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Identification of amino acids responsible for the oxygen sensitivity of ferredoxins from *Anabaena variabilis* using site-directed mutagenesis

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Abstract

The filamentous cyanobacterium *Anabaena variabilis* (ATCC 29413) possesses two molybdenum dependent nitrogenase systems, *nif1* and *nif2*. The *nif1* system is regulated by a developmental program involving heterocyst differentiation; the *nif2* system is expressed in all cells only under anaerobic conditions and the expression is controlled environmentally. The genes *fdxH1* and *fdxH2*, encoding two [2Fe–2S] ferredoxins, are part of the these two distinct and differently regulated *nif* gene clusters. The sensitivity of both ferredoxins to oxygen was different; the half-life of FdxH2 in air was only $\approx$1.5 h, while FdxH1 retained 80% of its nitrogenase activity after 24 h. We used site-directed mutagenesis to identify the role of individual amino acid residues responsible for oxygen sensitivity and found out that the FdxH2 double mutant I76A/V77L was much more resistant to oxygen than the wild-type ferredoxin (FdxH2) and similar to FdxH1. By modelling it was shown that the accessibility of the cavity around the iron–sulfur cluster was responsible for that.

1. Introduction

Cyanobacterial, plant-type [2Fe–2S] ferredoxins are small, acidic, iron–sulfur proteins that are widely distributed in Nature. They function as one electron carriers with a low (−400 mV) reduction/oxidation potential. Ferredoxins couple a number of reductive biosynthetic pathways to the photosynthetic electron transport chain. These are processes of global importance, such as CO_2-fixation, nitrogen assimilation and nitrogen fixation.

We discovered in nitrogen fixing strains of cyanobacteria two functionally specialized plant-type ferredoxins: PetF, which participates in electron flow through Photosystem I and a second ferredoxin, FdxH, with only 51% of sequence identity to PetF, which interacts specifically with nitrogenase reductase; the *fdxH* gene is coinduced with the nitrogenase system and the protein is synthesized only under nitrogen fixing conditions in heterocysts [1]. As shown by X-ray crystallographic analysis of ferredoxin, both iron atoms of the cluster are tetrahedrally coordinated by four cysteinyl-*S*-residues (Cys41, Cys46, Cys49 and Cys79) and two bridging inorganic sulfides to form a planar ring [2]. This redox center is bound near the surface of the protein with one edge of the iron–sulfur cluster more exposed. All high resolution structures of ferredoxin, including that of FdxH exhibit the same global folding pattern [3]. An

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additional feature of FdxH-type ferredoxins are two conserved lysine residues at positions 10 and 11, which are part of a flexible loop structure [4]. These residues seem to be necessary for specific interaction with nitrogenase reductase in vitro, as shown by site-directed mutagenesis [5].

To perform oxygen sensitive nitrogen fixation in the light simultaneously with oxygenic photosynthesis, many filamentous strains have developed a strategy of spatial separation. After nitrogen depletion, $\approx$7% of the vegetative cells, called heterocysts, are differentiated at semiregular intervals along a filament. Heterocysts, which lack oxygenic photosynthesis, are the sites of nitrogen fixation under external aerobic conditions. The *nif* genes of *Anabaena* sp. PCC 7120 (= *Anabaena* 7120), that are genes necessary for nitrogen fixation, are exclusively expressed in heterocysts and linked to the developmental program of differentiation.

However, the localization and regulation of this process in filamentous, heterocyst-forming cyanobacteria under anaerobic growth conditions has remained unclear. In studies more than 10 years ago with the heterocystous cyanobacterium *A. variabilis* (ATCC 29413), Hirschberg and coworkers [6,7] detected *nifHD* transcripts, encoding part of the structural genes of the nitrogenase system, already 2–3 h after onset of nitrogen starvation under anaerobic conditions, followed by nitrogenase activity 1 h later. This is about 10 h before the heterocyst development has started and aerobic nitrogenase activity commenced. The experiments suggested an environmental control of cyanobacterial nitrogen fixation. The lack of both oxygen and combined nitrogen in the environment was sufficient to induce anaerobic nitrogenase activity in *A. variabilis* [8,9].

