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Identification of amino acid residues associated with the [2Fe-2S] cluster of the 25 kDa (NQO2) subunit of the proton-translocating NADH-quinone oxidoreductase of *Paracoccus denitrificans*

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Abstract In order to identify the ligand residues of the [2Fe-2S] cluster of the 25 kDa (NQO2) subunit of the proton-translocating NADH-quinone oxidoreductase of *Paracoccus denitrificans*, we mutated individually all seven cysteine residues (C61, C96, C101, C104, C113, C137, and C141) and one conserved histidine residue (H92) to Ser or Ala and expressed them in *E. coli*. After purification of the mutated 25 kDa subunits, the effect of mutations on the iron-sulfur cluster were characterized by chemical analyses and UV-visible and EPR spectroscopy. All mutated subunits, especially mutants of conserved cysteines, contained lower amounts of non-heme iron than wild-type. The subunits of three non-conserved cysteine residues (C61, C104, and C113) mutated to Ser and a histidine residue (H92) mutated to Ala exhibited essentially the same spectroscopic properties as those of the wild-type subunit. In contrast, mutation of the four conserved cysteine residues (C96, C101, C137, and C141) to Ser or Ala considerably altered the UV-visible and EPR spectra from the wild-type subunit. These results indicate that the four conserved cysteine residues coordinate the [2Fe-2S] cluster in the *P. denitrificans* 25 kDa subunit.

Key words: Iron-sulfur cluster; NADH-quinone oxidoreductase; Expression; Site-directed mutation; *Paracoccus denitrificans*

1. Introduction

Paracoccus denitrificans is a Gram-negative soil bacterium and has been called 'a free-living mitochondrion' [1–3]. When grown aerobically, *P. denitrificans* bears the proton-translocating NADH-quinone oxidoreductase (NDH-1) [4,5]. The *P. denitrificans* NDH-1 appears to be composed of 14 dissimilar subunits [5] and contains non-covalently bound FMN [6] and at least 5 EPR-visible iron-sulfur clusters [7]. The structural genes encoding the subunits of the *P. denitrificans* NDH-1 constitute a single gene cluster [8–12] designated the *NQO1–14* genes [13]. By comparing the deducted primary structures of the *P. denitrificans* NDH-1 subunits against the known consensus sequences of the iron-sulfur clusters, and EPR analysis of the resolved bovine heart complex I fractions, it was possible to speculate as to which NDH-1 subunits bind iron-sulfur clusters [14–16]: the NQO1, 2, 3, 6, and 9 subunits are hypothesized to be associated with the iron-sulfur clusters [17]. It is important to clarify whether these subunits actually ligate the iron-sulfur clusters and, if that is the case, to determine the amino acid residues coordinating the iron-sulfur clusters in individual subunits. Two approaches appear to be applicable for this purpose. One is resolution of the NDH-1. The other is to express the gene encoding each subunit. As discussed in the previous paper [18], the latter approach appears superior to the former approach. However, host cells should be equipped with the machinery required for incorporation of the iron-sulfur clusters and native structural folding of the subunits. Therefore, we attempted to express genes encoding putative subunits bearing the iron-sulfur clusters of the *P. denitrificans* NDH-1 in *Escherichia coli* containing the NDH-1 enzyme [18–20].

In previous studies [18,21] it was shown that the expressed 25 kDa (NQO2) subunit carries a single binuclear cluster which is probably coordinated by only cysteine residues. In the present paper, utilizing site-directed mutagenesis, we have shown that the [2Fe-2S] cluster of the 25 kDa subunit is coordinated by C96, C101, C137, and C141.

