Kinetics and Thioredoxin Specificity of Thiol Modulation of the Chloroplast H^+-ATPase*

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The kinetics of thiol modulation of the chloroplast H^+-ATPase (CF_oCF_i in membrana) were analyzed by employing thioredoxins that were kept reduced by 0.1 mM dithiothreitol. The kinetics of thiol modulation depend on the extent of the proton gradient. The process is an exponential function of the thioredoxin concentration and reaction time and can be described by an irreversible second order reaction. The results indicate that the formation of the complex between thioredoxin and CF_oCF_i is slow compared with the subsequent reduction step. Furthermore we have compared the efficiencies of the Escherichia coli thioredoxin Trx and the two chloroplast thioredoxins Tr-m and Tr-f. The second order rate constants are 0.057 (Tr-f), 0.024 (Trx), and 0.010 s^{-1} μM^{-1} (Tr-m) suggesting that Tr-f rather than Tr-m is the physiological reductant for the chloroplast ATPase. The often employed artificial reductant dithiothreitol exhibits a second order rate constant in thiol modulation of 1.02·10^{-6} s^{-1} μM^{-1}.

The H^+-translocating ATPase of chloroplasts (CF_oCF_i, chloroplast ATP synthase) is a latent enzyme. Its physiological activation requires a transmembrane electrochemical proton potential difference (1–4). Hence, the proton gradient in addition to its role as the driving force of phosphorylation is a factor that controls CF_oCF_i activity. The obvious physiological meaning of this control mechanism is the suppression of unproductive ATP hydrolysis under conditions that would energetically allow this reaction, i.e. at low proton gradients (low light or dark) and high phosphate potentials.

A superimposed regulatory device is the so-called thiol modulation of CF_oCF_i. The structural basis for thiol modulation is a sequence motif of nine amino acids comprising two cysteines in the γ subunit of CF_i (5). This segment is present in higher plants (6) and green algae (7) but not in cyanobacteria (8–10) or in diatoms (11) suggesting that thiol modulation is an acquisition of the chlorophyll a + b plants only. In the demodulated (oxidized) state the two cysteines form a disulfide bond whereas the modulated state is obtained by reduction of this disulfide bridge. In vitro reduction can be achieved by dithiothreitol or other dithiols, but the natural reductant is a reduced thioredoxin. In chloroplasts at least two different thioredoxins occur, thioredoxin-m (Tr-m) and thioredoxin-f (Tr-f) (12). The former is thought to be involved in light/dark regulation of the chloroplast NADP-specific malate dehydrogenase, and the latter is responsible for the light/dark regulation of fructose bisphatase and other Calvin cycle enzymes (13, 14). The thioredoxins are reduced via ferredoxin and ferredoxin-thioredoxin reductase (15) by electrons from the photosynthetic electron transport chain. In most of the experiments carried out so far, however, thiol modulation of CF_oCF_i was conducted with the artificial reductant dithiothreitol, and in a few studies Escherichia coli thioredoxin (Trx) was used (16, 17). Little information is known about the action of the naturally occurring chloroplast thioredoxins on CF_oCF_i (18–20).

Thiol modulation requires illumination of the chloroplasts to allow reduction of the disulfide bridge. Apparently, the regulatory segment of the γ subunit, which is hidden in the dark, becomes accessible as a consequence of ΔμH^+−-induced CF_oCF_i activation (16, 21). Decay of the proton gradient in the dark leads to deactivation of the ATP synthase. The most significant difference between the reduced and oxidized active states concerns the velocity of deactivation. While the oxidized form is immediately deactivated upon relaxation of the gradient, deactivation of thiol-modulated CF_oCF_i takes several minutes. For this reason only chloroplasts with thiol-modulated CF_oCF_i are capable of hydrolyzing added ATP after transition from light to dark (22, 23).

Deactivation of the modulated enzyme may proceed with or without reoxidation of the dithiol group (16). Most likely the natural actual oxidant is the oxidized form of thioredoxin. Hence at least four CF_oCF_i forms may be discerned: the oxidized inactive (E^ox) and active (E^{oxa}) enzyme, and the reduced inactive (E^{red}) and active (E^{reda}) enzyme. Due to the lower deactivation rate, the equilibrium of active to inactive enzyme is shifted toward lower proton gradients when CF_oCF_i is in the reduced form (24). Fast deactivation of the modulated enzyme at light-dark transition is achieved by micromolar concentrations of ADP (25, 26) accompanied with tight binding of the nucleotide molecule to one of the three catalytic sites (27).

In the present paper the interaction of CF_oCF_i with thioredoxin is analyzed kinetically, and the thioredoxin specificity for thiol modulation is investigated. The efficiencies can be expressed by rate constants for the binding of the different thioredoxins.