Gene amplification by the polymerase chain reaction (PCR) with degenerate, *fdxH*-specific primers, led to the identification of two different *fdxH* genes in *A. variabilis*, *fdxH1* and *fdxH2* [8]. The *fdxH1* and *fdxH2* gene regions are part of two distinct and differently regulated *nif* gene clusters, *nif1* and *nif2*, both encoding Mo-dependent nitrogenase systems [9].

The *nif2* system of *A. variabilis* is immediately induced under anaerobic conditions in all cells, independent of heterocyst differentiation; it also lacks the *nifD*-element, which involves an 11 kb DNA rearrangement in the *nifHDK* region of heterocysts. A comparison of the *fdxH* gene regions from different filamentous nitrogen fixing cyanobacteria shows that the overall gene arrangement is similar in all cases, except that the anaerobic systems contain downstream an *fdxB* gene of unknown function.

An alignment of the derived amino acid sequences of FdxH-type ferredoxins shows that FdxH1 of *A. variabilis* is very similar to FdxH from *Anabaena* 7120 (95% identity); FdxH represents the ferredoxin originally identified in isolated heterocysts from *Anabaena* 7120. However, FdxH2 shares more residues with the oxygen sensitive FdxH from the non-heterocystous, anaerobically nitrogen fixing cyanobacterium, *Plectonema boryanum*, than with FdxH1 from the same cell [8,10]. The two proteins, FdxH1 and FdxH2, were 76% identical regarding their amino acid sequence, but there was little difference in their ability to transfer electrons to the nitrogenase system (derived from heterocysts) of *A. variabilis*.

Their sensitivity to oxygen, however, was different. By measuring the destruction of the iron–sulfur cluster by the absorbance decrease at 420 nm of aerobically incubated ferredoxins, the half-life of FdxH2 in air was only $\approx$1.5 h, while FdxH1 was comparatively stable, retaining approx. 80% of its absorbance after 24 h [8]. Some amino acid exchanges in FdxH1-type ferredoxins have obviously evolved to provide a higher oxygen tolerance that may be beneficial for nitrogen fixation within heterocysts compared to the drastic oxygen sensitivity of FdxH2. We used site-directed mutagenesis to identify the role of amino acids responsible for the oxygen sensitivity of this metalloprotein.

2. Materials and methods

2.1. Culture conditions and DNA manipulations

A. variabilis (ATCC 29413) was grown in glass tubes containing 200 ml Arnon medium without combined nitrogen. The cultures were bubbled with air enriched in 1% (v/v) CO_2 and illuminated with white fluorescent light (100 μE m^{-2} s^{-1}) at 30°C [11].

Escherichia coli strain MC 1061 [12] was used for propagation of plasmids as well as expression of recombinant *A. variabilis* ferredoxins. For selection of

plasmids, all strains were grown in Luria–Bertani medium containing 100 μg/ml ampicillin.

All DNA manipulations were performed following standard procedures [13]. The *fdxH1* and *fdxH2* genes from *A. variabilis* were isolated as described [8]. A 800 bp *Eco*RI/*Hpa*I DNA subfragment from pEKA8.2 carrying the *A. variabilis fdxH2* gene was subcloned into pUC18 resulting a hybrid plasmid pBBS1. Similarly, a 600 bp *Eco*RI DNA subfragment carrying *fdxH1* gene was subcloned into pUC18 resulting in pUN600. Both plasmids were subjected to protein overexpression using the *lacZ*-promoter. The yield of ferredoxin was up to 15 mg/l of *E. coli* culture. For the expression of *Anabaena* 7120 FdxH clone pAn321 was taken [14].

