Role of a Disulfide Bond in the \( \gamma \) Subunit in Activation of the ATPase of Chloroplast Coupling Factor 1*

(Received for publication, August 31, 1982)

Carlo M. Nalin† and Richard E. McCarty

From the Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

The relationship between activation of the latent ATPase activity of isolated chloroplast coupling factor 1 (CF\(_1\)) and reduction of a disulfide in the \( \gamma \) subunit has been assessed. The sulfhydryl residues involved in the disulfide bond are distinct from residues normally accessible to maleimide modification during incubation of thylakoids in the dark or the light. Dithiothreitol-induced activation is time dependent, and correlates with reduction of the disulfide. Sulphydryl residues exposed during activation can be reoxidized to disulfide by incubation with iodosobenzoate, with a concomitant loss of ATPase activity. Activation and deactivation are reversible, but deactivation is prevented by treatment of the reduced enzyme with N-ethylmaleimide.

Heat activation does not reduce the disulfide bond unless dithiothreitol is present during activation. Prior heating of CF\(_1\), which partially activates the enzyme, renders the disulfide more susceptible to subsequent dithiol reduction. The activity obtained when heat and dithiothreitol are used together is approximately equal to the sum of the partial activations obtained with heat or dithiothreitol alone. Iodosobenzoate has no effect on heat-activated CF\(_1\). Enzyme activated by heating in the presence of dithiothreitol can be partially deactivated, consistent with reversal of the activity attributable to the dithiol effect.

Fluorescence polarization of anilinonaphthalene-sulfonate bound to the reduced enzyme indicates that the sulphydryl residues involved in the disulfide are in a less rigid environment than the other two sulphydryl residues in the \( \gamma \) subunit. Polarization of anilinonaphthalene-sulfonate bound to these sulphydryls is reduced by heat treatment of CF\(_1\).

The increased susceptibility of the disulfide to reduction upon heat treatment, and the activation of ATPase activity with or without disulfide bond cleavage are indicative of conformational changes within the \( \gamma \) subunit that occur during the conversion of CF\(_1\), from a latent to an active ATPase. In addition the results are consistent with at least two distinct conformational forms of CF\(_1\), that can hydrolyze ATP.

\[ \text{CF}_1 \text{ catalyzes the synthesis of ATP using energy derived from the proton gradient across the thylakoid membrane (1-3). When isolated from the membrane, the enzyme loses its capacity for net ATP synthesis, but retains a latent ATP hydrolysis activity. This activity is expressed after treating the enzyme at room temperature with detergents (4), reducing agents (5), or proteases (6), or after heating CF}_1 \text{ in the absence or presence of reducing agents (6, 7). CF}_1 \text{ consists of five distinct subunits (\( \alpha-\gamma \)) in a stoichiometry that remains controversial. The molecular weight has recently been redetermined by various techniques (8); the value obtained (400,000) and the molecular weight of the subunits suggest a subunit stoichiometry of \( \alpha_3 \beta_5 \delta \gamma \). The role of sulphydryl residues in energy transduction remains a focus of investigation. At least one sulphydryl residue in the \( \gamma \) subunit is essential for ATP synthesis (9, 10). Modification of this residue in the membrane-bound enzyme under energized conditions (i.e. illuminated thylakoids) inhibits ATP synthesis and also inhibits ATP hydrolysis by both the membrane-bound and soluble CF\(_1\). In addition to the essential residue, a nonessential sulphydryl group is exposed under both energized and nonenergized conditions in the membrane-bound enzyme (10, 11). These residues have been shown to be distinct, and are not different conformers of the same reactive group (12). Controversy over the stoichiometry of sulphydryl residues is, in part, related to changes in the oxidation-reduction state of these residues that occur during activation of the latent ATPase activity. Dithiothreitol-induced activation causes reduction of a disulfide bond in the \( \gamma \) subunit of soluble CF\(_1\) (13). Recently, a model has been proposed for heat activation in which a disulfide exchange between the \( \gamma \) and \( \alpha \) subunits occurs, rather than a net reduction (13). Since ATPase activity can be expressed by heating CF\(_1\) in the absence of added reducing agents (6, 7), an activation mechanism that does not require net reduction bears consideration.

