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N-terminal truncation of the variable subunit stabilizes spinach ferredoxin:thioredoxin reductase

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Abstract The variable subunit of spinach ferredoxin:thioredoxin reductase (FTR) has an extended N-terminus compared to FTRs from other sources and this was proposed to contribute to the instability of the protein. We constructed two N-terminal truncation mutants of recombinant FTR by removing 16 or 24 residues from the variable subunit. The mutant proteins are readily expressed and show half-saturation values ($S_{0.5}$) for ferredoxin and thioredoxin *f* comparable to WT. However, truncation increases significantly their stability. Using the stabilized FTR an exposed Cys on its thioredoxin contact surface could be substituted without altering its properties, whereas the replacement of an active site Cys by Ser completely destabilized the protein.

Key words: Ferredoxin:thioredoxin reductase; Variable subunit; Spinach; Mutagenesis; Recombinant; Stability

1. Introduction

The ferredoxin:thioredoxin reductase (FTR) is the central enzyme of the ferredoxin/thioredoxin system, the light-dependent regulatory system in oxygenic photosynthesis [1–3]. The spinach FTR is a heterodimer of 25.6 kDa composed of a variable subunit (or subunit A) of 12.6 kDa [4] and a catalytic subunit (or subunit B) of 13 kDa [5]. Whereas the primary structure of the catalytic subunit is highly conserved in different species, the primary structure of the variable subunit is not. There are rather large differences in length of the polypeptide chain and in amino acid composition. The most striking aspect is the presence of a N-terminal tail in the variable subunit of the so far known higher plant FTR, which is absent from the cyanobacterial FTR. In the spinach FTR this N-terminal tail is unstable and gives rise to multiple bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Analyses of these bands had shown that they represent N-terminal truncated forms of the variable subunit [6]. Since this N-terminal tail is half as long in the variable subunit of the maize FTR compared to the spinach protein and missing in the cyanobacterial proteins we were interested to test whether its removal had some influence on the expression of the spinach enzyme, on its stability and on its catalytic properties. We constructed two N-terminal truncation mutants, removing respectively 16 and 24 residues, expressed them in *Escherichia coli* and tested them for stability and activity in the activation of spinach chloroplast fructose 1,6-bisphosphatase. In addition, based on such a truncated FTR we have constructed an active site mutant in view of forming a mixed disulfide with a target thioredoxin and we have replaced a conserved Cys, which is exposed on the contact surface for thioredoxin on the catalytic subunit to test whether this Cys has some function in the interaction with thioredoxins.

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Abbreviations: FTR, ferredoxin:thioredoxin reductase; 2-MET, 2-mercaptoethanol

2. Materials and methods

Restriction endonucleases were from Roche Diagnostics and Taq DNA polymerase was from Promega. They were used according to the manufacturer's instructions. The custom oligonucleotides were obtained from Microsynth AG (Balgach, Switzerland).

For the expression of the WT and mutant spinach FTRs the dicistronic construct described earlier was used [7]. Mutagenesis was performed by polymerase chain reaction (PCR). To truncate the N-terminus both at Ser16 and Glu24 (see Fig. 1), we designed a 31 bp upstream primer called SUAΔ16 and a 39 bp primer called SUAΔ24. *Nco*I restriction sites (underlined) were introduced in the positions just before the start codon. The downstream primer was the same as for the WT FTR containing a *Kpn*I restriction site.

Upstream primer I (SUAΔ16): 5′-atccatggcatcacctccagaagaagacgag-3′. Upstream primer II (SUAΔ24): 5′-aattccatggaattgaagaagaatctggag-3′. Downstream primer (SUA-MEG): 5′-ggtaccattagacttctactctgctatgatttcgaattc-3′.

The fragments generated by PCR were purified on agarose gels. The 313 bp SUAΔ16 and 290 bp SUAΔ24 were recovered and directly ligated into pBluescript SK+ (Stratagene) cloning vector used to transform *E. coli* strain XL1Blue. The ligated vectors were restriction digested with *Nco*I and *Kpn*I, the DNA fragments purified by electrophoresis and their sequence verified by automatic sequencing (LiCor). Finally the mutant genes were subcloned into *Nco*I/*Kpn*I-digested WT spinach FTR in pET-3d [7] and expressed in *E. coli* strain BL21(DE3)pLysS.