2. Materials and methods

Site-directed mutagenesis was performed using the Bio-Rad in vitro mutagenesis kit based on the method of Kunkel et al. [22]. Twelve oligonucleotides were synthesized for use in mutagenesis as follows:

1) CATCGAATATTCTGCCGACCTTC for the C61S mutation
2) CTCGGTCGCGGCTATCCAGATC for the H92A mutation
3) TCCAGATCAGCGGCACCACG for the C96S mutation
4) TATCCAGATCGCCGGCACCACG for the C96A mutation
5) CACCACGACCAGCATGATCTG for the C101S mutation
6) CACCACGACCGCCATGATCTG for the C101A mutation
7) CTGCATGATCAGCGGCGCCGAG for the C104S mutation
8) GATCCGGGTCAGCAAGGAAAAG for the C113S mutation
9) GAGGTCGAAAGCTTGGCG for the C137S mutation
10) GAGGTCGAAGCCTTGGCGC for the C137A mutation
11) CTTGGCGCCAGCACCAACGC for the C141S mutation
12) CTTGGCGCCGCCACCAACGC for the C141A mutation.

The underlined bases were those altered from the *P. denitrificans* DNA by the mutation.

As described previously [18], pTZ19(NQO2NdeI) was used as the template DNA for site-directed mutagenesis. Mutations were confirmed by dideoxy sequencing [23]. The plasmids carrying the mutations were digested with *Nde*I and *Bam*HI and the 1 kb DNA fragments of interest were subcloned into pET16b vector. These final constructs were verified by DNA sequencing of both strands. Expression and purification of these mutated 25 kDa subunits were carried out according to

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Abbreviations: NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase; [2Fe-2S], binuclear iron-sulfur cluster; Complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism.

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Yano et al. [18]. The mutated subunits all reacted with the antibody to the *P. denitrificans* 25 kDa subunit. Protein concentration was estimated by the method of Lowry et al. [24]. Non-heme iron was determined according to Doeg and Ziegler [25]. Immunoblotting experiments were carried out as described previously [26,27]. Any variations from the procedures and details are described in the figure legends.

3. Results

The expressed *P. denitrificans* 25 kDa subunit bears a single [2Fe-2S] cluster which exhibits a rhombic EPR signal with g values of $g_{x,y,z}$ = 1.92, 1.95, and 2.00 (g_{av} = 1.96) and a midpoint redox potential of −456 mV [18]. Furthermore, the resonance Raman and MCD studies of the expressed *P. denitrificans* 25 kDa subunit suggest that the iron-sulfur cluster is coordinated by only cysteine residues [21]. On the basis of the deduced amino acid sequence [9], the *P. denitrificans* 25 kDa subunit contains 7 cysteine residues (C61, C96, C101, C104, C113, C137, and C141): four of them (C96, C101, C137, and C141) are conserved among its homologues [9]. In order to verify the cysteine coordination of the [2Fe-2S] cluster, these residues have been mutated to serine or to alanine. The sequence analyses also show that the *P. denitrificans* 25 kDa subunit retains a single conserved histidine residue (H92). Except for cysteine, only histidine residues have been currently shown to coordinate [2Fe-2S] clusters in the native protein, as exemplified by Rieske iron-sulfur protein [28,29]. Therefore, in order to prove the expectation that only cysteine residues are involved in coordination of the [2Fe-2S] cluster of the 25 kDa subunit, we also replaced H92 with Ala. The mutated subunit protein was expressed in *E. coli* and purified as described previously [18]. The purified mutated subunits were subjected to chemical analysis of non-heme iron content and UV-visible and EPR spectroscopy.

