Mechanism of Activation of the Chloroplast ATP Synthase

A KINETIC STUDY OF THE THIOL MODULATION OF ISOLATED ATPase AND MEMBRANE-BOUND ATP SYNTHASE FROM SPINACH BY ESCHERICHIA COLI THIROEDOXIN

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The mechanism of thiol modulation of the chloroplast ATP synthase by Escherichia coli thioredoxin was investigated in the isolated ATPase subcomplex and in the ATP synthase complex reconstituted in bacteriorhodopsin proteoliposomes. Thiol modulation was resolved kinetically by continuously monitoring ATP hydrolysis by the isolated subcomplex and ATP synthesis by proteoliposomes. The binding rate constant of reduced thioredoxin to the oxidized ATPase subcomplex devoid of its ε subunit could be determined. It did not depend on the catalytic turnover. Reciprocally, the catalytic turnover did not seem to depend on thioredoxin binding. Thiol modulation by Trx of the ε-bearing ATPase subcomplex was slow and favored the release of ε. The rate constant of thioredoxin binding to the membrane-bound ATP synthase increased with the protonmotive force. It was lower in the presence of ADP than in its absence, revealing a specific effect of the ATP synthase turnover on thioredoxin-γ subunit interaction. These findings, and more especially the comparisons between the isolated ATPase subcomplex and the ATP synthase complex, can be interpreted in the frame of the rotational catalysis hypothesis. Finally, thiol modulation changed the catalytic properties of the ATP synthase, the kinetics of which became non-Michaelian. This questions the common view about the nature of changes induced by ATP synthase thiol modulation.

F₀F₁, proton ATPase (or ATP synthase) is responsible for ATP synthesis in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts, and the cytoplasmic membrane of bacteria (for review, see Ref. 1 and all papers in the same issue). The catalytic sector F₁, with subunits α, β, γ, δ, and ε (stoichiometry α₃β₂γδε₁) is a water-soluble entity bearing catalytic and noncatalytic nucleotide binding sites (for review, see Ref. 2). The X-ray structure of the major part of this subcomplex has been elucidated in the case of the bovine heart mitochondrial species (3). The F₀ sector is membranous and forms a proton-channeling device converting the energy of the electrochemical proton gradient ΔpH into mechanical energy. In chloroplasts, it contains the four subunits I, II, III, and IV in the probable stoichiometry I₁II₁III₉–₁₂IV₁. The number of ε subunits (subunit III equivalent) per complex was recently found to be 10 in crystals of the yeast mitochondrial ATP synthase (4). ATP hydrolysis probably results in the rotation of the γ subunit within the α₃β₂ε crown (5–8) coupled to the rotation of the oligomer of ε subunits (9). The whole F₀F₁ complex is thought to act as a rotatory proton-driven motor (for reviews, see Refs. 10 and 11), the stator containing subunits I, II, IV, α, β, and γ, and the rotor subunits III, γ, and ε in the case of the chloroplast enzyme.

It has been known for many years that the electrochemical proton gradient is necessary to activate CF₀CF₁, in addition to supplying it with energy for ATP synthesis (12, 13). In addition, reduction by a dithiol of a specific disulfide bridge located on the γ subunit (14) diminishes the magnitude of the ΔpH required to activate the enzyme (15, 16). This process is called thiol modulation. Thiol modulation itself is energy-dependent (13, 17). It is not yet possible to locate the two cysteines involved (γCys¹⁹⁹–γCys²⁰⁵) in the tridimensional structure because the only F₁ structure that has been published until now is that of the mitochondrial enzyme (3, 4), in which there is no domain homologous to that containing γCys¹⁹⁹ and γCys²⁰⁵. In fact, these two cysteines are included in an insertion specific of CF₁ (18). In vivo, the γ subunit reduction is achieved via a thioredoxin (Trx) by electrons diverted from photosystem I (19, 20). Two different Trxs, called f and m, are present in chloroplasts. On the basis of slightly better catalytic efficiency, Trx f has been proposed to be the physiological reductant of CF₁. However, Trx m is also able to reduce CF₁ with a good efficiency (21). Escherichia coli Trx, which is more similar in primary structure to Trx m than to Trx f, is more efficient than the former toward CF₁ reduction and has already been used for in vitro studies of its interaction with CF₁ (21–23). Binding properties of E. coli Trx to the isolated CF₁ subcomplex, devoid or not of its ε inhibitory subunit, and in different redox states, have been investigated using fluorescent techniques (23). The kinetics of thiol modulation of CF₀CF₁ by different Trxs in energized thylakoid membranes has been analyzed and the rate constant of binding determined in the presence of a high protonmotive force (21).

Because the domain of interaction of CF₁ with Trx belongs to the γ subunit, it is expected to rotate during the enzyme turn-
over. It is then of particular interest to study the Trx interaction in the isolated CF₁ subcomplex and in the membrane-bound CF₀CF₁ and to investigate carefully the effect of the protonotive force and of the enzyme turnover on this interaction. The present paper is a kinetic analysis of the interaction of E. coli Trx with isolated spinach CF₁, devoid or not of the ε subunit, and with complete CF₀CF₁ reconstituted into liposomes. The role of different effectors was investigated. In the whole complex, but not in the isolated ATPase, the enzyme turnover proved to have a specific effect on this interaction, antagonistic to that exerted by the membrane energization. The consequences for the mechanism of thiol modulation were examined in relation to the rotary mechanism hypothesis. The catalytic properties of oxidized and thiol-reduced CF₀CF₁ were also investigated, and it was shown that thiol modulation does not simply consist of an increase in the proportion of active ATP synthases at a given magnitude of the protonotive force.

