $\alpha\beta$(C263A) mutated protein, the $g = 2.01$ signal disappeared above −100 mV, due to the reduction of the [3Fe-4S] cluster (Figure 3a,b). The variations of the amplitude of this signal measured at $g = 2.02$ as a function of the redox potential (Figure 4b) are well fitted by a Nernst curve centered at −20 mV. In contrast with the wild type enzyme (Guigliarelli et al., 1992) and with the $\alpha\beta$(C26A) mutant (Figure 2), no other Fe-S signal was observed for potentials higher than −100 mV. The weak signal visible in Figure 3b is due to a small amount of Fe−NO species. Below −100 mV, a rhombic signal with peaks at $g = 1.89$, 1.93, and around 2.05 appeared (Figure 3c). A sharp derivative like feature was also visible at $g = 1.96$. At low potentials, broad lateral lines increased at $g = 2.08$ and 1.84 (Figure 3d) and the rhombic signal was progressively replaced by a broad featureless spectrum (Figure 3e), likely arising from magnetically coupled $[4Fe\text{-}4S]^{1+}$ clusters and resembling that given by the fully reduced wild type NR (Guigliarelli et al., 1992). No change of this spectrum was observed upon photoreduction of the $\alpha\beta$(C263A) protein in the presence of 5-deazaflavin. The variations of the $[4Fe\text{-}4S]^{1+}$ signal amplitude measured at $g = 1.89$ show two reduction steps characterized by midpoint potentials equal to −150 and −390 mV, respectively (Figure 4b). This suggests the presence of only two [4Fe-4S] clusters per molecule in the mutated enzyme, which is confirmed by a ratio of about 1:2 between the EPR signal intensities measured in the fully oxidized and fully reduced states of this mutant. Taken together, these spectral and redox properties are closely similar to those exhibited by the $\alpha\beta$-(C16A), $\alpha\beta$(C19A), and $\alpha\beta$(C19S) mutants of nitrate reductase in which the highest potential [4Fe-4S] center was completely removed by the Cys16 or Cys19 replacement

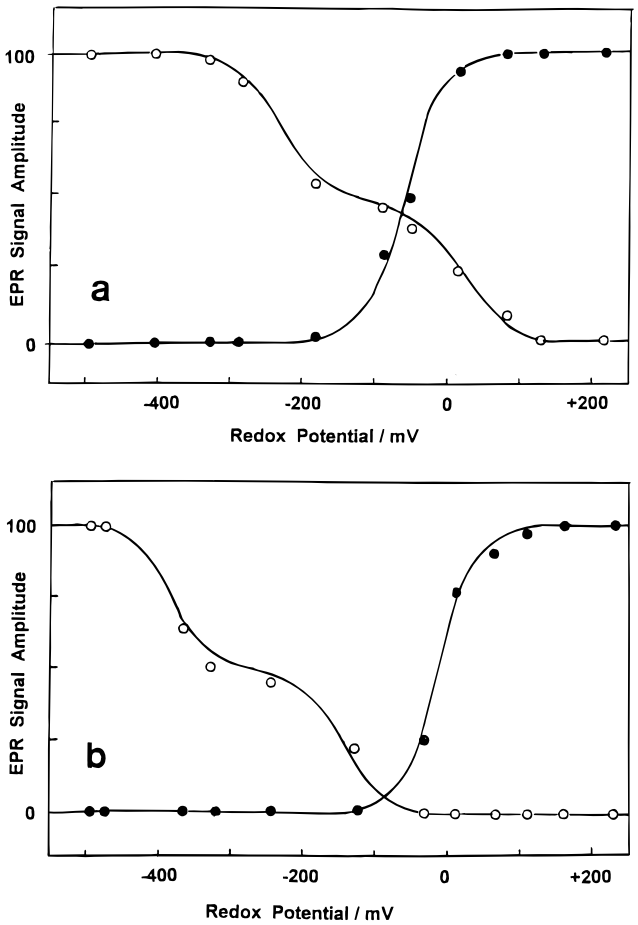

FIGURE 4: Normalized amplitude variations of the Fe-S signals as a function of the redox potential in mutated nitrate reductases: (a) $\alpha\beta$(C26A); (b) $\alpha\beta$(C263A). Experimental conditions as for Figure 2. Measurements were carried out at (●) $g = 2.02$ for the $[3Fe\text{-}4S]^{1+}$ signal and at (○) $g = 1.87$ (a) or $g = 1.89$ (b) for the $[4Fe\text{-}4S]^{1+}$ signals. Solid lines result from Nernst plot with the midpoint potentials listed in Table 5.