2.2. Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out following the unique site elimination method [15]. For this purpose both plasmids carrying the complete *fdxH1* and *fdxH2* gene were cloned into pUC19 with *lacZ*-promoter reading in the opposite direction to the ferredoxin gene, resulting in pBBS2 and pBBS3. Two oligonucleotides were used in this method, one which eliminates a non-essential *Nde*I restriction site called selection primer; the other primer led to desired mutation in the plasmid. The mutation was identified by the Sanger didesoxy chain termination sequencing method [13].

Chimeric proteins were formed by introducing into the nucleotide sequence of the *fdxH1* gene a *Bcl*I restriction site and eliminating one *Bcl*I-site nearby without alteration of the amino acid sequences. Both the genes were digested with *Bcl*I, ligated and the corresponding proteins were overexpressed in *E. coli*. The N-terminal amino acids (1–58) from FdxH1 were fused to the C-terminal amino acids (59–98) of FdxH2 and vice versa, resulting in chimeric protein I and II, respectively.

2.3. Purification of recombinant ferredoxins

Recombinant FdxH1 and FdxH2 were prepared as described [14]. *E. coli* cells, grown overnight, containing recombinant ferredoxin were washed in 20 mM Tris/1 mM EDTA buffer (pH 8.0), and broken in a precooled French pressure cell flushed with argon. Purification of the crude protein extract was achieved through anion-exchange chromatography on a Q-Sepharose column using a NaCl gradient (0–1 M NaCl). Samples having the absorbance ratio A_{422}/A_{276} more than 0.5 were collected and were used for the study. Whenever required, further purification was carried out using native preparative polyacrylamide gel electrophoresis (Biorad, 17% acrylamide/1.4% bisacylamide) and also by anion exchange chromatography Mono-Q column (Pharmacia), which was eluted with a 0–0.4 M NaCl gradient. Preparation of FdxH2 required an oxygen free tent in which all FdxH2 purification procedures were performed. The recombinant proteins were stored in liquid nitrogen.

2.4. Oxygen sensitivity test

Ferredoxins were dissolved in 20 mM Tris–HCl, 400 mM NaCl (pH 8.0) at an absorbance A_{422} of 0.7 [8]. Samples (2 ml) were incubated under air in a water-bath at 30°C in 8-ml vials. Control samples (2 ml) were flushed with argon for 10 min. At regular time intervals, the absorbance of the samples was determined spectrophotometrically at 422 nm.

2.5. Enzyme assays

The ferredoxin-dependent NADPH/cytochrome *c*-reduction and $NADP^+$-photoreduction assays were performed as described [16]. The modified reaction mixture for cytochrome *c* reduction contained 40 mM NaCl in addition to 4 nM recombinant FNR protein. The increase in absorbance of cytochrome *c* was observed at 550 nm. $NADP^+$-photoreduction was followed by the increase of absorbance at 340 nm.

Reconstitution of cyanobacterial nitrogenase activity with purified recombinant ferredoxins was performed with anaerobically prepared heterocyst homogenates from *A. variabilis* as described in [17]. The final volume of 200 μl contained 20 mM Hepes–NaOH (pH 7.5), 2.5 mM glucose 6-phosphate, 0.06 mM $NADP^+$, 0.2 mg bovine serum albumin and 10 μl heterocyst homogenate corresponding to 5 μg chlorophyll *a*. An ATP generating system was included in the reaction mixture, consisting of 5 mM ATP, 5 mM $MgCl_2$, 15 mM creatine phos-

phate and 20 μg creatine kinase, and 10 μM of the ferredoxins were added to measure nitrogenase activity.

