Ferredoxin:thioredoxin reductase (FTR) is a key regulatory enzyme of oxygenic photosynthetic cells involved in the reductive regulation of important target enzymes. It catalyzes the two-electron reduction of the disulfide of thioredoxins with electrons from ferredoxin involving a 4Fe-4S cluster and an adjacent active-site disulfide. We replaced Cys-57, Cys-87, and His-86 in the active site of *Synechoystis* FTR by site-directed mutagenesis and studied the properties of the modified proteins. Mutation of either of the active-site cysteines yields inactive enzymes, which have different spectral properties, indicating a reduced Fe-S cluster when the inaccessibly Cys-87 is replaced and an oxidized cluster when the accessible Cys-57 is replaced. The oxidized cluster in the latter mutant can be reversibly reduced with dithionite showing that it is functional. The C37S mutant is a very stable protein, whereas the C87A mutant is more labile because of the missing interaction with the cluster. The replacement of His-86 greatly reduces its catalytic activity supporting the proposal that His-86 increases the nucleophilicity of the neighboring cysteine. Ferredoxin forms non-covalent complexes with wild type (WT) and mutant FTRs, which are stable except with the C87A mutant. WT and mutant FTRs form stable covalent heteroduplexes with active-site modified thioredoxins. In particular, heteroduplexes formed with WT FTR represent interesting one-electron-reduced reaction intermediates, which can be split by reduction of the Fe-S cluster. Heteroduplexes form non-covalent complexes with ferredoxin demonstrating the ability of FTR to simultaneously dock thioredoxin and ferredoxin, which is in accord with the proposed reaction mechanism and the structural analyses.

**Site-directed Mutagenesis**

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The abbreviations used are: FTR, ferredoxin:thioredoxin reductase; Trx, thioredoxin; FNR, ferredoxin:NADP reductase; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; WT, wild type.

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Cys-87 attacks and cleaves the heterodisulfide linkage between FTR and Trx, thus liberating the reduced Trx. The closing of the active-site disulfide bridge completes the reaction cycle.

Alkylation of the active-site cysteine of the active-site disulfide (Cys-57 in Synechocystis) with N-ethylmaleimide provided a stable analogue of the one-electron-reduced heterodisulfide intermediate (10, 11). This modification is accompanied by a typical change of the visible spectrum (12) because of the oxidation of the cluster from its 2+ state in the resting enzyme to the 3+ state in the analogue of the reaction intermediate.

Recent spectroscopic analyses demonstrated that in the reaction intermediate Cys-87 is coordinated to the closest iron atom of the cluster (13). In addition there is partial bonding of the disulfide to this iron even in the resting state of the enzyme. This promotes charge buildup on this special iron making it an electron donor with increased ferrous character. The system is therefore primed and ready to accept an electron from ferredoxin to break the disulfide bond. The binding of an additional cysteine to the special iron, in the one-electron-reduced heterodisulfide intermediate, makes it more ferric, and the charge is drawn away from it. Thus Cys-87 appears to be a critical residue not only as a member of the disulfide bridge but also because it interacts with an iron atom of the cluster in the resting enzyme.

In this study we have produced and characterized mutant FTRs, based on the Synechocystis protein, to obtain further information on the function of three important residues. We individually replaced the redox-active cysteines, Cys-57 by serine or alanine, as well as His-86, proposed to increase the nucleophilicity of the active site, by tyrosine.

The choice of tyrosine was based on sequences of putative FTRs, based on the analyses of the heteroduplexes were done under non-denaturing conditions, and anion exchange chromatography. Ferredoxin:NADP reductase (FNR) from spinach leaves has been purified to homogeneity.

WT and mutant Synechocystis FTRs were expressed in the E. coli strain BL21(DE3)pLysS in the presence of 50 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 10 mM glucose. When the culture reached an A600/660 of 6–7, expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.2 mM, and 2 h later cells were harvested by centrifugation (2400 x g, 6 min, 4 °C). The purification of the FTR was done as described elsewhere (16).