**Experimental Procedures**

**Purification of CF₁, CF₁-ε, and CF₀CF₁ from Spinach Leaves—**The soluble chloroplast ATPase (CF₁) was extracted from spinach (Spinacia oleracea L., leaves) and purified under two forms, containing or not the ε subunit (24). Protein concentration was determined by UV absorption spectroscopy assuming an extinction coefficient of 0.48 cm²mg⁻¹ at 278 nm (25). The enzymes were stored at 5 °C in 34% ammonium sulfate. The CF₁, CF₁-ε, ATP synthase complex was extracted and purified from spinach leaves following the procedure described in Ref. 26. Its concentration was determined using the BCA protein assay reagent from Pierce. The purified complex was kept frozen in small vials under liquid nitrogen before use in reconstitution experiments.

**Purification of Thioredoxin from E. coli—**The E. coli thioredoxin overexpression plasmid pFPI (27) was used to transform a DH5a E. coli strain (Life Technologies). Protein purification to homogeneity was performed with 24-h bacterial cultures, essentially as described in (27). The main steps were: disruption of bacteria with a French pressure cell, heat-shock (3 min, 80 °C), ammonium sulfate fractionation, size exclusion chromatography on G-50, and ion exchange on DEAE-Sepharose (both from Amersham Pharmacia Biotech).

**Purification of Bacteriorhodopsin—**Purple membrane from Halobacterium salinarium was isolated according to Oesterhelt and Stoeckenius (28) and stored for months at −20 °C. Bacteriorhodopsin at a final concentration of 4–5 mg ml⁻¹ in the reconstitution buffer was monomerized by incubation in presence of 2% (31 ml) Triton X-100 for 20 h at 4 °C in the dark. The concentration was determined from the absorption at 560 nm (ε = 52, 800 M⁻¹cm⁻¹).

**Coreconstitution of Bacteriorhodopsin and CF₁, CF₁-ε, into Liposomes—**Unilamellar liposomes were made by reverse-phase evaporation from a mixture of 15 mM egg phosphatidylcholine, 2 mM egg phosphatidic acid, and 20 mM MOPS, pH 7.3, 50 mM Na₂SO₄, and 50 mM K₂SO₄. Then, 80 μl of 200 mg ml⁻¹ Triton X-100 was mixed with 60 μg of CF₁, CF₁-ε. 2 min later, 100 μl of 4 mg ml⁻¹ bacteriorhodopsin was added to the mixture, which was then supplemented with reconstitution buffer (final volume 1.5 ml). 0.5-ml liposomes were added and incubated for 10 min at room temperature. The detergent was then removed with three successive additions of SM-2 Bio-Beads (first one, 80 mg; second one, 80 mg 1 h later; third one, 160 mg 1 h after the second). Bio-Beads were then eliminated by rapid filtration on glass cotton. Proteoliposomes were stored on ice and in darkness. Their activity was stable for 1 day (for details, see Ref. 29).

**ATP Hydrolysis Measurements with the Isolated ATPase Complex—**CF₁ or CF₁-ε was diluted at a concentration of 10 μM (unless otherwise indicated) or 1.3 nM, respectively, in a 1-mL spectrophotometric microcuvette, stirred, and thermostatted at 37 °C. The reaction medium (1 ml) contained 50 mM Tris-Cl, 40 mM KHCO₃, pH 8.0, 4 mM MgSO₄, 1 mM P-enolpyruvate, 0.45 mM NADH, 20 units/ml pyruvate kinase, 50 units/ml lactate dehydrogenase. KHCO₃ was omitted when indicated. The absorbance at 340 nm was monitored continuously. The time response of the coupled system was checked by ADP injections. The ATPase activity was triggered by injecting 2 mM MgATP in the reaction medium. For studies of the ATPase reduction by Trx, a small aliquot of Trx preincubated with 2 mM DTNB was injected into the reaction medium supplemented with 0.1 mM DTNB. In some experiments, prereduced Trx was injected in the reaction medium devoid of MgATP, and the reaction was initiated later by injecting ATP. The first 10 s following each addition were discarded in the data analysis.

**Steady-state ATP Synthesis Measurements—**Proteoliposomes were diluted 4-fold in the reconstitution buffer, pH 7.3, supplemented with 2 mM MgSO₄, 1 mM ADP, and 10 μM diadenosine pentaphosphate. The 0.4-ml sample was contained in a transparent vessel, stirred, and thermostatted at 20 °C. It was illuminated continuously with a strong yellow light, obtained by filtering the light produced by a halogen lamp (filter band pass 500–650 nm, beam intensity 1.5 kW m⁻²). After 10 min of illumination, ATP synthesis was initiated by adding 50 mM KH₂PO₄ preadjusted at pH 7.3. Small aliquots were taken up at different times, quenched with trichloracetic acid, and assayed for ATP concentration using the luciferin-luciferase technique as in Ref. 29.

Energy-induced thiol modulation of CF₁,CF₁ was carried out by adding 3 μM Trx and 0.1 mM DTT before starting the illumination. Experiments with NEM-inactivated Trx were carried out as follows. Trx and DTT were mixed in the reaction medium 10 min before the addition of 0.25 mM NEM. 5 min later, NEM was neutralized by the addition of 0.2 mM DTT. Liposomes were then added, illuminated, and assayed for ATP synthesis as described above.

Thiol modulation of CF₁,CF₁ by Trx in darkness was achieved as follows. Liposomes were diluted in the reaction medium containing 3 μM Trx and 0.1 mM DTT. The reaction was stopped by adding 0.25 mM NEM at a given time. 5 min later, 0.2 mM DTT was added to neutralize NEM, and the experiment of ATP synthesis was carried out as described above.

