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Reduction of ferredoxin:thioredoxin reductase by artificial electron donors *

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Abstract

The ferredoxin:thioredoxin reductase is an essential enzyme of the light dependent regulatory system in oxygenic photosynthesis. It is composed of two dissimilar subunits and contains a 4Fe-4S cluster and a redox-active disulfide bridge. Artificial electron donors of redox potentials below −300 mV are capable of reducing the disulfide bridge. Based on our results we speculate that a group of more negative potential than the disulfide bridge is the first acceptor of the electrons in FTR. The chemical reduction of FTR has been used successfully for the detection of the enzyme during its purification.

Abbreviations: FBPase – fructose 1,6-bisphosphatase; FTR – ferredoxin:thioredoxin reductase; MV – methyl viologen

Introduction

Ferredoxin:thioredoxin reductase (FTR) is the key enzyme for the reductive regulation of photosynthetic regulatory enzymes (Buchanan 1992). It is a soluble, colored Fe-S protein, composed of two dissimilar subunits (Droux et al. 1987) each with a molecular mass of approximately 13 kD, encoded in the nucleus (Marc-Martin et al. 1993; Falkenstein et al. 1994). Whereas one subunit, the socalled variable subunit, has no known catalytic function (Iwadate et al. 1994), the second subunit, labelled the catalytic subunit, contains a 4Fe-4S cluster and a redox-active disulfide bridge (Chow et al. 1995). The disulfide bridge, with a midpoint potential of −230 mV is functional in the reduction of chloroplast thioredoxins, which have midpoint potentials of −210 mV (Salamon et al. 1995). The two electrons needed for their reduction are in vivo provided by reduced ferredoxin (Knaff and Hirasawa 1991), a one electron donor. Since FTR forms a 1:1 complex with ferredoxin (Hirasawa et al. 1988; De Pascalis et al. 1994) the complete reduction of the disulfide bridge by two electrons apparently has to proceed in two steps. It is not known whether the 4Fe-4S cluster has some function in the storage or in the transfer of the electrons from ferredoxin to the disulfide bridge of the FTR. Sofar only positive redox potentials of +410 mV (De La Torre et al. 1982) and +340 mV (Salamon et al. 1995) have been reported for this Fe-S cluster. They would exclude its involvement in the transfer of electrons from ferredoxin to the disulfide bridge. However, recent structural analyses have shown, that the ligation of the cluster in the protein is quite unique and that the cluster is very closely associated with the redox-active disulfide bridge (Chow et al. 1995). This arrangement may enable the cluster to be involved in the electron transfer to the disulfide bridge.

In the present report we show that FTR can be very efficiently reduced by artificial electron donors of sufficiently negative redox potential.

* Dedicated to Prof. D.I. Arnon.

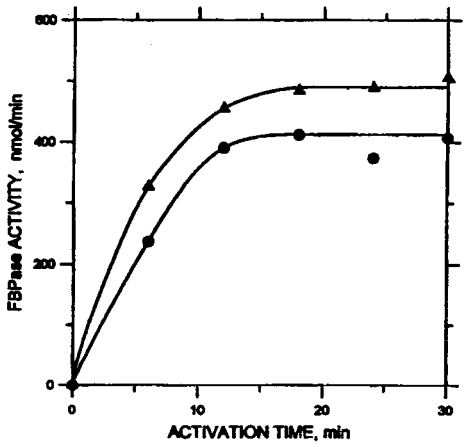

Fig. 1. Time course of FBPase activation with dithionite reduced ferredoxin (▲) and dithionite reduced MV (●). Experimental conditions were as given in Materials and methods, except that ferredoxin and MV were present at 2 μM concentration.

Materials and methods

All chemicals and biochemicals used were of analytical grade. FTR from spinach leaves (*Spinacea oleracea*) and thioredoxin *f* were purified as described in detail elsewhere (Schürmann 1995). FBPase was isolated and purified from spinach leaves essentially according to Schürmann and Wolosiuk (1978).