EXPERIMENTAL PROCEDURES

Chloroplast thylakoids were isolated from spinach leaves as described in Ref. 28. The reaction medium contained 25 mM Tricine buffer, pH 8.0, 50 mM KCl, 5 mM MgCl_2, 50 μM phenazine methosulfate, 2 mM phosphoenolpyruvate, 20 units/ml pyruvate kinase, 50 mM valinomycin (to cancel the electrical potential difference); the total volume was 2.5
ml. Further additions (dithiothreitol and thioredoxin) are indicated in the legends. The concentration of thioredoxin was equivalent to 25 μg of chlorophyll/ml.

Recombinant spinach chloroplast thioredoxins were produced as described in Ref. 29. All experiments were conducted at 20°C with clamped pH (30), which was measured by the 9-aminoacridine fluorescence quench (31) and monitored as described in Ref. 32. The concentration of 9-aminoacridine was 5 μM. The calibration of the fluorescence signal was done as in Refs. 33 and 34. The activity of reduced CF$_0$CF$_1$ was measured by the initial ATP-hydrolyzing activity at pH 0 reflecting the amount of active and reduced CF$_0$CF$_1$ since in all our experiments the thioredoxins were kept under the employed conditions; all enzyme molecules should become reduced at infinite reaction time independent of the extent of the proton gradient and the thioredoxin concentration. On the other hand, the enzyme activity should depend on the ΔpH to indicate the equilibrium between the reduced active and the reduced inactive form. For a general kinetic analysis we used commercially available Trx from E. coli. In Fig. 1, time courses of thiol modulation are shown at 3 μM Trx and three different extents of membrane energization. The final enzyme activities reflecting the equilibrium $E^\text{red} \leftrightarrow E^\text{ox}$ increase with increasing light intensity. At ΔpH 2.7 about 50% of the maximal activity is obtained. By acid-base transition Junesch and Gräber (24) found half-maximal activity of the reduced enzyme at ΔpH 2.2. At ΔpH 2.7 they found about 50% of the maximal phosphorylation rate and concluded that in case the enzyme is in the thiol-modulated form, phosphorylation is limited by the catalytic reaction whereas the activity of the ATP synthase is the limiting factor for phosphorylation when the enzyme is oxidized. More recent results, however, have shown that the "activating protons" cannot be discerned kinetically and that the activating protons show the same cooperativity as the "catalytic protons" (37). Hence activation may be a step of the catalytic cycle, and the two processes should have the same ΔpH profile. The apparent difference of the profiles may be due to the fact that the two processes, which have been measured under rather different experimental conditions, are affected differently by factors like ADP or phosphate concentrations (33).

The initial rates of thiol modulation likewise depend on the employed ΔpH (Fig. 1). According to the reaction scheme the rate should be dependent on the concentration of active oxidized enzyme molecules present at the beginning of thiol modulation, i.e., on the equilibrium $E^\text{ox} \leftrightarrow E^\text{red}$ established by preillumination. Accordingly, while the steady-state activities in Fig. 1 represent the activation equilibrium of the reduced form of CF$_0$CF$_1$, the initial rates of thiol modulation represent the activation equilibrium of the oxidized form. Compared with the reduced CF$_0$CF$_1$, the activation profile of the oxidized enzyme is shifted towards higher ΔpH values (24). The initial rates and the final levels of thiol modulation likewise depend differently on ΔpH in the expected manner (Table I).

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According to the results of Junesch and Gräber (24) both activation equilibria should be completely on the side of the active forms at ΔpH ≈ 4. To create clear-cut conditions for the action of thioredoxin, we employed illumination at saturating light intensity to achieve a saturating proton gradient. Saturation was ascertained by the fact that the activity of the oxidized enzyme could not be increased by a further increase of ΔpH. At light saturation the extent of the gradient is well above 4 ΔpH units, but in this range where the 9-aminoacridine calibration curve shows a progressive deflection from linearity, the ΔpH could not be precisely determined.

**RESULTS**

The combined reversible activation/thiol modulation process may be described by Scheme 1.

$$E^\text{ox} \Delta pH \rightarrow E^\text{red}$$

**SCHEME 1**

The transition of the active oxidized form to the active reduced form depends on the nature and concentration of the reductant and includes reversible binding of the reductant followed by reduction of the disulfide bridge in the γ subunit. The backward reaction (reoxidation by the oxidized form of thioredoxin (16)) is largely excluded under the employed conditions since in all our experiments the thioredoxins were kept reduced by excess dithiothreitol.

The kinetic measurements of thiol modulation are based on the suppositions that follow. 1) The ATP-hydrolyzing activity at ΔpH 0 reflects the amount of active and reduced CF$_0$CF$_1$ since only an active enzyme is able to catalyze ATP hydrolysis and only the reduced enzyme retains its activity after transition to ΔpH 0. 2) Thiol modulation requires the enzyme to be in its activated oxidized state.