The mutations did not affect the amount of protein expressed in *E. coli* on the basis of SDS-gel analysis. As Table 1 shows, however, the mutated subunit proteins contained considerably lower amounts of non-heme iron than the wild-type subunit. In C61S, C104S, C113S, and H92A mutants, the non-heme iron content decreased to 37–62% of the wild-type. Moreover, C96S, C96A, C101S, C101A, C137S, C137A, C141S, and C141A mutants contained only 12–27% of non-heme iron found in wild-type. The concentrations of the EPR-detectable iron-sulfur clusters in these mutants were determined based on spin quantitation of their EPR signals after reduction with dithionite at pH 8.0, and are compared with the value of the wild-type subunit. The non-heme iron content of these mutated subunits estimated from the spin quantitation are significantly lower than the iron contents determined by chemical analyses, whereas in the wild-type, all non-heme iron was detected as EPR-visible iron-sulfur clusters (Table 1). The decrease in cluster content was particularly remarkable in C96S, C96A, C101S, C101A, C137S, C137A, C141S, and C141A mutants. The disparity between the cluster content in the mutated proteins determined by these two independent methods may arise from the following two reasons: (i) expressed mutated subunits contain EPR-undetectable iron; (ii) the midpoint redox potentials of iron-sulfur clusters of these mutated subunits shift to an extremely low value and are thus not reducible under the experimental conditions used. These questions remain to be further clarified.

Table 1
Comparison of non-heme iron content between wild-type and mutated 25 kDa subunits

25 kDa subunit	Content of non-heme iron (nmol/mg protein)	
	Chemically determined	Based on spin quantitation
Wild-type	33.3	33.4
C61S	20.5	8.8
C104S	13.2	10.0
C113S	12.8	3.2
H92A	12.3	1.14
C96S	4.2	0.86
C96A	6.3	1.12
C101S	4.0	1.62
C101A	5.1	1.48
C137S	9.1	0.64
C137A	5.4	0.82
C141S	5.1	1.12
C141A	7.4	1.50

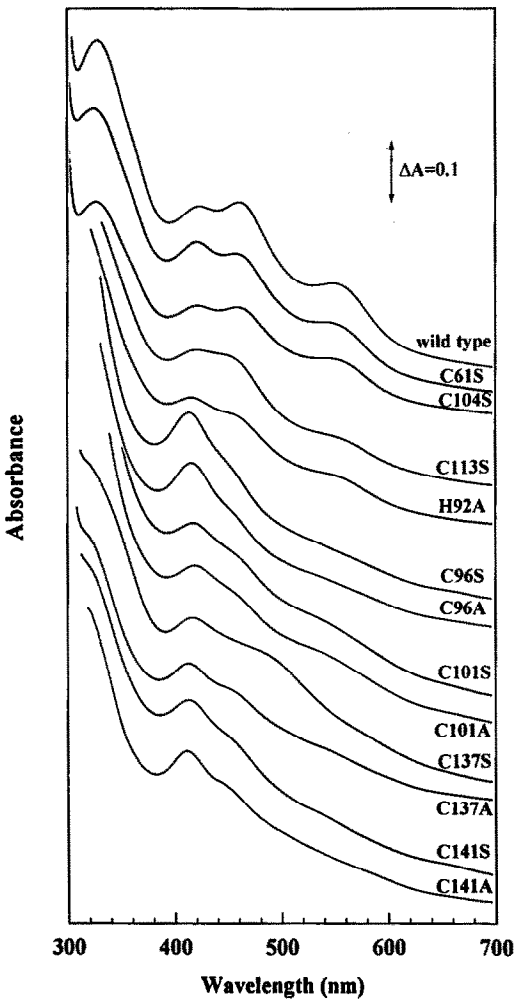

Fig. 1. UV-visible absorption spectra of wild-type and mutated 25 kDa (NQO2) subunits. The spectra were measured at room temperature using an SLM-Aminco DW-2000 spectrophotometer. The isolated subunits were diluted with 10 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol to 600 μM of the non-heme iron.

