Chemical Modification of the Active Site of Ferredoxin-Thioredoxin Reductase

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Abstract. Ferredoxin-thioredoxin reductase is an essential member of a light-dependent regulatory system in oxygenic photosynthesis. This enzyme is composed of two nonidentical subunits, contains a 4Fe-4S cluster of a yet unknown function, and a catalytically active dithiol group. We have used N-ethylmaleimide to modify specifically the active site thiols of the protein and show that their chemical modification completely abolishes the catalytic capacity of the enzyme and causes changes in the absorption spectrum of the protein.

Introduction

Light controls the activity of several enzymes in oxygenic photosynthesis by a regulatory system known as the ferredoxin/thioredoxin system [1-3]. In this system the light signal is transmitted in the form of electrons from the chlorophyll-containing thylakoid membranes via ferredoxin, a 2Fe-2S protein, ferredoxin-thioredoxin reductase and thioredoxins to the target enzymes which are activated or deactivated through the reduction of regulatory disulfide bridges. The first essential member in this regulatory thiol chain is the enzyme ferredoxin-thioredoxin reductase (FTR). FTR accepts electrons from ferredoxin, one at the time, for the reduction of a catalytically active disulfide bridge which in turn reduces thioredoxins, the ubiquitous small disulfide reductases. The FTR, with an apparent molecular mass of ca. 30000, is composed of two nonidentical subunits, contains nonheme Fe and elementary S in a 4Fe-4S cluster and, on the smaller subunit, the catalytically active thiol groups involved in catalysis [4]. So far no function has been demonstrated for the Fe-S cluster. We are interested to know whether the Fe-S cluster has a catalytic function in the transfer of the electrons from ferredoxin to the disulfide bridge in the active site of FTR or whether it plays a purely structural role. In this paper we describe the specific modification of the active site disulfide of FTR in order to obtain a protein with blocked electron-transfer capacity for further studies of the Fe-S cluster.

Results and Discussion

We have tested several reagents for their efficiency in inhibiting the FTR and found that N-ethylmaleimide (NEM) is very specific and, for our purposes, the most suitable. The results presented in Table 1 show that an incubation of oxidized FTR with NEM does not significantly alter the activity of the enzyme indicating that there is no free thiol present or accessible that would be important for enzyme activity. However, when the enzyme has first been reduced by ferredoxin we find that there is a complete loss of activity suggesting that NEM has specifically modified residues essential for catalysis. Since NEM under the experimental conditions applied reacts very specifically with thiol groups [5] and we know from earlier reports [6] that the active site contains a reducible disulfide bridge, we can conclude that NEM specifically blocks Cys residues of the active site of reduced FTR. Additional experiments showed that this reaction is complete in less than 2 min.

To quantify the number of modified thiol groups per enzyme molecule we have measured the incorporation of 14C-labelled NEM into FTR (Table 2). After incubation of the enzyme in the activation mixture in the dark, when no reduction of the active site disulfide occurs, only a minor amount of 14C-NEM is incorporated. This could be due to the modification of Cys residues which are only partially accessible and not important for catalytic activity since we observe no inhibition after incubation in the dark. According to our preliminary sequencing results [7] and unpublished the spinach FTR has two Cys residues, one on each subunit, which seem to be absent from the enzyme in other organisms suggesting that they do not have any particular function in catalysis. Unlike all the other Cys residues which we find at strictly conserved positions. After incubation in the light, when FTR has become reduced, 2 more equiv. of 14C-NEM are found per enzyme molecule. These results are in line with earlier reports [6] and suggest the appearance of a dithiol due to the reduction of the enzyme by ferredoxin. We, therefore, conclude that NEM blocks specifically the two Cys residues which constitute the active site of FTR.

Since the active-site disulfide of FTR has been located on the smaller subunit of the spinach enzyme [6] we investigated whether the 14C-NEM incorporated in the

| Table 1. Inhibition of Ferredoxin-Thioredoxin Reductase by N-Ethylmaleimide |
|-----------------------------|-------------------------------|
| Incubation conditions       | FTR activity in % (NEM-treated) |
| 10 min dark                 | 100                            |
| 5 min dark, 5 min light     | 100                            |

) FTR activity is measured as fructose 1,6-bisphosphatase activity after activation of this enzyme in the ferredoxin/thioredoxin system. 100% = 600 amol substrate hydrolyzed per min.