The reduction step is practically irreversible under the employed conditions; all enzyme molecules should become reduced at infinite reaction time independent of the extent of the proton gradient and the thioredoxin concentration. On the other hand, the enzyme activity should depend on the ΔpH to indicate the equilibrium between the reduced active and the reduced inactive form. For a general kinetic analysis we used commercially available Trx from E. coli. In Fig. 1, time courses of thiol modulation are shown at 3 μM Trx and three different extents of membrane energization. The final enzyme activities reflecting the equilibrium $E^\text{red} \leftrightarrow E^\text{ox}$ increase with increasing light intensity. At ΔpH 2.7 about 50% of the maximal activity is obtained. By acid-base transition Junesch and Gräber (24) found half-maximal activity of the reduced enzyme at ΔpH 2.2. At ΔpH 2.7 they found about 50% of the maximal phosphorylation rate and concluded that in case the enzyme is in the thiol-modulated form, phosphorylation is limited by the catalytic reaction whereas the activity of the ATP synthase is the limiting factor for phosphorylation when the enzyme is oxidized. More recent results, however, have shown that the "activating protons" cannot be discerned kinetically and that the activating protons show the same cooperativity as the "catalytic protons" (37). Hence activation may be a step of the catalytic cycle, and the two processes should have the same ΔpH profile. The apparent difference of the profiles may be due to the fact that the two processes, which have been measured under rather different experimental conditions, are affected differently by factors like ADP or phosphate concentrations (33).

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Fig. 2. Time course of thiol modulation at ΔpH > 4 with 0.5 and 3 μM reduced Trx. Experimental conditions are as shown Fig. 1.

Fig. 3. Thiol modulation as a function of reduced Trx concentrations at three different reaction times. Thiol modulation was conducted at ΔpH > 4. Further experimental conditions are as shown in Fig. 1.

Fig. 4. Thiol modulation as a function of the concentrations of reduced Tr-f, Trx, and Tr-m. Thiol modulation was conducted at ΔpH > 4. The ATP-hydrolyzing activity was determined after 10 s of thiol modulation. Further experimental conditions are as shown in Fig. 1.

Table II

| Reductant | k  |
|-----------|----|
| Tr-f      | 0.057 |
| Trx       | 0.024 |
| Tr-m      | 0.010 |
| Dithiothreitol | 1.02 · 10⁻⁶ |

Thiol modulation of CF₆CF₁ in membrana requires illumination of the thylakoids and a dithiol reductant to reduce the disulfide bond in subunit γ. Originally this complex interplay was expressed by the term “light-triggered ATPase” (22, 39).

Now it is clear that light is necessary for CF₆CF₁ activation and that it is the transmembrane proton gradient that is essential (1). As a consequence of energization a series of conformational changes occur in CF₆CF₁. Changes in the region of the nucleotide binding sites located in the α-β subunits lead to the release of tightly bound adenine nucleotides (1, 2, 28), γ subunit is accessible to chemical modification by pyridoxal 5'-phosphate (41). Likewise the so-called “light site” of the γ Subunit becomes accessible to chemical modification by pyridoxal 5'-phosphate (41).
subunit, a cysteine residue in position 89, becomes accessible to modification by o-phenylenediamine (42). Similarly the target sequence for thiol modulation, the disulfide bridge formed between Cys-199 and Cys-205 in the γ subunit, is exposed. This domain, which is hidden in the inactive enzyme, becomes available to tryptic cleavage upon activation (43) and accessible to reduction by dithiol reductants. At full CF$_0$CF$_1$ activation the pure kinetics of reduction can be measured, and a second order rate constant can be determined. The rate constant depends of course on the nature of the reductant and is much higher for the thioredoxins than for dithiothreitol.

In the basic work of Mills et al. (20) it was shown that Tr-f is active in unmasking the ATPase in thylakoids in the light. The result reported here suggests that Tr-f (rather than Tr-m) is indeed the natural reductant of CF$_0$CF$_1$ in the chloroplast. Tr-m is even less effective than Trx from E. coli as catalyst for the reaction between Trx and the enzyme, as first described by Dann and McCarty (16) by using fluorescent probes. This domain, which is hidden in the inactive enzyme, becomes available to tryptic cleavage upon activation (43) and accessible to reduction by dithiol reductants. The sequences of Trx from E. coli and human thioredoxin were 10 times less effective. They have the same general architectures as the other analyzed thioredoxins. Graphic analyses of the chloroplast thioredoxins confirm that they have the same general architectures as the other analyzed thioredoxins. However, due to their sequences the two chloroplast proteins show quite different surface structures. The surface area of Tr-f around the accessible active site cysteine is not as flat and hydrophobic as that of Trx but quite structured and surrounded by positive and negative charges. These charges are probably instrumental for the proper orientation during the protein-protein interaction. The corresponding surface area of Tr-m resembles much more the one of Trx, except for one boundary area. The fact that Tr-m is less efficient in thiol modulation than Trx may be due to some topological difference between these on the whole rather similar proteins. It might also be due to the replacement in the boundary area of Tr-m of three hydrophobic residues present in Tr-f and Trx by three charged residues: V86/R/A87/K/A88/E (notation of E. coli- Trx; the corresponding amino acids of Tr-m are in parentheses). These changes may reduce the binding of Tr-m to the coupling factor and therefore be responsible for its lower efficiency compared with Tr-f and Trx.

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