Until recently, the energy-dependent activation of thylakoid ATPase by thiols was generally considered to be a curiosity of little physiological significance. However, the finding that illumination of intact chloroplasts (14) or of leaves (15) causes a similar induction of the ATPase activity of thylakoids derived from them, has rekindled interest in the thiol-activated or reduced state of CF\(_1\). The active form of CF\(_1\), in \textit{vivo} is likely to be the reduced form.

In this paper, we compare the activation of the latent ATPase activity of soluble CF\(_1\) by dithiothreitol and by heat. Although heating in the presence of dithiothreitol promotes the reduction of a disulfide bond in the \( \gamma \) subunit, no change in the oxidation state of this subunit was detected when heating was carried out in the absence of the reducing agent.

ANM, N-(1-anilinonaphthyl-4)-maleimide; SDS, sodium dodecyl sulfate.

7275
While we agree that heat activation and reductive activation occur by different mechanisms (13), in our hands, only in the presence of an added reducing agent is the disulfide bond in the γ subunit reduced.

MATERIALS AND METHODS

Chloroplast thylakoids were prepared from market spinach (16). CF, was isolated essentially as described in (17). The sucrose gradient centrifugation step was omitted. The enzyme was determined to be more than 96% pure by SDS-polyacrylamide gel electrophoresis, with ribulose bisphosphate carboxylase being the major contaminating protein.

Modification of soluble CF, with ANM was performed in 50 mM Tris-Cl (pH 8.0), 1 mM EDTA, and 1 mM ATP buffer. CF, was incubated at 0.5–2.5 mg of protein/ml with 30–50 μM ANM for 3 min. Either 0.5 mM dithiothreitol or 1 mM N-acetylcysteine was added to stop modification and the samples were passed through a 1 x 10 cm Sephadex G-50 column, equilibrated with the Tris-EDTA-ATP solution. When small volumes were used, Sephadex centrifuge columns (18) were used to remove the unbound reagents. Where premodification of sulfhydryl groups with N-ethylmaleimide was required, CF, in the Tris-EDTA-ATP solution was incubated with 1 mM maleimide for 15–30 min prior to the treatments. Reduction of disulfide bonds was accomplished by incubating the latent enzyme with 50 mM dithiothreitol for 3–5 h, or 10 mM dithiothreitol for 15–30 min for denatured CF, Where SDS was added to denature protein, a final concentration of 1% was present.

Modification of membrane-bound CF, with maleimides in the light or the dark was performed as described (12). Heat activation (7), dithiothreitol activation (5), SDS-polyacrylamide gel electrophoresis (19), and assay of ATP hydrolysis (5) were performed by published procedures. P, was measured by a colorometric method (20). Protein was determined either by a spectrophotometric method (21) or by a modification of the Lowry technique (22). Fluorescence and fluorescence polarization measurements were performed on a Farrand 801 spectrophotofluorimeter or a Perkin-Elmer MPF-3 spectrophotometer using 355-nm exciting light. Photographs of fluorescent gels were made as described previously (12).

N-(1-Anilinonaphthol-4)-maleimide was purchased from Wako Pure Chemical Industries, Ltd., Japan, and used without further purification. N-Ethylmaleimide, none of the sulfhydryl residues will sub-

FIG. 1. ANM labeling of sulfhydryl residues in CF, SDS-polyacrylamide gel electrophoresis of CF, labeled with ANM. CF, at 1 mg of protein/ml was incubated with 50 μM ANM for 3–5 min followed by addition of 1 mM N-acetylcysteine to remove unreacted ANM. Fifteen μg of each sample was subjected to electrophoresis on a 12% acrylamide gel. Lane 1, latent CF; Lane 2, CF, in 1% SDS; Lane 3, CF, in SDS after prelabeling with 1 mM N-ethylmaleimide; Lane 4, as for Lane 3 with, in addition, 10 mM dithiothreitol for 30 min.