For the construction of the two further mutants the pET-3d FTRΔ24 plasmid was used. One mutation replaced the conserved Cys27 in the catalytic subunit by Ser and a second the active site Cys84 by Ser. The primers, with the mutation and restriction sites underlined, were:

Upstream sense primer I (SUB-MEG): 5′-ggtaccataaggaaacagacatatggcagatccttctgacaaatct-3′. Δ24C27S antisense primer: 5′-cttttatcaaacagaaaagtatgtatc-3′. Δ24C84S sense primer: 5′-gccactctatgctgttctgac-3′. Downstream antisense primer II (SUB-MIN): 5′-ggatcctacatatttgatgtaacttctcg-3′.

Two amplifications by PCR were needed to introduce the mutations

into the template cDNA. In the first we obtained the megaprimers, FTRΔ24 C27S or FTRΔ24 C84S, of 242 and 246 bp respectively, using the sense primer SUB-MEG with Δ24C27S antisense and the antisense primer SUB-MIN with Δ24C84S sense. In the second amplification, with the primers megaC27S and SUB-MIN, respectively SUB-MEG and megaC84S, we obtained fragments of 350 bp bearing the complete mutants. These *Nde*I–*Bam*HI fragments were ligated in pGEM-T Easy vector to be controlled by sequencing and finally transferred to the pET-3d vector, digested with the same enzymes, for production of the modified proteins.

Production and purification of the mutant proteins was achieved essentially by the methods developed for the WT FTR [7]. Cells were grown in LB medium in a 10 l fermentor (Model L1523, Bioengineering AG, Switzerland). When the culture reached a A_{600nm} of 0.8–1.0 the FTR expression was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After an additional 4 to 5 h the cells were harvested by centrifugation, resuspended in a minimum volume of 50 mM triethanolamine-Cl pH 7.3, 0.1 mM PMSF, 14 mM 2-mercaptoethanol (2-MET) and frozen. After thawing the viscous extract was liquefied by treatment with Benzonase (Merck) and clarified by ultracentrifugation at $150\,000 \times g$. Ammoniumsulfate was added to 50% saturation to the extract and the precipitated proteins removed by centrifugation. The supernatant was loaded on a Phenyl-Sepharose (Amersham-Pharmacia) column equilibrated in 100 mM K-phosphate buffer pH 7.3, 1 M $(NH_4)_2SO_4$, 14 mM 2-MET. The column was washed with the same buffer until the absorbance of the effluent was back to baseline. Then the retained, FTR containing protein fraction was eluted with 100 mM K-phosphate buffer pH 7.3, 14 mM 2-MET. The proteins were concentrated by precipitation with $(NH_4)_2SO_4$ (90% saturation), dissolved in column buffer and separated on a S-100HR Sephacryl (Amersham-Pharmacia) column equilibrated in 20 mM triethanolamine-Cl pH 7.3, 200 mM NaCl, 14 mM 2-MET. This step was followed by chromatography on a Q-Sepharose FF (Amersham-Pharmacia) column equilibrated in 20 mM triethanolamine-Cl pH 7.3, 100 mM NaCl, 14 mM 2-MET. The FTR was eluted with a gradient of 100–500 mM NaCl in the same buffer. The FTR containing fractions were concentrated and diafiltered by ultrafiltration with 20 mM triethanolamine-Cl pH 7.3 and loaded on a ferredoxin-Sepharose affinity column equilibrated with the same buffer. The column was rinsed with equilibration buffer and FTR eluted with the same buffer containing 300 mM NaCl. Before storage in liquid nitrogen the proteins were concentrated and diafiltered by ultrafiltration with 20 mM triethanolamine-Cl pH 7.3.