FTR reduction was tested by its capacity to activate chloroplast fructose 1,6-bisphosphatase in presence of thioredoxin *f* as described earlier (Schürmann and Jacquot 1979). The activations were performed in Eppendorf tubes at room temperature. The activation mixtures contained, if not otherwise indicated, in a final volume of 100 μl 100 mM Tris-Cl pH 7.3, 0.2 μM FTR, 1 μM recombinant thioredoxin *f*, 0.5 units chloroplast FBPase, 1 μM methylviologen, 14 mM 2-mercaptoethanol. After 5 min under argon, 5 mM dithionite dissolved in 100 mM Tris-Cl pH 7.9 and kept under argon was added anaerobically with a Hamilton Microlab pipettor. After 10 min activation in presence of dithionite an aliquot was withdrawn and injected into 1 ml reaction mixture. The reaction mixture contained 100 mM Tris-Cl pH 7.9, 0.1 mM EGTA-Na, 1.5 mM $MgSO_4$, 1 mM fructose 1,6-bisphosphate, 0.3 mM NADP, 1.75 units phosphoglucose isomerase, 0.7 units glucose 6-phosphate dehydrogenase and 14 mM 2-mercaptoethanol. The reaction was followed at 340 nm and 25 °C in a Perkin-Elmer Lamba 16 spectrophotometer. Activities are expressed as nmol/min per total activation mixture volume.

Table 1. Requirements for the activation of FBPase with dithionite. Experimental conditions were as given in Materials and methods

	nmol/min	% control
Complete	404	100
- dithionite	4	1
- MV	1.1	0.3
- FTR	0.8	0.2
- thioredoxin *f*	6.8	1.6
- FBPase	0.8	0.2
- 2-mercaptoethanol	388	96

Results and discussion

When testing the activity of the FTR it is routinely done in a two stage assay system. During an activation phase chloroplast FBPase is reduced and activated depending on FTR in the presence of saturating amounts of thioredoxin *f*. The FTR in turn is reduced using light, heated thylakoids (Jacquot et al. 1984) and ferredoxin, a rather complicated system (Schürmann and Jacquot 1979). In looking for alternative, simpler methods of reducing FTR we have realized that FTR can be efficiently reduced with dithionite and ferredoxin or dithionite in presence of certain mediators. Figure 1 shows the time course of FBPase activation with dithionite reduced FTR. This activation kinetic is entirely comparable to the kinetics obtained with light reduced ferredoxin and is strictly dependent on the presence of ferredoxin.

When ferredoxin is replaced by methyl viologen (MV), similar activation kinetics are observed, as shown in Fig. 1. This result indicates that MV can efficiently replace ferredoxin in the reduction of FTR. In Table 1 we present the requirements of the activation system where the light dependent reduction system has been replaced by dithionite and MV. The activation is strictly dependent on dithionite, MV and FTR, as well as on thioredoxin *f* and FBPase. The omission of one of these components abolishes the activation completely. However, the presence of 2-mercaptoethanol is not needed and we observed that its stimulating effect is variable. The stimulation may be due to the protection of FTR and FBPase during the preparation of the reaction mixtures when these two enzymes are present at rather low protein concentrations and exposed to a pH which is not optimal for their storage. In addition 2-mercaptoethanol stabilizes the activated FBPase (Schürmann and Jacquot 1979).

Table 2. Effectiveness of different mediators in the reduction of FTR. Experimental conditions were as given in Materials and methods. All electron donors were present at 1 μM concentration

	nmol/min	% of MV	E_m in mV
Methyl viologen	490	100	−430
Benzyl viologen	480	98	−311
Anthraquinone 2-sulphonate	10	2	−225
Anthraquinone 2,6-sulphonate	6.3	1.3	−185
Anthraquinone	1.4	0.3	−100
100 Duroquinone	1.4	0.3	+10
Ferredoxin	527	107	−420

Table 2 shows the effectiveness of different electron donors in the reduction of FTR. Only ferredoxin, as the natural electron donor with a midpoint potential of −420 mV, and the two viologens, methyl viologen and benzyl viologen, with redox potentials of below −300 mV, are able to reduce the FTR and thereby activate the FBPase. Electron donors with more positive redox potentials are unable to reduce the disulfide bridge of the FTR, which has a midpoint potential of −230 mV (Salamon et al. 1995). Although the present results provide no direct experimental evidence it seems unlikely that the viologens reduce the disulfide bridge directly. The fact that anthraquinone 2-sulphonate, which has about the same redox potential as the disulfide bridge of the FTR, is unable to catalyze the activation of FBPase, even up to a 500 fold excess over FTR, lets us speculate that a group with a more negative potential than the disulfide bridge is the primary electron acceptor in the FTR. This group could be related to the Fe-S cluster.

We have successfully used the described activation system, which includes dithionite and MV as described in Materials and methods, instead of the more complicated light dependent system, to follow FTR activity during its purification by column chromatography. The reactions were always strictly specific for the FTR and in no instance did we observe any unspecific reaction.

In summary, these results provide the first evidence that FTR can be efficiently reduced with viologens as artificial electron donors. This observation has been exploited to devise a simplified detection system for the FTR.

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