**Time-resolved Measurement of ATP Synthesis—**Proteoliposomes were diluted 5-fold in the reconstitution buffer, pH 7.3, supplemented with 50 mM KH₂PO₄, 4 mM MgSO₄, 10 μM diadenosine pentaphosphate, 4 mM glucose, 2 mM NADP⁺, 20 units/ml hexokinase, and 20 units/ml glucose-6-phosphate dehydrogenase. The 1-ml sample was contained in a spectrophotometric microcuvette, stirred, and thermostatted at 20 °C. The absorbance at 340 nm was monitored continuously. The time response and the proportionality between the rate of ATP production and the rate of NADP⁺ reduction were checked using myokinase at different concentrations in the presence of ADP and absence of diadenosine pentaphosphate. A small positive drift was detected in the absence of a known ATP-producing reaction. This drift was constant for 1 h; it did not depend on the presence of the liposomes and was not affected by the illumination. It was subtracted from the measured rates of NADP⁺ reduction. The sample was illuminated from the top of the cuvette with an optical fiber equipped with a yellow filter (filter band pass 500–650 nm, light intensity 0.8 kW m⁻²). The setup allowed injections into the cuvette to be made without interrupting the actinic light. The windows of the spectrophotometer were protected from the actinic light with highly selective filters transmitting at 340 nm. 10 min after the beginning of illumination, ATP synthesis was initiated by adding 1 mM MgADP to the liposome suspension. It was verified that the rate of ATP synthesis was practically constant for periods as long as 30 min. The reaction was allowed to proceed for 5 min, and then prereduced Trx was added in the presence of 0.1 mM DTT. ATP synthesis was then followed (time resolution 10 ms). Experiments were carried out by injecting NEM at a given time. 5 min later, NEM was neutralized by the addition of 0.2 mM DTT, and the reaction was initiated later by injecting MgADP. In studies at variable ADP concentrations, successive additions of substrate were made, separated by 4–5 min intervals. The first 10 s following each addition were discarded in all data analysis.

**δpH Measurements—**Proteoliposomes were diluted 20-fold in the reconstitution buffer, pH 7.3, supplemented with 50 mM KH₂PO₄, 1 mM MgSO₄, 4 μM 9-aminoacridine (both from Amersham Pharmacia Biotech). The suspension was contained in a fluorometric microcuvette, stirred, and thermostatted at 20 °C. Fluorescence (λexcitation = 400 nm, λemission = 450 nm) was recorded continuously. Illumination was provided by the same setup as for spectrophotometric measurements of ATP synthesis. 0.1 μM valinomycin was added during the illumination.

**Data Analysis—**Data were fitted to theoretical models by a nonlinear least squares iterative procedure based on Marquardt's algorithm, using program Origin 5.0.

**Reagents—**All reagents were of analytical grade. Egg phosphatidylcholine and egg phosphatidic acid were purchased from Avanti Polar Lipids. SM2 Bio-Beads came from Bio-Rad. FCCP, valinomycin, DTT, NEM, diadenosine pentaphosphate, and cholesterol were obtained from Sigma. ATP, ADP, P-enolpyruvate, NADH, NAD⁺, lactate dehydrogenase, and pyruvate kinase were obtained from Roche Molecular Biochemicals Ltd. Hexokinase and glucose-6-phosphate dehydrogenase were obtained from Calbiochem. The ATP-monitoring kit came from Bio-Orbit. 9-Aminoacridine was purchased from Fluka.
RESULTS

Rate of Activation of Oxidized CF1-ε by reduced thioredoxin—Fig. 1 shows how the rate of ATP hydrolysis catalyzed by isolated, oxidized CF1-ε changes after the addition of prereduced Trx into the reaction medium (a, direct curves; b, first derivatives). The ATPase starts to increase immediately after the addition of Trx and reaches a stable new value after a few minutes. Kinetics such as those displayed in Fig. 1b could be fitted satisfactorily with the equation

\[ V(t) = V_{eq} + (V_0 - V_{eq})e^{-kt_{on}} \]  

(Eq. 1)

where \( V(t) \) is the rate of ATP hydrolysis at a given time \( t \) after the addition of Trx, \( V_0 \) the rate of ATP hydrolysis at zero time (just before the addition of Trx), and \( V_{eq} \) is the final rate of ATP hydrolysis. \( V_0 \) (as a control), \( V_{eq} \), and \( k_{app} \) are plotted in Fig. 2 as a function of the concentration of reduced Trx. It can be seen that \( V_{eq} \) is practically independent of the concentration of reduced Trx. This indicates that thiol activation is irreversible under these conditions, as expected from the presence of 0.1 mM DTT to rereduce oxidized Trx (DTT alone did not reduce CF1-ε in this time range). The reduced form of CF1-ε has a catalytic turnover that is approximately doubled with respect to the oxidized form. Fig. 2 also shows that \( k_{app} \) is proportional to the concentration of reduced Trx, which demonstrates that the activation of CF1-ε is kinetically controlled by the formation of the supercomplex between oxidized CF1-ε and reduced Trx. The slope of the plot gives the value of the rate binding constant (\( k_{on} \)) of reduced Trx to oxidized CF1-ε,

\[ k_{app} = k_{on}[\text{Trx}_{red}] \]  

(Eq. 2)

where \([\text{Trx}_{red}]\) is the concentration of reduced Trx. The value of \( k_{on} \) here is \( 1.2 \times 10^5 \text{M}^{-1}\text{s}^{-1} \).