The FTR concentration was determined using a molar absorbency of 17 400 M^{-1} cm^{-1} at 408 nm [8]. The FTR activity was measured in an assay system using either dithionite-reduced ferredoxin or methylviologen as electron donor and the spinach chloroplast fructose 1,6-bisphosphatase as target enzyme as described elsewhere [9]. To assess stability the proteins were incubated in stoppered spectrophotometer cuvettes at 25°C in 20 mM buffers of different pH and absorbance readings recorded every 30 min with a Perkin-Elmer Lambda 16 spectrophotometer. Antibodies against FTR subunits have been produced as described earlier [7].

3. Results and discussion

Fig. 1 shows the sequence alignment for five FTR variable subunits, three from higher plants (two dicots and one monocot) and two from cyanobacteria. Earlier experiments had revealed that FTR preparations from spinach leaves contained, besides the full-length variable subunit, variable amounts of two N-terminal truncated forms, starting respectively at Asp7 and Ser18 [6]. These truncated forms migrate differently on SDS–PAGE and their amounts increase with repeated freeze–thaw cycles of the protein. To study possible functions of the long N-terminus present in the spinach FTR variable subunit and to eventually stabilize the protein we have constructed two N-terminal truncation mutants. In the first mutant, FTRΔ16, the variable subunit starts at position 17 where the first Ser residue has been replaced by Ala. This truncation removes one positive and three negative charges and a number of clustered Ser and Pro residues. The resulting subunit is one residue longer than the shortest form described earlier [6] and has a calculated molecular mass of 11069 Da. In the second truncation mutant, FTRΔ24, an additional seven residues of the variable subunit are removed, including four negative charges. The resulting subunit starts at position 25 with a conserved Asp and its mass is 10214 Da.

Fig. 1. Sequence alignment of variable subunits (subunits A) of FTR using ClustalX [14]. The N-termini of the two mutant constructs, FTRΔ16 and FTRΔ24, are indicated. In the mutant FTRΔ16 the terminal Ser has been replaced by Ala. The N-terminus of the *Arabidopsis* protein has been defined by similarity. SWISS-PROT accession numbers: Spinach (P38365), *Arabidopsis* (Q9FHL4), Maize (P80680), *Anacystis* (P24018), *Synechocystis* (Q55781).

The N-terminal truncation does not seem to influence the expression of the recombinant proteins. We did not observe any significant change in the amount of FTR produced. The yields of recombinant WT and mutant FTR were comparable at about 1.5 mg pure protein per liter of bacterial culture.

The mutant proteins could be purified by the same method used for the WT FTR, however, due to the removal of negative charges both mutants eluted earlier from the anion exchange column.

Gel electrophoretic analysis of the purified recombinant WT FTR shows a pattern very similar to that observed earlier with native FTR. SDS–PAGE separation followed by immunoblotting reveals the presence of three bands (Fig. 2) reacting with antibodies against variable subunit and only one reacting with antibodies against catalytic subunit. When the 16 or 24 N-terminal residues of the variable subunit are removed (mutants FTRΔ16 and FTRΔ24) only one band appears reacting with antibodies against the variable subunit indicating that no more N-terminal degradation is occurring. Due to their smaller sizes the truncated subunits also migrate further on the gel

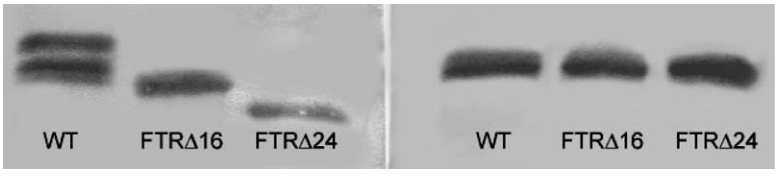

Fig. 2. Immunoblot of SDS–PAGE separated WT and mutant FTR. Lanes on the left-hand side: reaction with antibodies raised against the variable subunit. Lanes on the right-hand side: reaction with antibodies raised against the catalytic subunit.