Fig. 1 shows the absorption spectra of the mutated 25 kDa subunit. The wild-type 25 kDa subunit shows the absorption

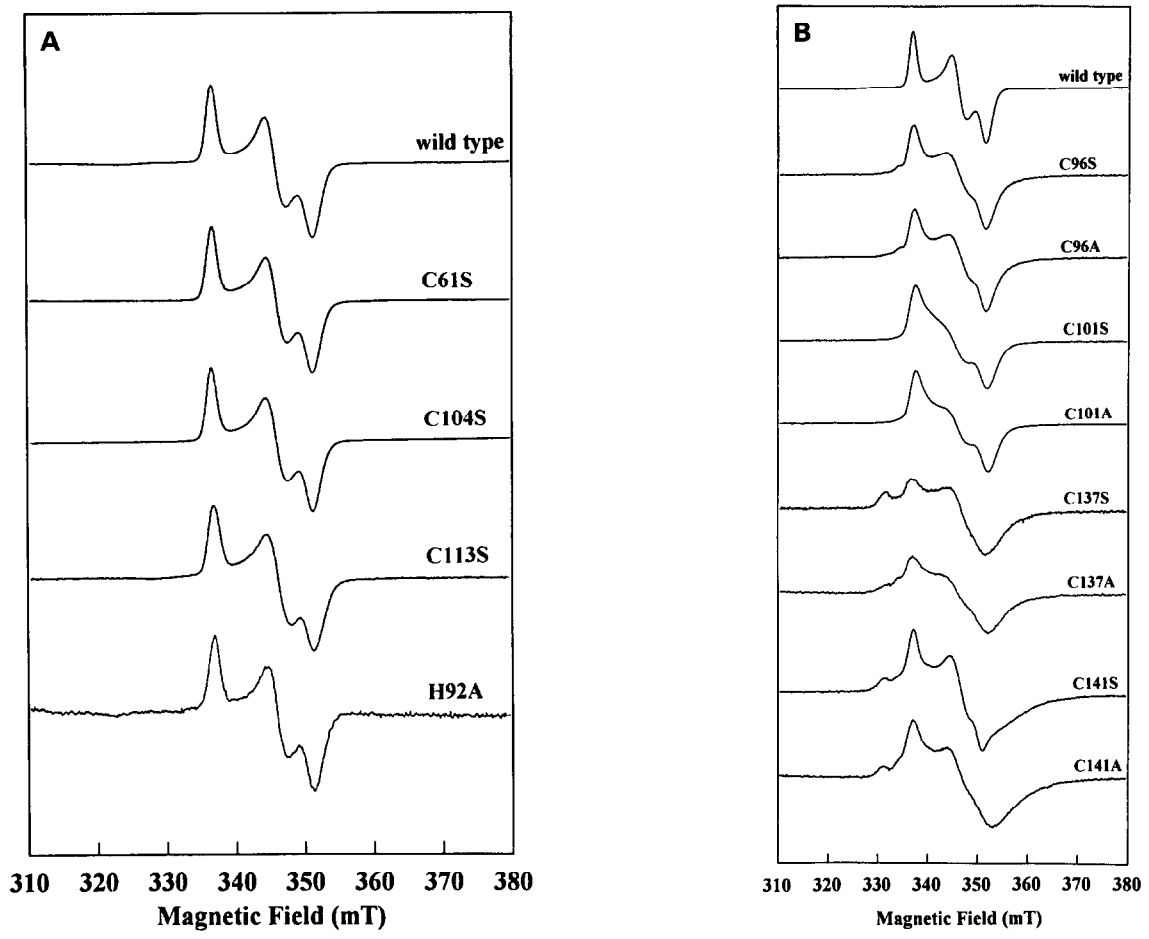

Fig. 2. EPR spectra of wild-type and mutated 25 kDa (NQO2) subunits of the *P. denitrificans* NDH-1. (A) Wild type (12 mg/ml), C61S (23.5 mg/ml), C104S (81 mg/ml), C113S (19.5 mg/ml), and H92A (22 mg/ml). (B) C96S (80 mg/ml), C96A (75 mg/ml), C101S (118 mg/ml), C101A (99.5 mg/ml), C137S (59.5 mg/ml), C137A (87.5 mg/ml), C141S (101 mg/ml), and C141A (85 mg/ml). The samples were reduced with 10 mM dithionite. EPR spectra were recorded under the following conditions: microwave frequency 9.44 GHz, microwave power 2 mW, modulation amplitude 1 mT, time constant 0.164 s, sample temperature 30 K. The spin concentrations of the clusters of wild-type and mutated 25 kDa subunits were determined using 0.5 mM Cu(II)-EDTA as standard.