Data of Figs. 1 and 2 were obtained in the presence of 40 mM bicarbonate ions, which activate the isolated ATPase. We have repeated the experiments in the absence of bicarbonate. The ATPase activity of oxidized CF1-ε was diminished by about 80%, but the residual activity was still doubled by thiol modulation. The rate of formation of the CF1-ε-Trx supercomplex, as defined by the \( k_{on} \) constant, was unchanged (results not
Fig. 3. Rate of ATP hydrolysis by CF$_1$-e as a function of time after the addition of reduced thioredoxin. Effect of the presence of ATP. Conditions were as described under "Experimental Procedures." Trx concentration, 0.025 $\mu$M. Continuous curve, instantaneous rate of ATP hydrolysis measured continuously after the addition of Trx in the presence of ATP. Closed symbols, initial rates of ATP hydrolysis triggered by injecting ATP at different times after the addition of Trx. At first sight, this binding rate does not depend on the catalytic turnover rate of CF$_1$-e.

We got more decisive evidence that the rate of formation of the supercomplex does not depend on the catalytic turnover. 25 nm reduced Trx was added to oxidized CF$_1$-e in the absence of ATP. The substrate was injected at different times after the addition of Trx, and the rate of ATP hydrolysis was measured immediately after the addition of ATP. Fig. 3, closed circles, shows how the initial rate of ATPase activity increases with the time of incubation of oxidized CF$_1$-e with reduced Trx. On the same graph is also displayed the continuous measurement of the ATPase activity after the addition of 25 nm reduced Trx in the presence of ATP. The two kinetics of activation are identical, showing that the working enzyme is reduced by Trx at the same rate as the resting enzyme.

Thioredoxin-induced Activation of Isolated CF$_1$ with the e Subunit—We have investigated the effect of the presence of the e subunit on the rate of activation of CF$_1$ by reduced Trx. Experiments were carried out exactly as previously, except that CF$_1$ containing its inhibitory e subunit was used. As expected, the rate of ATP hydrolysis by the oxidized enzyme, before the addition of Trx, was very low and probably represents a small subpopulation of CF$_1$-e because intact CF$_1$ has been proposed to be fully inactive (30, 31). After the addition of 2.5 $\mu$M reduced Trx, the rate of ATP hydrolysis increased progressively, and the kinetics of activation critically depended on the CF$_1$ concentration (Fig. 4a). Because the activation was more pronounced at low CF$_1$ concentrations, it can be deduced, in accordance with previous proposals (30, 31), that the reduction of the $\gamma$ subunit decreases the affinity of $\epsilon$ for the ATPase subcomplex. Fig. 4b shows the specific ATPase activity of the ATPase enzyme, before the addition of Trx and at the end of the activation process, as a function of the CF$_1$ concentration. In the initially oxidized state, the activity is independent of the enzyme concentration, which confirms that it is probably due to a small fraction of $\epsilon$-depleted enzymes. Comparison of this activity with that of the oxidized CF$_1$-e (Fig. 2b) suggests that this fraction represents about 5% of the total. After reduction, the specific activity decreases with the CF$_1$ concentration. The data could be correctly fitted with the equation

$$V = V_m \frac{K_d}{2[E_i]} \left(\frac{1}{1 + \frac{[E_i]}{K_d}} - 1\right)$$

where $V$ is the rate of ATP hydrolysis at a given ATPase concentration, $K_d$ is the dissociation constant of the $\epsilon$ subunit from the reduced ATPase complex, $[E_i]$ the total concentration (CF$_1$ + CF$_1$-e) of the ATPase complex, and $V_m$ is the specific activity of the CF$_1$-e complex. An equimolecular stoichiometry (i.e., one $\epsilon$ subunit/CF$_1$-e) was assumed, i.e., the 5% contribution of permanently $\epsilon$-depleted CF$_1$ was neglected. It was also assumed in Equation 3 that reduced CF$_1$ bearing the $\epsilon$ subunit was inactive, in accordance with Soteropoulos et al. (31) but not with Andralojc and Harris (32). The fit gives $V_m = 61.8 \pm 2.5$ s$^{-1}$ and $K_d = 4.4 \pm 0.7$ nM for details, see "Results." Panel c, rate of ATPase activity as a function of the time after the addition of reduced Trx at different concentrations. CF$_1$ concentration, 10 nM. Trx concentrations: 0.25 $\mu$M (curve 1), 0.5 $\mu$M (curve 2), 1 $\mu$M (curve 3), 2 $\mu$M (curve 4), 3 $\mu$M (curve 5), 4 $\mu$M (curve 6), and 5 $\mu$M (curve 7).
5.6 ± 1.9 s\(^{-1}\), and the two other parameters became \(V_{\text{m}} = 68.2 \pm 3.9\) s\(^{-1}\) and \(K_d = 2.3 \pm 0.7\) nM. In both cases the specific activity of the reduced CF\(_1\)-ε so extrapolated \((V_{\text{m}})\) is close to the value determined in Fig. 2b. The values of the dissociation constant determined here for the reduced form of CF\(_1\) are close that previously reported by Soteropoulos et al. \((K_d = 4\) nM\) (31).

Because of the complexity of the process of activation of intact oxidized CF\(_1\) by reduced Trx, it is not possible to describe its time course with simple kinetic equations. It is then difficult to know which steps limit the rate of formation of reduced CF\(_1\)-ε from oxidized CF\(_1\) in the presence of reduced Trx. Since the rate of activation depends on CF\(_1\) concentration even at the beginning of the kinetics (Fig. 4b), the dissociation and reassociation of the ε subunit are probably fast, and other factors probably control the rate of formation of reduced CF\(_1\)-ε, among them the binding of reduced Trx to CF\(_1\). Fig. 4c shows the kinetics of activation of 10 nM CF\(_1\) by reduced Trx at different concentrations (from 0.5 to 5 μM). It appears qualitatively that in this range, the concentration of Trx at least partially controls the rate of activation. In the initial phase of the kinetics, and for any Trx concentration below 3 μM, the instantaneous rate of ATP hydrolysis was indeed a unique function of the (time × Trx concentration) product (not shown). The analysis of this function\(^2\) allowed calculation of a gross value for the rate constant of binding of reduced Trx to oxidized CF\(_1\). It was estimated to be 3 × 10\(^3\) M\(^{-1}\) s\(^{-1}\), about 40 times lower than for CF\(_1\)-ε.