(Augier et al., 1993b). This indicates that center 1 of nitrate reductase is also missing in the $\alpha\beta$(C263A) mutant.

DISCUSSION

In a previous work, we have clearly established that the two first Cys of group I are ligands of the highest potential [4Fe-4S] center (center 1), and our results enabled us to propose a correspondence between each Fe-S cluster of the nitrate reductase and the first and second Cys of each of the four Cys groups present in the β subunit (Augier et al., 1993b). In the present work, the determination of the coordination of the four Fe-S centers and of their organization within NR was completed by successively replacing the fourth Cys residues of each Cys group of the β-subunit by Ala. For both Cys196 and Cys227, the Cys→Ala mutation gave inactive membrane-bound enzymes. This inactivation arose from the lack of the Mo-cofactor and of the Fe-S centers, which could not be inserted in the $\alpha\beta$* mutated complexes. These effects are identical to those previously observed for the Cys→Ala mutation of the first Cys residues of groups II and III (Augier et al., 1993a) and show that the Fe-S centers bound by these groups and/or the environment of these Cys residues have an important role in the enzyme stability. In contrast, the Cys26→Ala and Cys263→Ala mutations gave active enzymes carrying the Mo-cofactor and

Fe-S centers, but in both cases, EPR signal intensity measurements carried out on soluble NR showed that only three Fe-S clusters were present instead of four as in the native enzyme.

The $\alpha\beta$(C263A) mutant contained a [3Fe-4S] center characterized by an EPR signal and by a redox potential similar to those of center 2 of wild type NR. In contrast, center 1, the highest potential [4Fe-4S] cluster was not detected in this mutant. Below −100 mV, the two low-potential $[4Fe\text{-}4S]^{1+}$ clusters were observed with the same spectral and redox properties as in the native enzyme. These results are identical to those previously obtained in the study of the $\alpha\beta$(C16A), $\alpha\beta$(C19A), and $\alpha\beta$(C19S) mutants (Augier et al., 1993b) and show that Cys263 is a ligand of center 1. Thus, center 1 of NR, which is characterized by a unusually high redox potential (+80 mV), is bound to the β polypeptide chain by the first three Cys of group I (Cys 16, 19, and 22) and by the fourth Cys of group IV (Cys 263) in a coordination scheme identical to those found in ferredoxins.

The Cys26→Ala mutation had little effect on the high redox potential centers of nitrate reductase. Centers 1 and 2 were still present in the $\alpha\beta$(C26A) mutant, and their EPR characteristics were unchanged by comparison with the native enzyme. Their redox potentials are significantly lowered by the mutation but remain quite high for Fe-S centers. In contrast, below −200 mV, the Cys26→Ala mutation induces a strong effect on the redox behavior of the enzyme, which is interpreted in terms of the absence of one of the low-potential centers. Owing to the spin−spin interactions that develop between the reduced $[4Fe\text{-}4S]^{1+}$ clusters, the intrinsic EPR properties of centers 3 and 4 could not be completely determined in native NR (Guigliarelli et al., 1992), so the identification of the low potential cluster remaining in the C26A mutant is not straightforward. The spectral changes induced by the reduction of this low-potential cluster are very similar to those observed upon reduction of center 3 in the wild type enzyme, namely, the increase of a weak feature in the $g = 1.92$ region and the broadening of the lines of center 1, which were interpreted as arising from a weak magnetic coupling between centers 1 and 3 (Guigliarelli et al., 1992). Moreover, the midpoint potential of this center, −220 mV, is very close to that of center 3 in native NR. These results strongly suggest that center 3 remains coordinated in the mutant and that the lacking cluster is center 4. This is supported by the fact that the EPR lines of center 1 are still visible in the spectrum of the fully reduced $\alpha\beta$(C26A) mutant (Figure 2e), while they completely vanish upon reduction of center 4 when this center is present, due to intercenter spin−spin coupling (Guigliarelli et al., 1992; Augier et al., 1993a). Thus Cys26 is very likely the fourth Cys ligand of center 4.