Analytical Procedures—Spectrophotometry was performed with a PerkinElmer Lambda 16 instrument. Difference spectra were recorded in a thin-walled compartment cuvette with compartment path lengths of 0.438 cm. Thiol determinations were done according to Habeeb (21). The activity of FTR was measured by its capacity to activate fructose-1,6-bisphosphatase, using dithionite-reduced benzyl viologen as the electron donor for the FTR as described earlier (22). Gel electrophoretic analyses of the heteroduplexes were done under non-denaturing conditions. Site-directed Mutagenesis—The construction of the mutant proteins was based on the dicistronic construct for the expression of the FTR from Synechocystis sp. PPC6803 described earlier (6, 16).

Mutagenesis was performed by PCR. The three mutations C87S, C87A, and H86Y were introduced by directly amplifying the entire expression vector using the following oligonucleotides (mutations in bold): C87S antisense, 5'-AAACAACATAGCGTCACCTT-3'; H86Y antisense, 5'-AAAAACATAGCAGTCACCTT-3'; and SUB1 sense, 5'-TTAAACCCGA-GATAAGATTGGT-3'. For the introduction of the fourth mutation, C57S, two successive amplifications were necessary as described for the spinach FTR (7). In a first amplification, using the primers C57S antisense and C57S sense, the wild type C57 was replaced completely by C49S. In a second amplification using the primer C49Santisense and C57S sense, the C49S mutation was introduced. The resulting PCR product was digested with the restriction enzymes NotI and XhoI and ligated into a pET-3c vector. The resulting construct was transformed into E. coli strain BL21(DE3) (19). It was purified to homogeneity as described earlier (20). Recombinant Synechocystis ferredoxin was constitutively expressed in E. coli cells (strain DH5α from Invitrogen), transformed with the expression vector pCKS/19 (gift from Herbert Bohme, University of Bonn), and grown overnight in the presence of 50 μM FeSO4 and 100 μg/ml ampicillin. The purification of the FNR was achieved through hydrophobic interaction, size exclusion, and anion exchange chromatography.

RESULTS

Production and Purification—The WT and mutant Synechocystis FTRs were well expressed in E. coli and could be purified by our standard procedure except for the C87S mutant. This mutant, although produced in E. coli as evidenced by immunoblotting, could not be purified. The Cys to Ser mutation apparently generates an unstable protein, whereas the C87A mutation yields a more stable protein. We obtained yields of purified proteins in the range of 7–27 mg/liter of bacterial culture.

Spectral Characterization—WT FTR has a typical UV-visible
applied successfully to the spinach FTR (7). These results (data used to compare their stability in solution, an approach already
shifts back to its original oxidized form. During oxidation of the added dithionite, because of the presence of oxygen in the cuvette, the spectrum of the C57S mutant
also the FTR C57S mutant did not form a heteroduplex, because its interacting Cys had been replaced. We observed, however, that in the presence of Trx
as their capacity to activate fructose-1,6-bisphosphatase and C49S a new band
formed. This was verified by following the oxidation of the Fe-S cluster to its 3+
redox state. This has been verified by treating WT and mutant proteins with dithio-
the formation of such heteroduplexes. Their presence could be demonstrated by chromatography and by native gel electrophoresis followed by immunoblotting. SDS-PAGE was not
suitable for these analyses because of artifactual S–S bond formation upon denaturation under oxidizing conditions leading to multiple bands. When the WT FTR as well as the mutants C87A and H86Y are incubated with Trx
acids). These complexes are stabilized by electro-
static interactions and have a high affinity, because concentrations above 200 mM NaCl had to be added to observe a deviation from linearity in the formation of the complexes. Curiously, with the mutant C87A the absorbency difference due to the complex started to decrease when ferredoxin above an equimol-
lar ratio was added (Fig. 2B).