**Thioredoxin-induced Activation of CF\(_0\)CF\(_1\) in Proteoliposomes**—Fig. 5a shows the light-induced formation of ATP driven by bacteriorhodopsin-CF\(_0\)CF\(_1\) proteoliposomes, in control conditions or a few minutes after the addition of 3 μM reduced Trx prior to illumination. The rate of ATP synthesis was more than doubled after Trx treatment, likely because the amount of activated CF\(_0\)CF\(_1\) at a given magnitude of the protonmotive force was increased (16). It is then possible to follow the thiol reduction of the membrane-bound CF\(_0\)CF\(_1\) by measuring the rate of ATP synthesis. Fig. 5b shows the rate of light-induced ATP synthesis measured with proteoliposomes treated with reduced Trx for different times in darkness (the reaction was stopped by derivatizing Trx active-site thiols with NEM, and the excess of NEM was neutralized further by a small addition of DTT). The activity increased progressively after the addition of Trx, confirming that in the absence of protonmotive force, the reduction of the γ disulfide bond by Trx is effective, but slow.

The method used here to measure the rate of reduction of CF\(_0\)CF\(_1\) in darkness cannot be applied to study its reduction in the light because the arrest of the reaction by NEM is not fast enough. We have then developed a method similar to that used in the case of the isolated complex, based here on the continuous monitoring of photophosphorylation. This was achieved by coupling the reaction of ATP synthesis to the reduction of NADPH via the hexokinase/glucose-6-phosphate dehydrogenase system (33). As for ATP hydrolysis, the instantaneous reaction rate was computed directly from the first derivative of the spectrophotometric measurement at 340 nm. Fig. 6 shows the time course of ATP-dependent NADPH reduction in the presence of illuminated proteoliposomes before and after the addition of reduced Trx (a) and the time-dependent rate of ATP synthesis after the addition of Trx (b). The kinetics of reduction of ATP synthase were found to be monophasic, and the rate constant of the process was proportional to the concentration of reduced Trx (Fig. 6c). The thiol activation of the membrane-bound ATP synthase can therefore be considered limited by the binding of Trx, which allows the determination of the rate binding constant \(k_{\text{on}}\) (Equations 1 and 2). Here, \(k_{\text{on}} = 6.4 \times 10^3\) M\(^{-1}\) s\(^{-1}\). Using different proteoliposome preparations, its value ranged between 6 × 10\(^3\) and 7.5 × 10\(^3\) M\(^{-1}\) s\(^{-1}\).

**Effect of Partial Uncoupling and Enzyme Turnover on the Rate of Energy-induced Thioredoxin Modulation of the Membrane-bound ATP Synthase**—We have checked the sensitivity of the rate of CF\(_0\)CF\(_1\) thiol modulation to a limited decrease of the electrochemical proton gradient. This was achieved by monitoring the rate of ATP synthesis before and after the addition of reduced Trx, as in Fig. 6, but at a unique Trx concentration and in the presence of FCCP at different concentrations. The progression curves were submitted to the same numerical analysis as above (Equation 1), and three parameters were drawn from each experiment: the initial rate of ATP synthesis \((\text{b CF}_0\text{CF}_1\text{ in its oxidized state})\), the final rate of ATP synthesis \((\text{by CF}_0\text{CF}_1\text{ in its reduced state})\), and the rate constant \(k_{\text{app}}\) of the transition between these two forms. These three parameters are plotted as a function of the FCCP concentration in Fig. 7. The rate constant \(k_{\text{app}}\) decreases when the uncoupler concentration increases. Its decrease is less pronounced than the loss of activity of the oxidized CF\(_0\)CF\(_1\) but more pronounced than the loss of activity of the reduced CF\(_0\)CF\(_1\).

In the data of Figs. 6 and 7, the reduction of the membrane-
bound ATP synthase by Trx proceeded during continuous ATP synthesis. To try to discriminate between the respective roles of the electrochemical proton gradient and of the enzyme turnover in the exposure of the γ-disulfide bond, we also studied this process in the absence of ADP. The principle was identical to that used previously to investigate the possible role of enzyme turnover in the thiol modulation of isolated CF1-ε (see Fig. 3). Here, reduced Trx (0.4 mM) was added to illuminated proteoliposomes, and then ADP was added at different times. The rates of ATP synthesis revealed after ADP addition were compared with those obtained at the same time after the addition of Trx, but with ADP present from the beginning. The results are presented in Fig. 8a, which shows the rates of ATP synthesis as a function of the time after the addition of Trx under the different conditions. Obviously, the different kinetics cannot be superimposed. When the time between the addition of Trx and ADP is increased, it becomes clear that in the absence of ADP the thiol modulation of ATP synthase was accelerated. For each curve, the rate of ATP synthesis was extrapolated to the time of the addition of ADP (Fig. 8a, symbols). Its time dependence allowed estimation of the rate constant of thiol modulation in the absence of ADP. It was about double of that obtained in the presence of ADP. Because the rate of Trx binding depends on the protonmotive force, we have verified that ADP did not affect its magnitude. This was achieved by comparing the light-induced quenching of 9-aminoacridine, related to the ΔpH (34), in the presence of 1 mM ATP and 1 mM ADP (Fig. 8b). No difference was observed.
the absence and in the presence of valinomycin, which shows that neither $\Delta\psi$ nor $\Delta\Pi$ was affected by ADP. We can then conclude that contrary to the isolated CF$_1\epsilon$, the activated membrane-bound ATP synthase is thiol-modulated at a rate that is modified by the enzyme turnover.