RESULTS

Location of Sulfhydryl Residues and the Disulfide Bond in Isolated CF,:—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of CF, reacted with a fluorescent maleimide such as ANM provides a simple, visual means of locating the sulfhydryl residues and disulfides in the enzyme. Fig. 1 shows an example of this approach. Soluble, nondenatured CF, contains ANM-reactive groups (presumably sulfhydryls) that are predominantly in the γ and ε subunits (Refs. 10 and 11; Fig. 1, Lane 1). Trypsin treatment of this enzyme (23) releases a peptide from the γ subunit having M, ~ 6000 and containing all the fluorescent label (not shown). A similar pattern of digestion of γ was observed using CF, isolated from thylakoids modified with ANM in the dark (12). The similarity in the size of the ANM-labeled peptides released by trypsin digestion suggests that the reactive sulfhydryl in the γ subunit of the soluble enzyme is the same one that is accessible on the membrane-bound enzyme in the deenergized state. The fluorescent label associated with the ε subunit is presumably bound to the single sulfhydryl residue (11, 17, 24) in that subunit. When CF, is denatured in SDS (Fig. 1, Lane 2), additional sites in α, β, and γ subunits become accessible to labeling. Only the δ subunit does not show any covalent incorporation of ANM, consistent with another published report that this subunit does not contain sulfhydryl groups (25). When denatured CF, is first exposed to a large excess of N-ethylmaleimide, none of the sulfhydryl residues will sub-

sequently react with ANM (Lane 3). However, if after incubating with N-ethylmaleimide, the enzyme is treated with dithiothreitol prior to ANM addition, ANM is incorporated specifically into the γ subunit (Lane 4). These results are indicative of the presence of a disulfide bond only in the γ subunit. No evidence for intersubunit disulfide bonds was obtained, in agreement with previous reports (13, 25). The increased electrophoretic mobility of the γ subunit observed after modification is likely caused by bound ANM, and not by cleavage of the polypeptide chain.

The sulfhydryl residues on the γ subunit exposed by dithiothreitol reduction are distinct from the dark- or light-accessible sulfhydryl residues of the membrane-bound enzyme (9, 10). Thylakoids were incubated with N-ethylmaleimide in the light or the dark and CF, was isolated. The purified CF, preparations were denatured in 1% SDS and incubated in the presence (·) and absence (−) of 10 mM dithiothreitol. Following removal of the dithiothreitol, the preparations were incubated with ANM. Although some ANM was incorporated into the δ subunit of CF, from thylakoids incubated with N-ethylmaleimide in the dark, increased ANM incorporation was seen after dithiothreitol reduction (Fig. 2, Lanes 1 and 2). Much less ANM was incorporated into the γ subunit of CF, from thylakoids incubated with N-ethylmaleimide in the light (Lane 3), but substantial ANM fluorescence was seen after reduction (Lane 4). Since the prior alkylation of the sites accessible to N-ethylmaleimide in the light did not affect ANM incorporation after reduction, it is unlikely that these groups are part of the disulfide bond in the γ subunit and that the disulfide bond is reduced upon illumination of thylakoids. Consistent with this result, dithiothreitol reduction is still required to elicit trypsin sensitivity of the γ subunit (23) of enzyme released from the membrane after modifying in the dark or the light with N-ethylmaleimide (data not shown).
Dithiothreitol treatment can be modified by maleimides with acrylamide gel. Note that only the p~ANM was added for 3 min followed by 1 mM N-acetylcysteine. was added as noted. After 30 min, dithiothreitol was removed, and 50 enzyme-bound nucleotides in the activation process. Both the these observations do not rule out possible involvement of ATP hydrolysis requires treatment with dithiothreitol, its soluble form, CF, is a latent ATPase. Expression of ATP had no effect on the efficacy of iodosobenzoate, nor is there a significant difference in the deactivation after short or long incubation with dithiothreitol (data not shown).