2.6. Modelling of FdxH1 and FdxH2

Comparison of the amino acid sequences of FdxH from *Anabaena* 7120 and FdxH1 and FdxH2 from *A. variabilis*, respectively, showed that FdxH was 95% identical to FdxH1 and 76% to FdxH2; both proteins, FdxH1 and FdxH2, showed a high similarity (100% and 90%, respectively). Computer models of FdxH1 and FdxH2 were obtained using the information of the crystallization data obtained with FdxH from *Anabaena* 7120 (1FRD) [3]. The overall main chain conformation of FdxH1 and FdxH2 was nearly the same. The side chains were exchanged using the SCWRL programme [18]. This structure was further used for energy minimization (AMBER [19]) keeping the [2Fe–2S] cluster fixed to four cysteinyl residues (41, 46, 49 and 79) [20]. The minimized structure underwent a molecular dynamics calculation to obtain the final structure. The final model structures were analyzed for further studies with the BRAGI programme per RMS-fitting [21] and presented graphically.

3. Results and discussion

The objective of this study was to reveal the structural basis of oxygen tolerance or sensitivity of ferredoxins and determine the amino acids involved. As shown in Fig. 1, FdxH1 was found to be stable towards oxygen and only 3% of decrease in its absorbtion spectrum was observed after 2 h of incubation

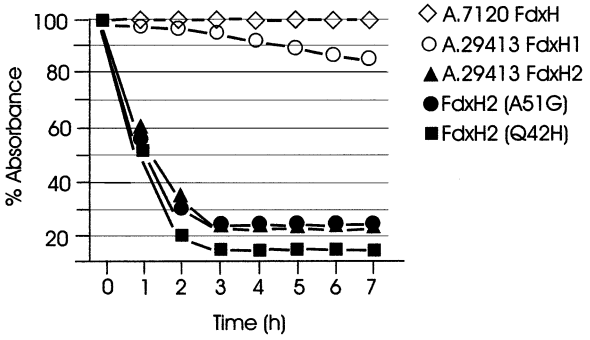

Fig. 1. Differential oxygen sensitivity of cyanobacterial FdxH-type ferredoxins and substituted ferredoxin proteins. Ferredoxin solutions, 1 ml in 20 mM Tris–HCl (pH 8.0), 400 mM NaCl, were incubated as described in Section 2. At times indicated, samples were taken for absorbance measurements at 422 nm. At time zero, 100% absorbance was 0.7 at 420 nm for all ferredoxin solutions. A.7120 FdxH (◇); A.29413 FdxH1 (○); A.29413 FdxH2 (▲); A.29413 FdxH2 (A51G) (●); A.29413 FdxH2 (Q42H) (■). Absorbance values stayed above 10% because of increasing turbidity produced by ferredoxin denaturation.

with oxygen. On the other hand a sharp loss (70%) at 422 nm of the absorbtion spectrum, indicative of the iron–sulfur cluster, of FdxH2 was observed in the same time range in the presence of oxygen. This absorbance change was not reversible. Instead the metalloprotein was denatured under aerobic conditions. FdxH, the heterocyst ferredoxin from *Anabaena* 7120, showed no decrease in its absorbtion spectrum during this time.

The first step to probe oxygen sensitivity by mutagenesis was to compare the amino acid sequence of FdxH1 and FdxH2 and unique amino acid exchanges possibly making FdxH1 more oxygen tolerant (Fig. 2). The comparison shows that glutamine 42 and alanine 51 of FdxH2 fulfil these criteria, and

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                1       10        20        30        40        50
A. 7120 FdxH  ASYQVRLINK KQDIDTTIEI DEETTILDGA EENGIELPFS CHSGSCSSCV
A.29413 FdxH1  T      S   EN
A.29413 FdxH2 TT          RA  I  PV   N     A   QQD       Q
                                  **                     *
                        60        70        80        90        98
A. 7120 FdxH  GKVVEGEVDQ SDQIFLDDEQ MGKGFALLCV TYPRSNCTIK THQEPYLA  100% identity
A.29413 FdxH1                     V                                 95% identity
A.29413 FdxH2 A           E V   E    A   IV    S    D   R        V   76% identity
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A. = *Anabaena*

Fig. 2. Amino acid sequence alignment of *Anabaena* 7120 FdxH with FdxH1 and FdxH2 of *Anabaena* 29413. Positions of residues unique to FdxH2 and subject to site-directed mutagenesis were marked by an asterisk. Amino acids not shown are identical to the *Anabaena* 7120 FdxH-sequence.