**Kinetic Properties of Oxidized and Thiol-modulated CF$_0$CF$_1$ in Proteoliposomes**—For the sake of simplicity, it is generally assumed that oxidized and thiol-reduced CF$_0$CF$_1$ differ only in their ability to be activated by the electrochemical proton gradient and have the same catalytic properties. A simple way to check this proposal is to examine parameters that do not depend on the amount of active enzyme, e.g. Michaelis constants. Fig. 9 shows the result of a typical experiment where the light-induced rate of ATP synthesis catalyzed by oxidized and thiol-reduced CF$_0$CF$_1$ was plotted as a function of ADP concentration.

In the case of the oxidized CF$_0$CF$_1$, the data were fitted satisfactorily with simple Michaelis-Menten kinetics from 1 to 1,000 $\mu$M. The case of the reduced enzyme was different. The data could not be fitted with simple Michaelis-Menten kinetics, but rather with a model with two independent classes of sites (Fig. 9, dotted curve). This does not prove that the thiol-modulated CF$_0$CF$_1$ works with two independent classes of sites because a lot of different models could probably accommodate the data. However, it is clear that under our conditions, the thiol-modulated CF$_0$CF$_1$ works under a different mode than the oxidized CF$_0$CF$_1$. If one restricts the kinetic analysis to the 1–100 $\mu$M range and imposes a Michaelis-Menten fit (Fig. 9, solid curves), one finds that the $K_m$ (ADP) of the reduced form is approximately the double of the $K_m$ (ADP) of the oxidized form. Actually, the $K_m/V_{\text{max}}$ ratio is approximately the same for both forms. Table I shows that this pattern was confirmed with different preparations of proteoliposomes. Even though some variations were found in the kinetic parameters $V_{\text{max}}$ and $K_m$ (calculated in the 1–100 $\mu$M ADP concentration range), $K_m$ was always higher in the reduced form than in the oxidized form. Moreover, in the 1–1,000 $\mu$M concentration range, the saturation curve of the oxidized form was always monophasic, and the saturation curve of the reduced form biphasic (not shown). These experiments show that thiol modulation modifies not only the proportion of active CF$_0$CF$_1$, but also its catalytic properties.

**DISCUSSION**

**Thiol Modulation of the ATPase Subcomplex and of the CF$_0$CF$_1$ Complex by Thioredoxin**—To get a clear picture of the mechanism of CF$_0$CF$_1$, thiol modulation *in situ*, a prerequisite is to study the interaction between Trx and the y subunit in the isolated ATPase subcomplex and in the whole, membrane-bound, ATP synthase complex. The present study is a new step in this investigation. Surprisingly, it reports for the first time kinetic data about the interaction between Trx and the isolated CF$_1$ (with or without its $\epsilon$ subunit). Previous data included qualitative studies of the interaction between CF$_1$ and different Trxs (22, 23), quantitative studies of this interaction at equilibrium using fluorescent probes (23), and kinetic studies of this interaction in thylakoids (21). These different approaches are complementary. From the rate of activation of oxidized CF$_1\epsilon$ by reduced Trx, we were able to measure the rate constant of formation of the supercomplex between these two species. It was about $10^{5}$ mole$^{-1}$ s$^{-1}$ at pH 8 and 37°C. Because of the quasi-irreversibility of the reaction, neither the dissociation constant of this supercomplex nor the characteristics of further steps of ATPase reduction could be determined.

The $K_d$ of the ATPase-Trx supercomplex was estimated previously to be about 1 $\mu$m, regardless of the redox state of CF$_1$ and Trx (23), but changes in kinetic binding parameters with the redox state remain a *priori* possible. We have found that $k_{\text{on}}$ = 1.2 x $10^{5}$ mole$^{-1}$ s$^{-1}$ if Trx is initially reduced and CF$_1\epsilon$ initially oxidized. If $K_d$ is close to 1 $\mu$m, $k_{\text{off}}$ can be estimated to 0.1 s$^{-1}$. We have tried to know if the occupancy of the binding site of oxidized CF$_1\epsilon$ by oxidized Trx (up to 4 $\mu$m) could delay its reduction by reduced Trx. No delay was observed within our resolution time (a few seconds, data not shown), consistent with a $K_{\text{on}}$ of 0.1 s$^{-1}$, or higher, for the release of oxidized Trx from the oxidized CF$_1\epsilon$. At the present time, it seems reasonable to consider that, like the $K_d$, the kinetics of formation and dissociation of the ATPase-Trx supercomplex are redox-independent. At first sight, this should exclude large redox-dependent conformational changes of the interacting domains of ATPase and Trx. Such changes are unlikely indeed in the case of *E. coli* Trx (35).

If one assumes a $K_d$ of 1 $\mu$m for the ATPase-Trx supercomplex (23), this entity is predominant for Trx concentrations of several $\mu$m. No variation of ATPase activity of CF$_1\epsilon$, oxidized or reduced, was detected upon addition of several $\mu$m Trx in the

**TABLE I**

| Experiment | $V_{\text{max}}$ | $K_{\text{m}}$ (ADP) | $\text{Red/ox ratio}$ |
|------------|------------------|-----------------------|-----------------------|
| Oxidized   | Reduced          | $	ext{nmol min}^{-1}$/mg | $\mu$m | Oxidized | Reduced | $\mu$m |
| I          | 178 ± 4          | 398 ± 14              | 2.23                   | 1.7 ± 0.15 | 4.3 ± 0.35 | 2.53 |
| II         | 193 ± 5          | 446 ± 21              | 2.31                   | 3.2 ± 0.4 | 7.7 ± 1.3 | 2.41 |
| III        | 298 ± 6          | 511 ± 18              | 1.71                   | 1.4 ± 0.15 | 3.43 ± 0.5 | 2.45 |