The new data obtained in the present study on the ligands of the Fe-S clusters enable us to progress significantly in the knowledge of their organization within the enzyme. Taken together, the effects of the mutations carried out on the first (Augier et al., 1993a,b) and fourth Cys residues of each Cys group show clearly that the four Fe-S clusters of NR are coordinated by the four Cys groups of the β subunit. If we consider that the binding of all Fe-S centers is analogous to that found in bacterial ferredoxin, then two types of organization can be defined. Either the Fe-S clusters are coordinated *in pairs* in which two Fe-S centers are bound by only two Cys groups (see for instance the model of Figure 5a) or the coordination of two Fe-S clusters involves at least three Cys groups, which leads to an arrangement in which the four Cys groups and the four clusters are completely interconnected (see for instance Figure 5b). At this point, it is interesting to compare NR to the dimethyl sulfoxide reductase from *E. coli*, a molybdoenzyme closely related to NR and whose the DmsB subunit is very similar to the β subunit. DmsB contains four groups of four Cys residues and carries four [4Fe-4S] clusters with redox potentials ranging from −50 to −330 mV (Cammack & Weiner, 1990). The coordination of the four Fe-S centers in the DmsB subunit was tentatively described by Weiner et al. (1992) as resulting from a duplication of a 2 × [4Fe-4S] ferredoxin structure, with two Fe-S clusters bound by the first and the second Cys groups and the two other bound by the third and the fourth Cys groups of DmsB. The finding that center 1 of NR is bound by the Cys groups closest to the N- and C-terminus parts of the β polypeptide chain strongly restricts the number of possible coordination schemes and show clearly that the model proposed for dimethyl sulfoxide reductase is not convenient for NR.

In the β subunit, the group III of Cys contains only three Cys residues (Figure 1) and is very similar to the Cys motif which binds the [3Fe-4S] clusters in ferredoxins, where the second cysteinyl residue is either missing or not used for the iron coordination (Holm, 1992). This led us to propose that Cys217 and Cys223 of group III are ligands of center 2 in NR (Augier et al., 1993a), which is supported by the fact that in DmsB, the substitution of Cys102, the second Cys of the third Cys group, induced the transformation of a [4Fe-4S] center into a [3Fe-4S] cluster (Rothery & Weiner, 1991). If one takes into account the fact that Cys26 is a ligand of center 4, only two coordination models can then be proposed for the Fe-S centers in NR. They are shown in Figure 5. One of the major differences between them concerns the nature of the low-potential [4Fe-4S] cluster (center 3 or 4) bound by the first Cys residues of group II. In a previous work (Augier et al., 1993a), we have shown that the replacement of Cys184 of this group with Ser produced a mutated enzyme containing the four Fe-S clusters, and EPR studies concluded that Cys184 was a ligand of center 3. Therefore we propose that the four Fe-S clusters of NR are coordinated according to the *in pairs* model shown in Figure 5a, in which centers 1 and 4 are bound by groups I and IV and centers 2 and 3 are bound by groups II and III.