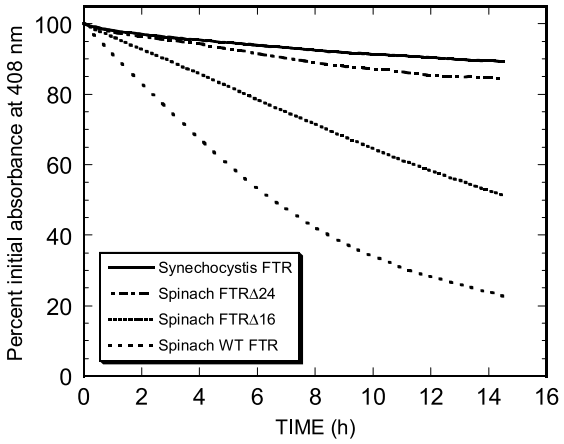

Fig. 3. Decrease of absorbance of FTR as a measure of stability. The proteins, at 8.5 μM, were incubated in 20 mM Tris–Cl buffer pH 8.0, in spectrophotometer cuvettes kept at 25°C.

than the WT variable subunit (12683 Da) or the catalytic subunit (12959 Da).

We have observed that the FTR from *Synechocystis*, whose variable subunit lacks the extended N-terminus, is significantly more stable than the spinach FTR [10]. This suggests that the N-terminus of the variable subunit might influence the stability of the heterodimeric protein. We compared therefore the stability of WT and truncated spinach FTR with that of the *Synechocystis* enzyme. Since the visible absorption peak at 408 nm, which is due to the Fe–S cluster of FTR, is a good indicator for an intact and functional enzyme correlating well with enzyme activity, we monitored the absorbance changes at this wavelength during incubations of the proteins in different buffers. Fig. 3 shows a typical result obtained at pH 8.0. In all buffer systems, tested in the range from pH 7 to 8, the stability of the truncation mutants was clearly improved compared to the WT spinach protein. The shortest mutant, FTRΔ24, was the most stable, comparable to *Synechocystis* FTR. Whereas the *Synechocystis* FTR was equally stable between pH 7 and 8, showing only about 10% absorbance decrease over 15 h, the WT spinach enzyme was less labile at neutral pH (50% decrease over 15 h).

Reduction of the active site disulfide of FTR is achieved with electrons donated by ferredoxin. Ferredoxin as a negatively charged protein has to interact twice with FTR to deliver consecutively the two electrons needed. If in the spinach FTR the N-terminal tail of the variable subunit comes close to the ferredoxin interaction area then its charged residues might have some influence on the interaction. In Fig. 4A we compare the activation of fructose 1,6-bisphosphatase with WT and mutant FTR as a function of ferredoxin concentration to obtain the half-saturation concentration $S_{0.5}$ for ferredoxin. It can be seen that the removal of the N-terminus of subunit A does not significantly alter the affinity for ferredoxin although the truncations remove a number of mainly negative charges making the protein more positively charged, which might favor the interaction with the negatively charged ferredoxin. These results are in agreement with the structural analysis of the FTR from *Synechocystis*, which revealed that all charged residues possibly involved in interaction with ferredoxin are located on the catalytic subunit and are conserved in all reported FTR sequences [11].

Likewise we determined the affinity of WT and mutant FTR for thioredoxin *f* (Fig. 4B). Since spinach thioredoxin *f* is rather positively charged one would expect a higher affinity of the mutant proteins if the N-terminal tail is involved in interaction with thioredoxin. However, we do not observe a significant difference in the affinities of WT or mutant FTR for thioredoxin *f*.

The present results clearly show that the removal of up to 24 N-terminal residues of the variable subunit stabilizes the dimeric protein and does not change any of its catalytic properties. The truncated FTR has also been used in spectroscopic studies where it behaved like the WT protein [12]. Since no crystal structure of a higher plant FTR has yet been solved the conformation of the spinach FTR N-terminal tail is not known nor can it be reasonably modeled due to the lack of data. It is possible that the N-terminal region is rather flexible, sticking out from the rest of the subunit, thus rendering the dimeric protein less stable leading to the separation of the