Interaction with Thioredoxins through Formation of Covalent Heteroduplexes—Reduction of Trxs proceeds via the forma-
tion of a transient, covalent heteroduplex between FTR and Trx. This intermediate complex can be stabilized using muta-
tants in which one or both non-accessible Cys of the participating disulfide bridges are modified. We used mutant Trxs fC49S and m C40S as well as WT and all three mutant FTRs to study the formation of such heteroduplexes. Their presence could be demonstrated by chromatography and by native gel electrophoresis followed by immunoblotting. SDS-PAGE was not
suitable for these analyses because of artifactual S–S bond formation upon denaturation under oxidizing conditions leading to multiple bands. When the WT FTR as well as the mutants C87A and H86Y are incubated with Trx f C49S a new band appears upon electrophoretic analysis. This band is colored, clearly visible during migration like the band representing FTR, and reacts with antibodies against Trx f indicating that it represents the heteroduplex. Similar results were obtained with WT FTR and mutant Trx m. When dithiothreitol is added to the electrophoresis samples the heteroduplex band disappears. Upon incubation of the FTRs with WT Trx f, no heteroduplex is formed. Also the FTR C57S mutant did not form a heteroduplex, because its interacting Cys had been replaced. We observed, however, that in the presence of Trx f C49S, the C57S mutant was degraded. This was verified by following the A408/A278 absorbency ratio of an equimolar mixture of the two proteins. Fig. 3A shows that the ratio decreases because of the disintegration of the Fe-S cluster leading to a degradation of the protein, whereas the ratio of FTR incubated alone does not change.

Changes of the spectra of the four proteins over time were used to compare their stability in solution, an approach already
applied successfully to the spinach FTR (7). These results (data not shown) indicate that the C57S mutant has the most stable
conformation. The WT and H86Y mutant are about equally stable but less than the C57S. The C87A mutant is signifi-
cantly more labile, progressively losing its color during incubation, which indicates that the Fe-S cluster disintegrates.

Thiol Determinations—The accessible and total thiols have been determined by reacting the proteins with 5,5’-dithiobis(2-
nitrobenzoic acid) in the absence and presence of SDS to verify the correctness of the mutations and the intactness of the structures. For the WT and the mutants H86Y and C57S the experimentally determined values correspond to the theoreti-
cally expected numbers, i.e. one accessible and five total thiols in the WT and H86Y FTR, and one accessible and six total thiols in the C57S mutant. For the C87A mutant the expected two accessible thiols were found; however, there were only four instead of six total Cys residues. This might be because of the formation of an artifactual disulfide bond in this less stable mutant under our aerobic assay conditions.

Activity—The catalytic activity of the mutants was measured as their capacity to activate fructose-1,6-bisphosphatase and compared with that of the WT protein. As expected, the two mutations modifying the active-site disulfide, C57S and C87A, completely abolish activity. The mutant H86Y had only ~10% of the WT activity when tested under comparable conditions.

Non-covalent Interaction with Ferredoxin—Synechocystis FTR and ferredoxin form a 1:1 complex resulting in a typical difference spectrum with a peak at 460 nm and a trough at 410 nm (Fig. 2A, inset). We titrated the Synechocystis FTR mutants with ferredoxin to verify whether the mutations influence this protein-protein interaction. The results show that at a low salt concentration all mutants form a 1:1 complex like the WT protein (Fig. 2A). These complexes are stabilized by electro-
static interactions and have a high affinity, because concentrations above 200 mM NaCl had to be added to observe a deviation from linearity in the formation of the complexes. Curiously, with the mutant C87A the absorbency difference due to the complex started to decrease when ferredoxin above an equimolar ratio was added (Fig. 2B).

Interaction with Thioredoxins through Formation of Covalent Heteroduplexes—Reduction of Trxs proceeds via the forma-
tion of a transient, covalent heteroduplex between FTR and Trx. This intermediate complex can be stabilized using muta-
tants in which one or both non-accessible Cys of the participating disulfide bridges are modified. We used mutant Trxs fC49S and m C40S as well as WT and all three mutant FTRs to study the formation of such heteroduplexes. Their presence could be demonstrated by chromatography and by native gel electrophoresis followed by immunoblotting. SDS-PAGE was not
suitable for these analyses because of artifactual S–S bond formation upon denaturation under oxidizing conditions leading to multiple bands. When the WT FTR as well as the mutants C87A and H86Y are incubated with Trx f C49S a new band appears upon electrophoretic analysis. This band is colored, clearly visible during migration like the band representing FTR, and reacts with antibodies against Trx f indicating that it represents the heteroduplex. Similar results were obtained with WT FTR and mutant Trx m. When dithiothreitol is added to the electrophoresis samples the heteroduplex band disappears. Upon incubation of the FTRs with WT Trx f, no heteroduplex is formed. Also the FTR C57S mutant did not form a heteroduplex, because its interacting Cys had been replaced. We observed, however, that in the presence of Trx f C49S, the C57S mutant was degraded. This was verified by following the A408/A278 absorbency ratio of an equimolar mixture of the two proteins. Fig. 3A shows that the ratio decreases because of the disintegration of the Fe-S cluster leading to a degradation of the protein, whereas the ratio of FTR incubated alone does not change.
The heteroduplexes were separated from the unreacted proteins by chromatography. Whereas gel filtration did not completely resolve heteroduplex and FTR, because of the relatively small difference in molecular mass, ion exchange chromatography provided quantitative separation of all proteins engaged in the reaction (Fig. 4). This approach enabled us to purify and characterize the heteroduplexes and to use them for interaction studies with ferredoxin.