This model explains rather well the main characteristics of the intercenter magnetic interactions within NR. Centers 1 and 4, which are close together, are strongly coupled, as revealed by the replacement of the center 1 EPR lines by a complex interaction spectrum when center 4 becomes reduced. In contrast, the coupling is smaller between centers 1 and 3 which are more distant, and no magnetic interaction was observed between center 1 and 2 (Guigliarelli et al., 1992), which seem to be the farthest centers on the β polypeptide chain (Figure 5a). Centers 2 and 3 are not EPR detectable in the same potential range, but the broadening of the center 3 spectrum might arise from the spin−spin coupling with the reduced $[3Fe\text{-}4S]^{0}$ cluster which is paramagnetic ($S = 2$), since the two centers are maintained in a close proximity by groups II and III. Such a spectral broadening has also been observed in other enzymes containing reduced [3Fe-4S] cluster (Cammack, 1992). Finally, the notable interaction that is observed between centers 3 and 4

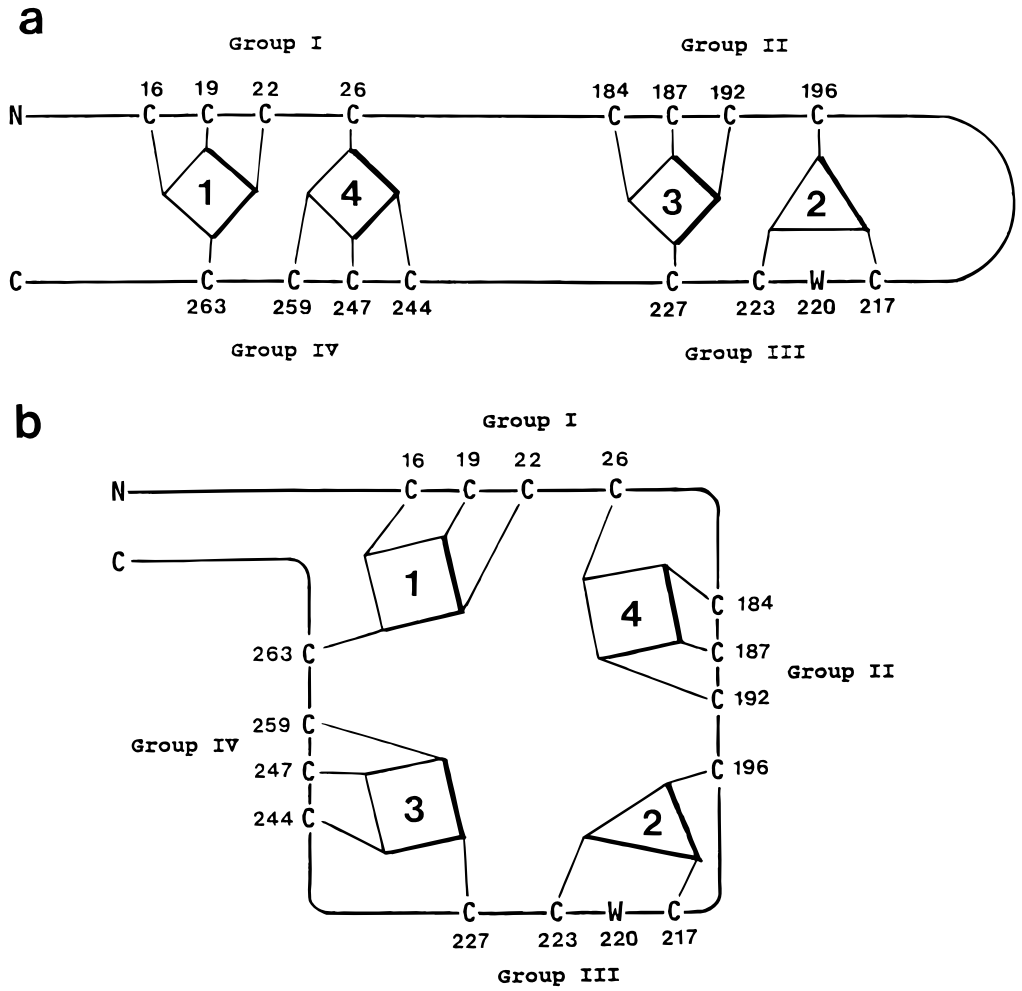

FIGURE 5: Proposed models for the coordination of the Fe-S centers in the β subunit of nitrate reductase: (a) *in pairs* model; (b) complete interconnection of the four clusters.

was not anticipated since these centers belong to different pairs, but it might result from a short intercenter distance induced by the folding of β.