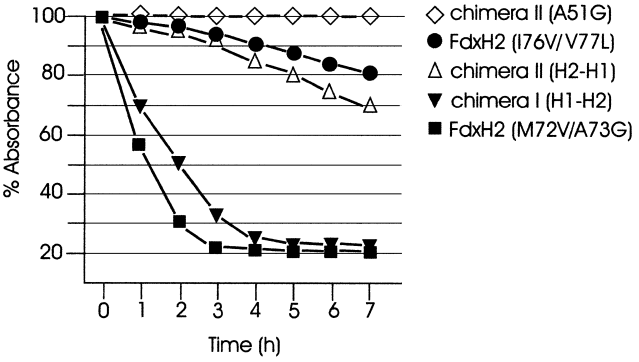

Fig. 3. Differential oxygen sensitivity of cyanobacterial FdxH-type ferredoxins and substituted ferredoxin proteins. Chimeric ferredoxin I (▼); chimeric ferredoxin II (△); chimeric ferredoxin II (A51G) (◇); FdxH2 (I76V/V77L) (●); FdxH2 (M72V/A73G) (■).

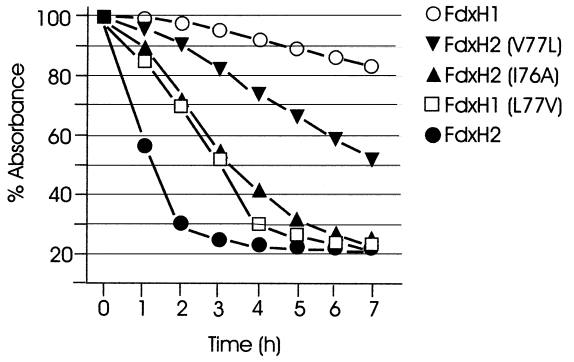

Fig. 4. Differential oxygen sensitivity of cyanobacterial FdxH-type ferredoxins and substituted ferredoxin proteins. FdxH1 (○); FdxH1 (L77V) (□); FdxH2 (●); FdxH2 (V77L) (▼); FdxH2 (I76A) (▲).

they are in addition near cysteines 41 and 49, which are part of the iron–sulfur cluster. A third candidate for site-directed mutagenesis of FdxH2 was proline 19, which is also present at that position in the moderately oxygen-sensitive *Plectonema* FdxH [10]. This proline may alter the main chain conformation and thereby indirectly destabilize the iron–sulfur cluster in the presence of oxygen.

The mutated FdxH2 ferredoxins were isolated as recombinant proteins. The proline 19 mutant could not be expressed in *E. coli*, as was the later constructed proline–valine (19/20) double exchange mutant. Glutamate and isoleucine were the corresponding residues in FdxH1 (compare Fig. 2). Both amino acid residues seem to be required to maintain the structure of the protein. Compared to the wild-type (wt) FdxH2 and the mutation FdxH2(A51G), only the mutant protein Q42H showed an effect in this assay; it was more oxygen-sensitive than wt FdxH2. The loss in absorbance at 422 nm was 80% within 2 h of exposure to air (Fig. 1).

To find out which region or domain confers insensitivity to oxygen, we constructed two chimeric proteins. By site-directed mutagenesis we introduced a *Bcl*I restriction site into the *fdxH1* gene sequence and eliminated a nearby *Bcl*I restriction site, whereas in *fdxH2* this site was already present. A hybrid protein consisted of the 58 N-terminal amino acids from one ferredoxin and the residual 40 C-terminal amino acids from the other ferredoxin.