<table>
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<tr>
<th>Table 2. Light-Dependent Incorporation of 14C-N-Ethylmaleimide into Ferredoxin-Thioredoxin Reductase</th>
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<tr>
<td>Incubation conditions</td>
</tr>
<tr>
<td>mol NEM/mol FTR</td>
</tr>
<tr>
<td>dark, FTR oxidized</td>
</tr>
<tr>
<td>light, FTR reduced</td>
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<td>light minus dark</td>
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light is found also in the smaller subunit of FTR. For that purpose the 14C-NEM modified enzyme was subjected to polyacrylamide gel electrophoresis under denaturing conditions, the separated subunits transferred electrophoretically to nitrocellulose and the membrane exposed to X-ray film. The results of the autoradiography confirmed that the light-dependently incorporated radioactivity is located in the smaller, catalytic subunit only. The small amount of radioactivity incorporated into the oxidized FTR appears to be evenly distributed between the two subunits which suggests that the 14C-NEM has reacted with the two unconserved Cys residues mentioned above and that these free Cys are relatively inaccessible.

We were further interested to know whether the chemical modification of the active site had any influence on the spectral properties of the protein. For that purpose we developed a method for the rapid purification of NEM-modified enzyme from contaminating components of the reaction mixture by two dimensional chromatography. The NEM-modified FTR showed the same chromatographic behaviour as the untreated protein. It was tested for activity and for changes in its spectral properties. As expected from a covalent modification the modified FTR showed no activity also after chromatographic separation. The Figure presents the absorption spectra of FTR before and after modification with NEM. The visible absorption of the native FTR is due to the Fe-S cluster and is typical for a 4Fe-4S protein with a peak at ca. 410 nm. The modification with NEM causes the visible absorption of the protein to increase, as can be seen from the difference spectrum, and a new peak at 330 nm to appear. The general aspect of the spectrum, however, is unchanged. These observations suggest that the Fe-S cluster is undamaged by the modification of the active site. However, the increase in absorbance might be due to a spacially close contact between the Fe-S cluster and the active site disulfide. Further experiments are under way to characterize the Fe-S cluster in the native and NEM-modified enzyme and to look for a possible function of the cluster in catalysis.

Experimental

Materials. Chemicals and biochemicals, purchased either from Fluka, Boehringer, or Sigma, were of anal. grade. FTR, thioredoxin, ferredoxin, and fructose 1,6-bisphosphatase were purified from spinach leaves according to our earlier described procedures [8][9]. Enzyme reactions and spectra were recorded with a computer-controlled Perkin Elmer Lambda 17 spectrophotometer. Polyacrylamide gel electrophoresis was performed according to [10].

Modification of FTR with NEM was done in the following reaction mixture (100 μl total volume): 100 mM NaK-phosphate buffer pH 7,0, 10 mM Na-acetate, 0.1 mM dichlorophenolindophenol, 30 μM ferredoxin, heated thylakoids equivalent to 10 μg chlorophyll [11]. 12.6 μM FTR. The gas phase was Ar and the temperature 25°. After 5 min equilibration of the reaction mixture with Ar, light was turned on to reduce the enzyme. After another 5 min NEM was added to 1 mM and 2 min later the reaction was quenched by the addition of a large excess of 2-mercaptoethanol. Before the addition of NEM and at the end of the incubation period 1 μl aliquots were removed from the incubation mixture and injected into 20 μl of 20 mM 2-mercaptoethanol. The quenched samples were subsequently assayed for FTR activity by measuring their capacity to catalyze chloroplast fructose 1,6-bisphosphatase activation in the complete ferredoxin/thioredoxin system under standard assay conditions [12]. The activity of 2.5 units of FBPase was measured after 5 min activation in saturating red light at 25°.

To quantify the number of modified Cys residues 14C-labelled NEM of known specific activity replaced the NEM in the above reaction mixture and its incorporation into the protein was determined after incubation in the dark and in the light. The quenched reaction mixtures were centrifuged for 10 min at 27 000 g to remove the thylakoid membranes. To the clear supernatant 200 μg bovine serum albumine were added as carrier protein and the total protein precipitated by addition of an equal volume of cold 20% trichloroacetic acid. The precipitate was collected by centrifugation, 5 min 27 000 g, washed three times with 10% trichloroacetic acid, dissolved in 200 μl 100 mM Tris-Cl buffer pH 7.9 containing 0.1% sodium dodecyl sulfate and the specific activity determined in a Betamatic liquid scintillation counter.

Purification of NEM-modified enzyme by two-dimensional chromatography (FPLC): several quenched reaction mixtures were combined, centrifuged as above to remove thylakoid membranes and chromographehd automatically through a G-25S fraction (1 x 10 cm) and a Mono Q (0.5 x 5 cm) column connected in series on a FPLC (Pharmacia) equipment. The columns were equilibrated with 20 mM triethanolamine-Cl buffer pH 7.3 containing in addition 100 mM NaCl for the desalting step. The proteins were eluted from the anion exchange column with a 20 ml 0-600 mM NaCl gradient. The FTR obtained was pure as judged by gel electrophoresis.

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Figure. Absorption spectra of native (solid line) and NEM-modified FTR (dashed line). From the absorption spectra, normalized at 278 nm, the difference spectrum, NEM-modified minus native FTR (dotted line), has been calculated.