Interestingly, we can see that the removal of center 1 in the C16A, C19A, C19S, and C263A mutants and of center 4 in the C26A mutant does not change greatly the spectral and redox properties of the three remaining Fe-S clusters, and does not affect their relative arrangement. This suggests that centers 1 and 4 does not play an important structural role in NR. Moreover, the results concerning the substitution of Ala for the ligands of center 4 (Figure 5a) show that it is important to distinguish the structural role of a Fe-S cluster and that of the Cys ligands. Thus, the replacement of Cys244 and Cys247 by Ala or Ser led to inactive enzymes, lacking all the metal centers (Augier et al., 1993a), which indicates that these residues are required to obtain a protein conformation allowing the insertion of other centers and leading to an enzymatic activity. For centers 2 and 3, the mutation of the first (Augier et al., 1993a) as well as the fourth Cys ligands induces the loss of all metal centers, which emphasizes the strong structural role of these Fe-S clusters and/or of their Cys ligands in the NR folding. These observations suggest the presence of two polypeptide regions within the β subunit having different behavior upon amino acid replacements, which is consistent with the *in pairs* coordination model we propose for the Fe-S centers (Figure 5a). Thus, the pair of centers 1 and 4 is likely located in a polypeptide domain allowing significant structural changes, whereas centers 2 and 3 are bound by a region where few conformational changes are allowed, and which is probably strongly constrained as indicated by the impossibility of converting the [3Fe-4S] cluster into a [4Fe-4S] cluster when Trp220 is replaced by Cys (Augier et al., 1993a).

It is worth noting that in each pair of centers defined in the model shown in Figure 5a a low-potential cluster and a high-potential cluster are associated. The very negative values of the redox potentials of centers 3 and 4, −200 and −400 mV respectively, apparently precludes their involvement in the electron exchange between quinols and nitrate, which raises the question of the role of these Fe-S centers in the enzyme. Such a question is not restricted to NR since a number of respiratory enzymes or electron transfer proteins have been found to contain centers with redox potentials out of the range defined by the electron donor and acceptor of the system. This situation is encountered, for instance, in dimethyl sulfoxide reductase (Cammack & Weiner, 1990), fumarate reductase (Hederstedt & Ohnishi, 1992), nickel-containing hydrogenase (Volbeda et al., 1995), and cytochrome c_3 (Bertrand et al., 1994).

In NR, the quinol-nitrate reductase activities of the mutated enzymes we obtained are differently affected, depending on the Fe-S cluster, with high or low redox potential, that is lost. Thus, in the β(C26A) mutant, both duroquinol and menaquinol-nitrate reductase activities are significantly and equally affected (Table 3), but the removal of center 4 does not preclude enzymatic activity. In contrast, the β(C263A)

mutant and the other mutants lacking center 1, β(C16A) and β(C19A) (data not shown), cannot accept electrons from menaquinols, which are, however, preferentially used by the wild type enzyme. Strikingly, the cytochromes *b* of these mutants, which are completely reduced by the two types of quinols, cannot be reoxidized by nitrate when the electron donor is menaquinol. Assuming that only the high-potential centers are involved in the electron pathway, the nitrate reductase activity of mutants devoid of center 1 results from an electron transfer through the high-potential cluster of the other pair, namely, center 2. Moreover, these results indicate that center 1 is an obligatory intermediate in the electron transfer from menaquinols to nitrate and suggest the presence of parallel electron pathways in the enzyme through the two pairs of clusters 1-4 and 2-3.

The preparation of mutants lacking centers 2 or 3, or in which the redox or spectral properties of these centers are selectively modified would be useful to confirm the sharing out of the four Fe-S clusters in two pairs and to specify the electronic pathway within the nitrate reductase complex. Site-directed mutagenesis experiments are in progress in this aim.

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