As shown, the first chimeric protein (the N-terminal position FdxH1 fused to the C-terminal portion of FdxH2) exhibited a similar oxygen sensitivity as the wild-type FdxH2, whereas the second hybrid protein (FdxH2 fused to FdxH1) was found to be more stable to air for at least 7 h (Fig. 3). The second hybrid protein, with an additional exchange of A51 for G51, showed 100% tolerance to air exposure, more than the wild-type heterocyst ferredoxin, FdxH1. Different to alanine, glycine shows a higher flexibility which may, e.g., stabilize the ferredoxin molecule by assuming a tighter packing in this area. The chimeric proteins allowed the conclusion that some of the 40 C-terminal amino acid residues of FdxH1 strongly contributed to the difference in oxygen tolerance.

To get more detailed insight into which amino acid residues may have caused this dramatic effect towards oxygen, we further searched FdxH2 for amino acids distinct to FdxH1 at the C-terminal end and in the vicinity of the fourth cysteine-ligand of the

Table 1
Nitrogenase activities of heterocyst homogenates reconstituted in vitro by the addition of different FdxH-type ferredoxins and mutant proteins thereof from *Anabaena variabilis*

Ferredoxin	Nitrogenase activity (μmol C_2H_4 (μg Chla^{-1}) h^{-1}
FdxH1	8.4
FdxH2	10.0
Chimera I (FdxH1-H2)	7.7
Chimera II (FdxH2-H1)	11.7
FdxH2 (176A/V77L)	8.8
FdxH2 (176A)	8.1
FdxH2 (V77L)	8.7
FdxH1 (L77V)	8.3

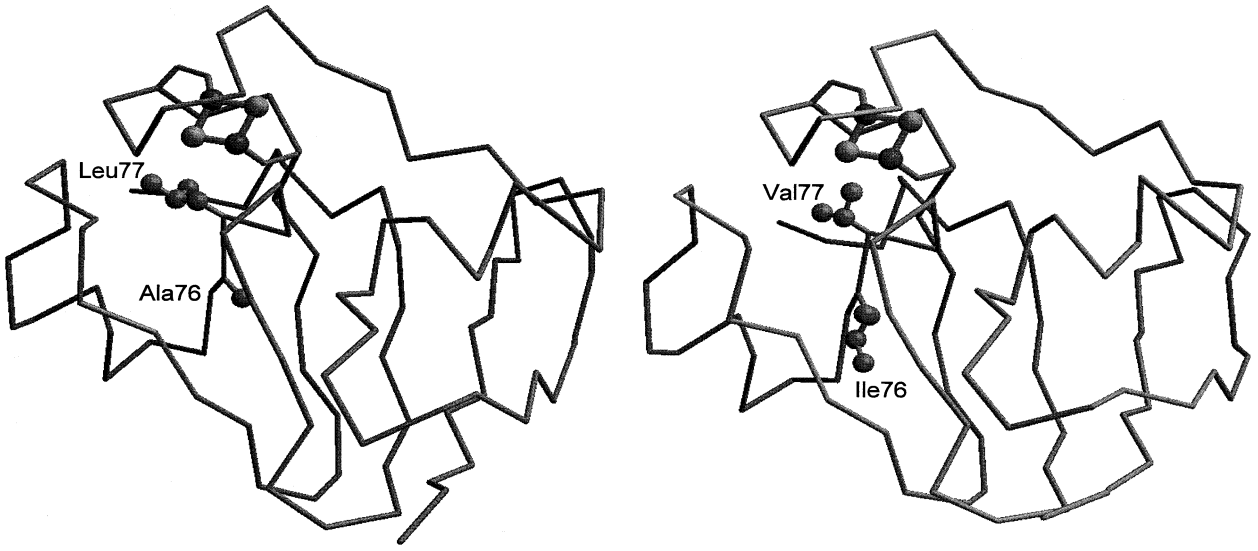

Fig. 5. Models of FdxH1 (left) and FdxH2 (right) with the side chains of leucine 77 and alanine 76 (FdxH1) and valine 77 and isoleucine 76 (FdxH2), respectively, highlighted by a ball-and-stick representation.