**Fig. 9.** Rate of ATP synthesis by proteoliposomes as a function of ADP concentration. Effect of thiol modulation. Conditions were as described under "Experimental Procedures." $\square$, CF$_0$CF$_1$, in the oxidized form; $\square$, CF$_0$CF$_1$, in the reduced form. Dotted curves, fits obtained using the data from 1 to 1,000 $\mu$m. Solid curves, fits obtained using the data from 1 to 100 $\mu$m. For the oxidized form of CF$_0$CF$_1$, a simple Michaelis-Menten model was used in both cases (solid curve, $V_{\text{max}}$ = 189 ± 3 nmol min$^{-1}$ mg$^{-1}$ of protein, $K_m$ = 3.05 ± 0.3 $\mu$m; dotted curve, $V_{\text{max}}$ = 193 ± 5 nmol min$^{-1}$ mg$^{-1}$ of protein, $K_m$ = 3.25 ± 0.4 $\mu$m). For the reduced form of CF$_0$CF$_1$, a simple Michaelis-Menten model was used in the 1–100 $\mu$m range for the solid curve, $V_{\text{max}}$ = 526 ± 16 nmol min$^{-1}$ mg$^{-1}$ of protein, $K_m$ = 12.5 ± 2 $\mu$m) and a model with two independent classes of sites was used in the 1–1,000 $\mu$m range (dotted curve, $V_{1}$ = 338 ± 32 nmol min$^{-1}$ mg$^{-1}$ of protein, $K_1$ = 4.64 ± 0.9 $\mu$m, $V_{2}$ = 262 ± 25 nmol min$^{-1}$ mg$^{-1}$ of protein, $K_2$ = 152 ± 55 $\mu$m). For details, see "Results."
same redox state (data not shown). Binding of Trx has apparently no effect in itself on the ATPase activity, although comparisons of \( k_{\text{on}} \) (0.1 \( s^{-1} \)) and catalytic turnover rate (40 to 80 \( s^{-1} \)) indicate that the ATPase-Trx supercomplex remains stable during several hundreds of enzyme turnovers. This situation is depicted in Scheme 1a. This rules out possible contacts or collisions of Trx with any part of the stator during the rotation of the \( \gamma \)-Trx entity in \( \text{CF}_{1}-\epsilon \). This is also consistent with the lack of effect of the enzyme turnover on the rate of binding of Trx to isolated \( \text{CF}_{1}-\epsilon \).

It was also reported that the presence of the \( \epsilon \) subunit increases the distance between \( \gamma \)-Cys322 and Trx in the ATPase-Trx supercomplex without changing its dissociation constant (23). In the present work, the kinetic study of the reduction of intact \( \text{CF}_{1} \) by Trx was complicated by the release of the \( \epsilon \) subunit. However, the kinetics obtained at limited Trx concentrations suggests that the binding of reduced Trx to \( \text{CF}_{1} \) is slower than its binding to \( \text{CF}_{1}-\epsilon \).

Observations about the activation of the isolated ATPase complex are relevant to the mechanism only if studies are also made with the membrane-bound ATP synthase complex. Until now, thiol modulation of the membrane-bound \( \text{CF}_{0}\text{CF}_{1} \) was always revealed by the rate of ATP hydrolysis immediately after the collapse of the proton motive force (16, 36, 21). The present work shows that it is also possible to follow the thiol modulation by measuring the increase of the ATP synthesis capacity. When a coupled enzymatic assay is used, this approach allows a continuous monitoring of the ATP synthase capacity. When a coupled enzymatic assay is used, this approach allows a continuous monitoring of the ATP synthase capacity. When a coupled enzymatic assay is used, this approach allows a continuous monitoring of the ATP synthase capacity.

Therefore, the ATPase activity of the ATP synthase is only two times higher, was obtained at a very high proton motive force, estimated to more than 4 pH units. Although we did not affect its rotation. Reciprocally, the rotation induced by ATP hydrolysis does not change the rate of Trx binding to \( \text{CF}_{1}-\epsilon \), as shown in Fig. 3. Panel b, the membrane-bound ATP synthase complex exists under three different functional states: top, resting (without proton gradient); middle, proton-activated (with proton gradient but without substrates); bottom, working (driving ATP synthesis).

The rate constant \( k_{\text{on}} \) of Trx binding is different for the three states. Its approximate values, drawn from the present report, are indicated. 