The visible absorbency spectra of free and Trx-complexed FTR C87A are identical (compare in Table I). By contrast, the spectra of the heteroduplexes formed with WT or H86Y mutant FTR are comparable with the spectra of N-ethylmaleimide-modified FTR or mutant C57S (Fig. 1). This suggests that the redox state of the cluster is altered. The spectral change, which is most pronounced at 345 nm, provided a useful measure to follow complex formation over time as documented in Fig. 3B for the reaction with Trx m C40S. Similar results were obtained with Trx f C49S. A comparison of the kinetics between WT FTR and mutant H86Y reveals that the mutant FTR reacts more slowly.

**Table 1**

Spectral properties of WT and mutant FTRs and of FTR-Trx heteroduplexes

<table>
<thead>
<tr>
<th>FTR</th>
<th>Color</th>
<th>408 nm</th>
<th>408/278 nm</th>
<th>408/345 nm</th>
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</thead>
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<tr>
<td>WT</td>
<td>Brown-green</td>
<td>17,400</td>
<td>0.44</td>
<td>0.94</td>
</tr>
<tr>
<td>C87A</td>
<td>Brown-green</td>
<td>17,400</td>
<td>0.44</td>
<td>1.00</td>
</tr>
<tr>
<td>H86Y</td>
<td>Brown-green</td>
<td>17,400</td>
<td>0.41</td>
<td>0.96</td>
</tr>
<tr>
<td>C57S</td>
<td>Brown-red</td>
<td>18,700</td>
<td>0.47</td>
<td>0.78</td>
</tr>
<tr>
<td>WT-Trx f</td>
<td>Brown-red</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-Trx m</td>
<td>Brown-red</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C87A-Trx f</td>
<td>Brown-green</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2.** Complex formation between FTR and ferredoxin. WT (A) and mutant (B) FTR were titrated with ferredoxin in 20 mM triethanolamine-Cl buffer, pH 7.3, at 25 °C. *Inset,* difference spectrum of the complex FTR-ferredoxin at 23 μM. Difference spectra were recorded using dual compartment cuvettes. To the solution of about 50 μM FTR in the measuring cuvette small aliquots of ferredoxin were added. In the reference cuvette corresponding aliquots of ferredoxin were added to buffer in one compartment and aliquots of buffer to the solution of FTR in the second compartment. After 5 min of equilibration, three spectra from 550 to 350 nm were recorded for each data point, and the difference was calculated from the averaged absorbencies at 408 and 410 nm corrected for dilution. *Upper axis* represents the ferredoxin/FTR ratio calculated with the exact protein concentrations.

**Fig. 3.** Kinetic analyses of FTR-Trx interactions. A, change of the absorbency ratio during incubation of an equimolar mixture of FTR C57S and Trx f C49S in 20 mM triethanolamine-Cl buffer, pH 7.3, at 25 °C (squares). As a control, FTR C57S was incubated alone under the same conditions (circles). Absorbency readings at 408 and 278 nm were taken automatically at 100-min intervals. B, comparison of the rate of heteroduplex formation between WT or H86Y mutant FTR and Trx m C40S. 15 nmol of FTR were incubated with 20 nmol of Trx m C40S in 1 ml of 20 mM triethanolamine-Cl buffer, pH 7.3, at 25 °C in spectrophotometer cuvettes. The reaction mixture contained, in addition, 14 mM 2-mercaptoethanol to keep the mutant Trx monomeric. The percentage of heteroduplex formed was calculated from the change of the 408/345 nm absorbency ratio. Continuous line, FTR WT-Trx m C40S; broken line, FTR H86Y-Trx m C40S.
On the Trx interaction side of the FTR, Cys-30 represents an accessible thiol. A titration of accessible thiols in the heteroduplexes by 5,5'-dithiobis(2-nitrobenzoic acid) can therefore provide some information about the coverage of this surface by Trx. In the FTR-Trx \(_f\) complex one thiol was found; however, none was found in the complex with Trx \(_m\). Because Trx \(_f\) contains an accessible Cys on its surface and Trx \(_m\) has none (25), this result suggests that Cys-30 on the FTR surface is masked by the bonded Trx.