[2Fe–2S] cluster. Among the possible candidates were A72, I76 and V77 (Fig. 2).

As shown in Fig. 3, a double mutant of FdxH2, M71V/A72G, did not show any remarkable difference in oxygen tolerance compared to the wt FdxH2 protein, whereas the double mutant I76A/V77L of FdxH2 was found to be more stable than the non-mutated protein. In fact its stability towards oxygen was comparable to wt FdxH1. The exchange of individual amino acids (I76A) and (V77L) of FdxH2 confirmed the above results and showed that substitution of valine for leucine at position 77 was more critical than isoleucin 76 exchanged for alanine (Fig. 4).

To find out whether the same is true for the reverse mutation, leucine 77 of FdxH1 was exchanged for valine (L77V), which was at the same position in FdxH2. The mutated protein showed a decreased oxygen stability, which led to a complete loss of the iron–sulfur cluster and protein denaturation within 5 h of incubation (Fig. 4). Leucine at the position 77 may be one amino acid determining the oxygen stability in FdxH proteins. An exchange of leucine to valine, being smaller in size, makes the [2Fe–2S] cluster to become more susceptible to oxygen.

Data obtained by X-ray crystallography of recombinant heterocyst ferredoxin [3] showed that the binding pocket of the iron–sulfur cluster was amphipathic, with one side (Fe I) containing hydrophilic residues; the other side was lined with hydrophobic residues, among which was leucine 77, where Fe II of the iron–sulfur cluster was situated. Leucine 77 was present within 4 Å of the electron transferring iron–sulfur cluster.

To assay the flow of electrons through ferredoxin, we used the cytochrome *c* reduction and the $NADP^+$-photoreduction assay (data not shown). It showed that the rate of cytochrome *c* reduction and $NADP^+$-photoreduction are independent processes and not related to oxygen stability. We also checked whether these mutants are able to show in vitro nitrogenase activity or not (Table 1). The nitrogenase complex was reconstituted in vitro with 10 μM of different ferredoxins. Again we conclude from these data that oxygen sensitivity are independent processes and not related to electron donation to nitrogenase by ferredoxins.

Modelling of FdxH1 and FdxH2 clearly showed that a cavity was formed near the iron–sulfur cluster, close to Cys49. This was caused by a change of interactions between Val50 and the exchanged residue 77 (Val or Leu, respectively); in FdxH2 the cavity provided sufficient space for dioxygen to enter and stay until the iron–sulfur cluster was oxidized and removed from the protein (Fig. 5). Although an iron–sulfur cluster is also present in FdxH1, it is less accessible to oxidants, such as dioxygen. The

cavity of FdxH1 is relatively small and thus does not facilitate the entry of oxygen. Leucine 77 in the FdxH1 molecule is able to fill the cavity partially, at least to such an extent that the entrance of oxygen is blocked. Valine at position 77, as in the FdxH2 molecule, cannot cover this cavity and thus the movement of oxygen is not restricted. Moreover, the loop was pushed towards the outside by valine 77, thereby increasing the size of the cavity for the entrance of the oxygen molecule, as shown by modelling. The exchange of valine by leucine lead to an increase of one side chain by a $-CH_2$ group, which prevents an easy access of oxygen to the iron–sulfur cluster. One has to remember that the discussion of the cavity size is strictly valid only for the rigid protein structure as obtained from X-ray analysis and modelling. During the temperature-induced fluctuation of the molecule the size of the cavity is going to change and will be accessible to the oxygen molecule part of time. Nevertheless, the argument is still valid as this will be the case much less frequently in the Leu variant, and the affinity for oxygen will be much lower.

Acknowledgements

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