Heat treatment of CF, in the absence of added dithiothreitol...
also partially activates the latent ATPase (7). However, in our hands, this does not cause reduction of the disulfide bond, or formation of an alternate disulfide (i.e. disulfide exchange). This was determined by denaturing CF, and blocking free sulphydryl residues with N-ethylmaleimide. Subsequent dithiothreitol treatment and exposure to ANM would label residues that had been part of a disulfide linkage. Both the latent enzyme (Fig. 5, Lane 1) and the heat-activated enzyme (Lane 2) contain a disulfide bond in the γ subunit alone. No disulfide bond is detectable in the α subunit in either enzyme (the small, fluorescent band at the leading edge of the β subunit is due to a ribulose bisphosphate carboxylase contamination). In contrast to heat-activated enzyme, dithiothreitol-activated CF, does not contain any disulfide bonds (Lane 3). Iodosobenzoate treatment of dithiothreitol-reduced enzyme reforms a disulfide bond in γ (Lane 4).

Consistent with its effect as a sulphydryl-oxidizing reagent, iodosobenzoate has no effect on the Ca**+-ATPase activity of the heat-activated enzyme, and only a partial effect on CF, heated in the presence of dithiothreitol (Table I). However, heating in the presence of iodosobenzoate further activates CF, although heat activation alone does not reduce the disulfide bond, it causes a permanent conformational change in the γ subunit. The rate of further activation of the ATPase and, presumably, dithiothreitol reduction of the disulfide bond after heat activation is faster than is the rate of dithiothreitol activation of the latent enzyme (Fig. 6). This suggests that heat-induced conformational changes make the disulfide more accessible to the medium.

Conformational changes induced by activation have also been examined by fluorescence polarization of ANM bound to the sulphydryl residues. Polarization and anisotropy of bound ANM (Table II) indicate that the sulphydryl residues exposed by dithiothreitol treatment are in a less rigid envi-

![Fig. 5. Activation effects on the disulfide bond of CF,](image)

**TABLE I**

| Method of activation | ATPase activity | µmol-min**-1-mg**-1 |
|----------------------|-----------------|---------------------|
| No addition          | -IBZ            | 0.8                 |
| Dithiothreitol       | +IBZ            | 1.4                 |
| Heat                 |                 | 1.7                 |
| Heat + 5 mm dithiothreitol |            | 12.1                |
| Heat + 0.5 mm IBZ    |                 | 13.9                |

**FIG. 6. Dithiothreitol treatment of heat-activated CF,** Latent CF, and heat-activated CF, at 1 mg of protein/ml were incubated in 50 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1 mM ATP buffer with 10 or 50 mM dithiothreitol at room temperature. At the times indicated, 5-µl aliquots were removed and assayed for ATPase activity. □, latent CF, ○, heat-activated CF; □, ○, 10 mM dithiothreitol; ■, ○, 50 mM dithiothreitol.

**TABLE II**

Fluorescence polarization of ANM-labeled CF, fluorescence of ANM-labeled CF, was measured as described under "Materials and Methods." Dark-accessible and light-accessible refer to CF, modified while bound to thylakoids, followed by isolation of CF, (12).

| Site(s) modified      | λ**max** (corrected) | Polarization | Anisotropy |
|-----------------------|----------------------|--------------|------------|
| Dark-accessible       | 441                  | 0.42         | 0.32       |
| Light-accessible      | 430                  | 0.44         | 0.34       |
| Dithiothreitol-accessible | 438                 | 0.36         | 0.27       |
| Heat + dithiothreitol-accessible | 438               | 0.31         | 0.23       |

**DISCUSSION**

In this paper, we present a comparison of two different methods of activating CF, and the relationship between activation and the oxidation-reduction states of sulphydryl residues in the enzyme. There are two striking observations made.
in these experiments. The first involves the existence of a disulfide bond in the γ subunit that is distinct from the two sulfhydryl residues that are accessible in the membrane-bound enzyme in the light or dark. The second observation is related to the apparent differences between methods of activating the latent ATPase and their effects on the disulfide bond.

Previous determinations of the stoichiometry of sulfhydryl residues in the γ subunit were based on the incorporation of radioactive labeled sulfhydryl reagents or by quantitation of cysteic acid after oxidative treatment (7, 11, 13, 25). Stoichiometries of 2–4 mol of cysteine/mol of γ have been reported, based on the molecular weight of CF1, being 325,000 (27). This molecular weight has recently been shown to be an underestimate, with 400,000 being a more accurate value (8). The results in this paper and the accompanying reports (28, 29) provide strong evidence that the γ subunit of soluble CF1 contains four sulfhydryl residues, two of which are linked in a disulfide bond. Previous discrepancies in stoichiometry are likely due to incomplete modification, and to underestimation of the molecular weight of the enzyme (28). In addition, steric constraints, nearest neighbor effects, and/or detergent effects may have caused abnormal reactivity of these residues.