The heteroduplex between WT FTR and Trx represents a stable reaction intermediate with an oxidized Fe-S cluster. Its reduction should dissociate Trx from FTR, which can be shown by gel filtration. It is assumed that in the chloroplast, light-reduced ferredoxin delivers the electron necessary to reduce the Fe-S cluster and thereby breaks the disulfide bond linking the two molecules. We have therefore incubated the WT FTR-Trx heteroduplex with ferredoxin, thylakoids, and ascorbate/dichlorophenol-indophenol as electron source and observed complete dissociation in the light. However, control experiments showed that ferredoxin is not required and that even in the dark, partial dissociation is obtained. In vitro ferredoxin can also be reduced via FNR with an excess of NADPH + H\(^+\). In this reduction system both FNR and NADPH + H\(^+\) in a 50-fold excess over the heteroduplex were needed to obtain dissociation. Here again, the presence of ferredoxin was not required.

We have also compared the effect of different chemical reductants on the dissociation of the WT FTR-Trx \(_f\) or \(_m\) heteroduplex by gel electrophoresis and spectrophotometry. Reduced glutathione had no effect and mercaptoethanol had only a weak effect at concentrations of a 1000-fold excess over the heteroduplex. DTT, at a 200-fold excess, was more efficient, showing a dissociation kinetic with a 50-min lag phase. Dithionite was the most efficient. A 40-fold excess immediately broke the disulfide bond linking the two proteins and reestablished the spectrum of free WT FTR. When the FTR(C87A)-Trx \(_f\) heteroduplex was similarly treated with dithionite, Trx \(_f\) was not dissociated.

Redox Potentials of the Intermolecular Disulfide Bonds—The
above results suggest that it should be possible to estimate the redox potential of the intermolecular disulfide bridge by comparing the dissociation of the FTR-Trx heteroduplex at different ambient redox potentials. We equilibrated the heteroduplexes in incubation mixtures of known redox potentials and subsequently analyzed them by gel electrophoresis. Fig. 5 shows the results obtained with the heteroduplexes between WT FTR and Trx \( \text{f} \) or \( m \), which indicate a potential of \(-270 \pm 10 \text{ mV}\) for the complex with Trx \( \text{f} \) and \(-280 \pm 10 \text{ mV}\) for the one with Trx \( m \). With the FTR\( (C87A) \)-Trx \( f \) duplex we observed only partial dissociation down to \(-390 \text{ mV}\).

Formation of a Triple Complex among FTR, Trx, and Ferredoxin—According to the proposed reaction mechanism, the FTR should be able to interact simultaneously with Trx and ferredoxin. We therefore were looking for evidence of complex formation between the heteroduplex FTR-Trx and ferredoxin by gel filtration and spectrophotometry. When an equimolar mixture of FTR-Trx \( m \) heteroduplex and ferredoxin was chromatographed at low ionic strength (Fig. 6), a single peak (triplex) eluted from the column at a lower elution volume (9.3 ml) than either the heteroduplex (9.7 ml) or ferredoxin (11.16 ml). When the molar ratio in the mixture was modified to 2:1 in favor of ferredoxin, a peak of free ferredoxin appeared, and when the ratio was changed in the opposite direction, a shoulder on the profile of the triplex indicated free heteroduplex. Identical elution profiles were obtained with the FTR-Trx \( f \) heteroduplex. These observations are corroborated by difference spectroscopy. Both heteroduplexes were titrated with ferredoxin at 0 \( \times \) NaCl, and we obtained titration curves identical to the one shown in Fig. 2A indicating a non-covalent complex between one ferredoxin and one heteroduplex molecule. These results clearly demonstrate that FTR is capable of interacting simultaneously with Trx and ferredoxin forming a 1:1:1 complex.