Panel a, isolated \( \text{CF}_{1}-\epsilon \) subcomplex. Panel b, membrane-bound \( \text{CF}_{0}\text{CF}_{1} \). Left, ATPase (a) and ATP synthase (b) complexes free of Trx. Right, the same complexes with bound Trx. Some values of the rate constants of binding (\( k_{\text{on}} \)) and release (\( k_{\text{off}} \)) of Trx are indicated in the middle. Rotation rates of the \( \epsilon \) subunit during enzyme turnover (\( \alpha \), ATP hydrolysis; \( \beta \), ATP synthesis) are indicated below the complexes. Binding and release of Trx are assumed to be independent of the redox state of Trx and \( \gamma \) subunit. In panel a, the isolated ATPase complex and the ATPase-Trx supercomplex, both present at \( \mu M \) concentrations of Trx, hydrolyze ATP at the same rate. The rotation rates (in turns/s) were deduced directly from the enzyme turnover of the reduced state of \( \text{CF}_{1}-\epsilon \) in Fig. 2. The rate of formation of the ATPase-Trx supercomplex (\( k_{\text{on}} = 10^{4} \text{ M}^{-1} \text{ s}^{-1} \)) comes from the data of Fig. 2. The rate of dissociation of the ATPase-Trx supercomplex (\( k_{\text{off}} \)) was calculated from \( k_{\text{on}} \) and \( K_{D} \), assuming \( K_{D} = 1 \mu M \) (23). The comparison between the rotation rate and the dissociation rate of the ATPase-Trx supercomplex indicates that this supercomplex may experience a lot of turns without being dissociated. The quasi-permanent presence of Trx bound to the rotor of \( \text{CF}_{1}-\epsilon \) does not affect its rotation. Reciprocally, the rotation induced by ATP hydrolysis does not change the rate of Trx binding to \( \text{CF}_{1}-\epsilon \), as shown in Fig. 3. Panel b, the membrane-bound ATP synthase complex exists under three different functional states: top, resting (without proton gradient); middle, proton-activated (with proton gradient but without substrates); bottom, working (driving ATP synthesis).
not carry out absolute measurements of the protonmotive force in our proteoliposomes, we know that it is much lower (37). This is confirmed by the low maximum rates of ATP synthesis (200–300 nmol of ATP min^{-1} mg^{-1} of oxidized CF_{0}CF_{1} ), 30–50 times lower than that observed when these liposomes are submitted to an artificial protonmotive force (29). These comparisons suggest that the rate of binding of Trx to membrane-bound CF_{0}CF_{1} saturates at moderate values of the protonmotive force, contrary to the rate of ATP synthesis. Accordingly, when the protonmotive force is lowered with FCCP (Fig. 7), the binding rate diminishes, but less than the rate of ATP synthesis catalyzed by oxidized CF_{0}CF_{1}. If the decrease of \( k_{a} \) with the protonmotive force directly reflects the diminution of the number of activated CF_{0}CF_{1} (21), this would mean that the more pronounced decrease of the rate of ATP synthesis by oxidized CF_{0}CF_{1} is due to the combined lowering of activation and catalytic turnover.\(^3\) The situation is actually complex because \( k_{on} \) itself is affected by enzyme turnover (Fig. 8). Understanding in more quantitative terms the modulating effect of the catalytic turnover, at different magnitudes of the protonmotive force, is a challenge for future studies.

It is of interest to have an estimation of the rate of dissociation of the CF_{0}CF_{1}/Trx supercomplex and to correlate it to the enzyme turnover. If the rotary mechanism is exact, it seems difficult indeed to imagine that the lifetime of this supercomplex exceeds the time of rotation of the enzyme because of the presence of the external stalk. The center-to-center distance between the external stalk and the \( \gamma \) internal stalk seems to be about 3 nm (38). The space between these two stalks is probably too narrow to be readily crossed by the bound Trx at each revolution of the rotor (the diameter of Trx is not far from 3 nm). If the \( K_{a} \) of the CF_{0}CF_{1}/Trx supercomplex is 1 \mu M, as for the isolated ATPase (23), since the \( k_{a} \) is about 6 \times 10^{-3} m^{-1} s^{-1} \) (present work), the \( k_{off} \) should be 6 \times 10^{-3} s^{-1}, which is actually much lower than the apparent catalytic turnover (2 s^{-1} in the present conditions). In this case, the supercomplex should be very stable. Consequently, it should be unable to rotate and inactive. This is depicted in Scheme 1b. With \( K_{a} = 1 \mu M, 33\% \) of CF_{0}CF_{1} should be inactivated by 0.5 \mu M Trx, 50\% by 1 \mu M Trx, and 67\% by 2 \mu M Trx. Because this was not observed, it is probable that the \( K_{a} \) is much higher than 1 \mu M in the case of CF_{0}CF_{1} or that the binding parameters depend on the redox state of the partners. This problem obviously deserves further investigations.

Catalytic Properties of Oxidized and Thiol-reduced CF_{0}CF_{1}—We have compared the ATP synthesis activity of oxidized and thiold-induced CF_{0}CF_{1} at various ADP concentrations. Surprisingly, this is the first time, to our knowledge, that such a comparison is made without ambiguity. Because of the negligible contribution of ATP synthesis to the total proton flow in the present conditions, our measurements were made at a constant value of the protonmotive force, which avoids side effects generally encountered in this kind of study, neglected by most investigators, but stressed by others (39–41). For the sake of simplicity, it was assumed previously that the functional characteristics of the catalytic reaction did not depend on the redox state of the enzyme, and it was concluded from this postulate that contrary to the maximum activation, the maximum rate of catalytic turnover of the oxidized CF_{0}CF_{1} was reached at a rather low protonmotive force magnitude (16). The present data do not allow a refined kinetic study, but they are sufficient to show that oxidized and thiol-reduced CF_{0}CF_{1} actually have different catalytic properties. Because they respond in a different way to the ADP concentration, there is no reason to believe that they respond in an identical way to variations of the protonmotive force, and the conclusions based on this postulate should be questioned. The non-Michaelian pattern observed here in the case of thiol-reduced CF_{0}CF_{1} differs from previous observations in thylakoids where ATP synthesis catalyzed by the thiol-reduced ATP synthase obeyed simple Michaelis-Menten kinetics (41, 42). Although different reasons could be invoked to explain this discrepancy (different magnitudes of the protonmotive force or different pH, for example), at the present time the actual reason is not clear, and a detailed comparative kinetic study, out of the present scope, would be necessary to solve this problem.

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Mechanism of Activation of the Chloroplast ATP Synthase: A KINETIC STUDY OF THE THIOL MODULATION OF ISOLATED ATPase AND MEMBRANE-BOUND ATP SYNTHASE FROM SPINACH BY ESCHERICIA COLITHIOREDOXIN

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