peaks at 325, 425, 460, and 550 nm in the oxidized form. Four of the mutated subunits (C61S, C104S, C113S, and H92A) exhibit almost the same absorption spectra as the wild-type subunit, except for the slight blue-shift observed in the peak at 460 nm to 455 nm in the C113S mutated subunit. As shown in Fig. 2A, C61S, C104S, C113S and H92A mutated subunits also demonstrate identical EPR spectra to that of the wild-type subunit ($g_{x,y,z}$ = 1.92, 1.95, and 2.00). These results indicate that the substitutions of non-conserved cysteines (C61, C104, and C113) and of a conserved histidine residue (H92) scarcely affects the spectroscopic properties of the [2Fe-2S] cluster of the wild-type subunit.

In contrast, when the four conserved cysteines C96, C101, C137, and C141 were mutated individually to Ser or to Ala residues, the absorption spectra of these mutated subunits were considerably altered (see Fig. 1). A major absorption peak at 415 nm was commonly observed and the broad peak at 550 nm seen in the wild-type subunit was diminished or broadened in all 8 mutated subunits. Furthermore, a peak at 460 nm in the wild-type was seen as a small shoulder in the mutated subunits, except for the C137S mutated subunit which exhibited a broad shoulder at 490 nm. Significant changes were also observed in the EPR spectra of these mutated subunits (Fig. 2B). Although the EPR signals of these mutated subunits showed g-values close to those of the wild-type subunit, their line widths became much broader and with discernible additional signals (Fig. 2B). Taken together with the fact that these four cysteine residues are conserved among their counterparts in other organisms [30–35], it can be concluded that the four conserved cysteine residues, C96, C101, C137, and C141, are involved in coordinating the [2Fe-2S] cluster to the 25 kDa subunit protein.

The probabilities of non-cysteinyl ligand to [2Fe-2S] clusters in site-directed mutated proteins have been reported so far: [2Fe-2S] cluster in *E. coli* fumarate reductase [36], *Clostridium pasteurianum* [2Fe-2S] ferredoxin [37], and *Anabaena variabilis* 7120 vegetative ferredoxin [38]. The oxygen ligand from the constructed serine seems to coordinate the iron-sulfur cluster, because the g_{av} values of C→S mutants were shifted from 1.96 to 1.88–1.92. These g_{av} shifts observed in C→S mutants may be interpreted in terms of the ligand-field model based on the results of spectroscopic studies of synthetic compounds of [2Fe-2S] cluster [39,40]. However, such drastic g_{av} shifts were not observed in any of the EPR spectra of the *P. denitrificans* C96S, C101S, C137S, and C141S mutated subunits (Fig. 2B). In order

to confirm whether the constructed Ser coordinated the iron-sulfur cluster, we constructed Ala mutants C96A, C101A, C137A, and C141A, because replacement by Ala excludes any possibility that the substituted amino acid residues are involved in coordination of the [2Fe-2S] cluster. The UV-visible and EPR measurements of these Ala mutants revealed that all four Ala mutants (C96A, C101A, C137A and C141A) were almost identical to the Ser mutants (C96S, C101S, C137S, and C141S, respectively) (Figs. 1 and 2B). These results strongly support the above speculation that the constructed Ser does not ligate to the [2Fe-2S] clusters in these Ser mutated subunits. Rather, it seems likely that these Ser and Ala mutated subunits bear new [2Fe-2S] clusters which are coordinated by other recruited amino acid residues, most likely non-conserved cysteines located in their proximity. Moreover, heterogeneity of iron-sulfur clusters was clearly observed in C96, C137, and C141 mutated subunits. A small signal (g_z = 2.02) is detected in the C96S and C96A mutated subunits. For instance, the power saturation study of the C141S mutated subunit revealed that there are at least two populations of iron-sulfur clusters: one exhibits EPR signals with g-values of $g_{x,y,z}$ = 1.92, 1.95, and 2.00 (g_{av} = 1.96) and a half saturation parameter ($P_{1/2}$) of 9 mW at 25 K. The other one shows g-values of $g_{x,y,z}$ = 1.93, 1.95, and 2.04 (g_{av} = 1.97) and $P_{1/2}$ = 26 mW. The relative ratio of these different species may vary depending on preparation.