DISCUSSION

The FTR has structural features that are unique and enable this enzyme to reduce disulfides with electrons from a Fe-S cluster. This reduction is possible because of the close proximity and the interactions of the two important catalytic structures, the 4Fe-4S cluster and the redox-active disulfide bridge.

The structural analyses show that Cys-57, which is part of the redox-active disulfide, is close to the protein surface and should act as the attacking nucleophile in the reduction of Trx \( (5, 8, 9) \). We have replaced this Cys with a Ser residue, which leaves Cys-87, the buried partner residue of the disulfide bridge, as a free thiol. The mutant protein is no more active and has the same spectral characteristics as the N-ethylmaleimide-modified FTR. This indicates that the Fe-S cluster is in its oxidized state with one of its iron atoms forming a fifth ligation to the sulfur of Cys-87. This appears to be a favorable conformation rendering the mutant by itself more stable than the WT protein. The mutant protein can be reversibly reduced with dithionite, and this is accompanied by a change of the spectrum to that of the resting WT enzyme. Thereby Cys-87 becomes reduced and switches from being a cluster ligand to a thiol or thiolate. The ferredoxin interaction surface appears to be unaltered, because the protein forms the 1:1 non-covalent complex with ferredoxin like the WT FTR. However, with Trxs no complex formation is observed, confirming that Cys-57 is part of the intermolecular disulfide and that Cys-30 on the Trx interaction surface is not involved in any covalent interaction. Surprisingly, in the presence of the mutant Trx \( f \) C49S, the FTR C57S loses color indicating a degradation of the Fe-S cluster. We do not know whether this is caused by the non-covalent contact of the two proteins or by some interaction between the accessible cysteines on the protein surfaces.

The partner residue of the redox-active disulfide, Cys-87, is very close to an iron atom of the cluster and apparently crucial for its stability. In the resting state of the enzyme the sulfur shows some weak interaction with this iron and becomes its fifth ligand in the one-electron-reduced intermediate (13). Replacing the sulfur by a hydroxyl in the C87S mutant perturbs this interaction and renders the cluster, and as a consequence the whole protein, unstable. This has also been observed with the spinach FTR (7). By contrast, the introduction of a hydrophobic side chain, the C87A mutation, appears to be compatible with the cluster. However, this enzyme is significantly less stable than the WT or the other mutant FTRs. This mutant shows a spectrum very similar to the resting WT FTR. The small deviation in the 408/345 absorbency ratio might be because of the missing interaction between the cluster iron and residue \( 87 \). Through its free Cys-57 this mutant FTR forms covalent heteroduplexes with Trxs.

His-86 is located between a Cys liganding the Fe-S cluster and the buried inaccessible Cys of the active site. Based on its properties and on structural considerations we proposed that it might increase the nucleophilicity of the active-site Cys-57 (8). Our present results seem to confirm this hypothesis. The H86Y mutant showed a low catalytic activity in the activation of fructose-1,6-bisphosphatase, and the kinetic of heteroduplex formation with Trx was clearly slower than with the WT FTR. The other properties of this mutant were comparable with those of the WT protein.

An interesting aspect of these studies was the formation of
Ferredoxin:Thioredoxin Reductase Active-site Mutants

protein–protein complexes among ferredoxin, FTR, and Trx. A non-covalent complex between FTR and ferredoxin has already been reported for the spinach proteins (26, 27). We observed identical high affinity 1:1 complexes between Synechocystis ferredoxin and WT, as well as all three mutant FTRs. These complexes were stable except for the one with the C87A mutant FTR. Here a disintegration of the cluster was observed when a 1:1 ratio of the two proteins was reached. Because the interaction between a cluster iron and the sulfur of Cys-87 is missing, the cluster is probably more labile and not able to withstand the slight deformation due to the electrostatic forces stabilizing the complex.