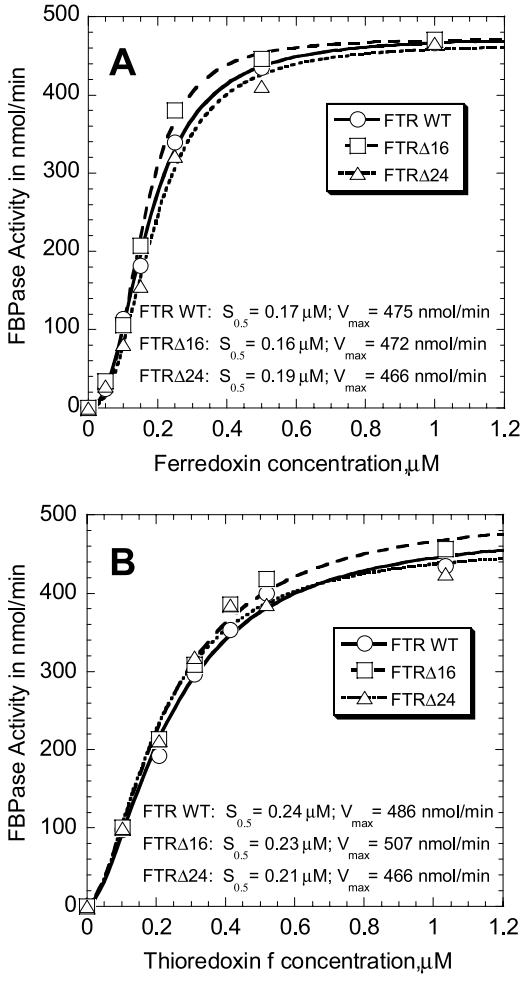

Fig. 4. Activation of fructose 1,6-bisphosphatase with WT and truncated FTR. A: Ferredoxin-concentration dependency. The curves are averages from three experiments. The fructose 1,6-bisphosphatase (0.5 U) was activated during 5 min in presence of 5 mM Na-dithionite, varying concentrations of ferredoxin, 0.16 μM FTR and 2 μM thioredoxin *f*. B: Thioredoxin-concentration dependency. The curves are averages from two experiments. The fructose 1,6-bisphosphatase (0.5 U) was activated during 5 min in presence of 5 mM Na-dithionite, 1 μM methylviologen, 0.4 μM FTR and varying concentrations of thioredoxin *f*. The results have been evaluated using a simplified Hill equation [15].

subunits and loss of the Fe–S cluster. From earlier experiments in our laboratory, expressing only the catalytic subunit in *E. coli* [13], we know that this subunit by itself is inactive. In absence of the variable subunit the essential cluster is apparently missing, which may be due to the fact that several residues in close vicinity of the cluster are, in the dimer, involved in the interaction with the variable subunit.

We used the stabilized, truncated FTR to explore the functions of two cysteine residues of the catalytic subunit. We replaced Cys27, a conserved, surface exposed residue on the thioredoxin interaction side, as well as Cys84, the inaccessible residue of the disulfide bridge, by Ser. Both mutants were produced at about the same level as the WT protein as judged by SDS–PAGE and immunoblotting. However, the C84S mutant was extremely labile and disintegrated very rapidly during the first steps of purification. The presence of an OH group in the close vicinity of the Fe–S cluster seems to destabilize the cluster resulting in a separation of the subunits and a denaturation of the protein. By contrast the FTRΔ24C27S mutant could be purified by the method used for the truncated FTR. This mutant was perfectly capable of activating the FBPase and showed a thioredoxin *f*-concentration dependency comparable to the FTRΔ24. This result suggests that the conserved Cys on the thioredoxin contact surface has no function in the interaction with thioredoxin.

In conclusion, we have obtained a significantly more stable recombinant spinach FTR by removing 24 residues from the N-terminus of its variable subunit. This truncated protein showed the same high affinities for ferredoxin and thioredoxin *f* with $S_{0.5}$ values between 0.15 and 0.25 μM as observed with the WT protein. Using the truncated FTR as template, the surface exposed additional Cys27 on the catalytic subunit was replaced by Ser without any effect on the properties of this mutant. However, substituting Cys84 in the active site disulfide by Ser yielded an extremely labile protein, which could no longer be purified, indicating that the presence of an OH group close to the Fe–S cluster was strongly destabilizing it.

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