4. Discussion

In the present paper we have shown that four conserved cysteine residues (C96, C101, C137, and C141) ligate the [2Fe-2S] cluster of the *P. denitrificans* 25 kDa subunit. All of the results presented herein are consistent with previous data of the wild-type 25 kDa subunit obtained by UV-visible, resonance Raman, MCD spectroscopy, which predicted the coordination of the iron-sulfur cluster by only cysteine residues [21]. The arrangement of the four cysteine residues of the 25 kDa subunit shows the novel binding motif, $Cx_4Cx_{25}Cx_3C$, whereas the $Cx_4Cx_2Cx_{29}C$ motif is observed in all plant-type [2Fe-2S] ferredoxins, and the $Cx_5Cx_2Cx_{36/37}C$ motifs are found in hydroxylase-type ferredoxins [41]. As previously reported, the spectroscopic properties of the *P. denitrificans* 25 kDa subunit are very similar to those of [2Fe-2S] ferredoxin of *Clostridium pasteurianum* [21,41]. The *C. pasteurianum* [2Fe-2S] ferredoxin contains five cysteine residues, C11, C14, C24, C56, and C60, which belong to a unique subclass of the [2Fe-2S] ferredoxin family [41–43]. Unfortunately, no homologues of this subclass have been found to date, and even the ligand residues of the [2Fe-2S] cluster in *C. pasteurianum* ferredoxin are not completely identified. According to Crouse et al. [21], comparison of the cysteine arrangements between the *P. denitrificans* 25 kDa subunit and the *C. pasteurianum* [2Fe-2S] ferredoxin may suggest that C96, C101, C137, and C141 of the 25 kDa subunit correspond to C11, C14, C56, and C60, respectively, of the *C. pasteurianum* ferredoxin. Recently, Meyer and co-workers demonstrated that C56 and C60 are involved in the cluster ligation on the basis of results of site-directed mutation (C→S) studies [37]. Further experiments to identify two other ligand residues in the *C. pasteurianum* ferredoxin are in progress (J. Meyer, personal communication).

Our results suggest that substitution of these ligand residues of the [2Fe-2S] cluster by Ser or Ala (Table 1, Fig. 2B) leads (at least in a fraction of protein molecules) to the formation of new [2Fe-2S] clusters, probably coordinated by non-conserved cysteine residue(s) of the 25 kDa subunit. It seems likely that the ligation of iron to non-conserved cysteine residues rather than to the constructed Ser is preferred by these mutated 25 kDa subunits. This view may imply that the conformation of the 25 kDa subunit is so flexible as to allow the rearrangement of cluster coordination, as seen in *Azotobacter vinelandii* FdI C20A mutation [44]. Presumably, not only the polypeptide structure surrounding the iron-sulfur cluster (its unique cysteine arrangement) but also the fact that the subunit is expressed solely in the absence of the neighboring subunits in situ may potentially contribute to the flexibility of the 25 kDa subunit. It should be noted that the structure of the *P. denitrificans* 25 kDa subunit is further stabilized by assembly with the NQO1 subunit in the *P. denitrificans* NDH-1 enzyme complex (Yano et al., unpublished results).

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