We have obtained stable covalent heteroduplexes between active-site mutant Trxs (Trx f C49S or Trx m C49S) and FTR, WT, or mutants H86Y and C87A but not mutant C57S. The visible absorbency spectrum of the heteroduplex with the active-site mutant C87A is indistinguishable from the spectrum of the free mutant FTR. The absence of any change suggests that the cluster stays in its 2+/H11001 state and that the covalent attachment of Trx f has no influence on the cluster. By contrast, heteroduplexes formed with either WT or H86Y FTR have visible absorbency spectra that superimpose with the spectrum of the C57S mutant or N-ethylmaleimide-modified FTR. This implies that the cluster has become oxidized, and the sulfur of Cys-87 has connected to an iron atom of the cluster as a fifth ligand thus stabilizing the heteroduplex.

The mechanism of heteroduplex formation with either mutant C87A or WT FTR must be different. In the case of the mutant C87A FTR a disulfide bond is made between two thiolates with the concomitant removal of two electrons by an oxidant, which in our system is dissolved oxygen (Scheme 1). In the case of WT FTR one disulfide bond, the active-site disulfide of FTR, has to be opened before another one, between FTR and Trx, can be formed, accompanied by the removal of one electron (Scheme 2). The active-site disulfide of the FTR may be broken by the attacking Trx, helped by the cluster draining the electron. We have indications that oxidants, e.g. ferrocyanide, or mediators with more positive redox potentials than the FTR, e.g. anthraquinone 2,6-disulfonic acid, accelerate the heteroduplex formation. Further experiments are needed to better understand the mechanism of heteroduplex formation.

According to the proposed reaction mechanism for Trx reduction by FTR, the heteroduplex represents the one-electron-reduced reaction intermediate. This intermediate is thought to be dissociated by an electron provided by ferredoxin. We incubated the heteroduplexes with ferredoxin, reduced either photochemically with light and thylakoids or enzymatically with NADPH and FNR. Surprisingly, in both reduction systems the dissociation was independent of ferredoxin, i.e. FNR or thylakoids were capable of delivering electrons directly to the heteroduplex and releasing Trx. With the thylakoids we even observed dissociation in the dark, which, however, was increased in the light. Thylakoids appear to contain some “reductants” capable of interacting with the FTR through the Fe–S cluster or acting directly on the mixed disulfide bond. We do not know whether these ferredoxin-independent reductions occur in vivo or only under our in vitro conditions. Our experiments do not provide any kinetic information. It may well be that in vivo the reduction by ferredoxin is by far the most efficient and therefore the preferred mechanism.

Among the chemical reductants dithionite was the most efficient and therefore in reducing the mixed disulfide bond and dissociating Trx. This corroborates earlier reports (11) and the results obtained with mutant C57S showing that dithionite alone is capable of reducing the Fe–S cluster in analogues of the one-electron-reduced intermediate. The electrons are probably funneled through the cluster to Cys-87, which becomes the attacking nucleophile. Thereby the cluster is reduced as seen by the spectral change. In the C87A mutant the reduction by dithionite is not possible because of the already reduced cluster and the absence of Cys-87, and the mixed disulfide bond cannot be broken. The reduction of the reaction intermediates or their analogues without the need of ferredoxin contrasts with the fact that the disulfide bridge of resting FTR can only be opened by reduced ferredoxin or some reduced mediators (10, 22), a difference we cannot currently explain with our results.

The redox potentials of the mixed disulfide bonds between FTR and Trxs are 40–50 mV more positive than the potential of the active-site disulfide of FTR (midpoint redox potential (Em)), pH 7.0 = −320 mV). These significantly more positive redox potentials make the second step of Trx reduction, the liberation of reduced Trx, a more favorable reaction. The heteroduplex between mutant FTR C87A and Trx f C49S displayed quite a different behavior. At potentials lower than −280 mV, down to −390 mV, only a partial dissociation was observed. This is probably because this mutant FTR cannot reform an active-site disulfide. Further spectroscopic studies will provide more information on the properties of the different mutant FTRs.

The heteroduplexes were able to interact with ferredoxin non-covalently, thus forming triple complexes with a 1:1:1 component ratio and confirming nicely the proposed interaction scheme (8). Titrations of the heteroduplexes with ferredoxin yielded difference spectra that were identical to those obtained with FTR alone, and the affinities were also essentially the same. These observations suggest that the FTR is not subjected to major structural changes following the binding of Trxs. Detailed information on these aspects will be provided by the structural analyses of the different protein–protein complexes.

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