Dithiothreitol-induced activation of the latent ATPase has been correlated with reduction of the disulfide bond in the γ subunit. Fig. 7 is a simple model that depicts a possible relationship between the ATPase activity and oxidation-reduction of sulfhydryl residues in γ. The location of the sulfhydryl groups in the model is based on reactivity with dithiothreitol and iodosobenzoate, and on fluorescent properties of ANM bound to these sites. Sulfhydryl residues shown close to the center of the circle are considered to be less exposed to the medium, and hence less reactive. Dithiothreitol-induced activation of CF1 occurs by a reversible mechanism. It is sensitive to oxidizing agents that convert reduced vicinal sulfhydryls to a disulfide. Dithiothreitol-induced activation is distinct from heat activation, which does not reduce the disulfide and is insensitive to oxidizing agents. In addition, heat activation is irreversible, causing a permanent change in the exposure of the disulfide bond to the medium. When used together or sequentially, dithiothreitol plus heat transform CF1 into a still more active form, which is only partially sensitive to oxidizing agents.

We cannot explain the discrepancy between our results and those of Arana and Vallejos (13), who found that heat activation caused an apparent disulfide-dithiol exchange between the α and γ subunits. No exchange was detected after heating, as determined from the thiol content of the subunits using [3H]N-ethylmaleimide, and using this reagent only the γ subunit was found to contain a disulfide bond.2 Recently, the amino acid sequence of the α subunit of CF1 from tobacco chloroplasts was deduced from the gene sequence (30). Only a single cysteine residue per copy of the α subunit was observed, in agreement with one published report for the Escherichia coli α subunit (31), but in contrast to previous measurements for CF1 (17, 32) and E. coli (33). The presence of only one cysteine residue per α subunit and the lack of evidence for intersubunit disulfides in CF1 would make a disulfide exchange mechanism appear unlikely.

The differences between the two methods of activation compared in this paper are important to consider in studying the catalytic mechanism and regulation of CF1. Although heating in the presence of dithiothreitol yields the most active preparation of the soluble ATPase, this treatment causes permanent changes in the structure of the enzyme. Therefore, attempts to study regulatory properties of CF1 after heat treatment may produce results that are unrelated to properties of the membrane-bound enzyme. Indeed, it has previously been demonstrated that heat-activated enzyme will not reconstitute photophosphorylation in thylakoid membranes stripped of endogenous CF1 (34) whereas the dithiothreitol-activated enzyme is more effective than oxidized CF1. Since the reduced enzyme is the physiologically significant form (14, 15, 29), dithiothreitol treatment clearly should be the method of choice for activating CF1 in experiments designed to study mechanistic features.

Finally, the importance of conformational changes in the γ subunit during activation bears comment. The direct correlation between reduction of the disulfide bond and ATPase activity suggests that reduction causes conformational changes in γ which are transmitted to the β subunit and the catalytic site. Considering the possible role of the γ subunit as the proton gate in the mechanism of ATP synthesis, an understanding of these conformational changes in the soluble enzyme may be crucial to our understanding of the membrane-bound enzyme and to the coupling mechanism. The nature of the signal between the γ subunit and the catalytic sites on β subunits remains to be identified.

REFERENCES

1. McCarty, R. E., and Carmeli, C. (1982) in Energy Conversion in Plants and Bacteria (Govindjee, ed) pp. 647–695, Academic Press, New York
2. Nelson, N. (1982) in Electron Transport and Photophosphorylation (Barber, J., ed) pp. 81–104, Elsevier Biomedical Press, Amsterdam
3. Cross, R. L. (1981) Annu. Rev. Biochem. 50, 681–714
4. Pick, U., and Bassilain, S. (1981) in Energy Coupling in Photosynthesis (Selman, B., and Selman-Reimer, S., eds) pp. 251–260, Elsevier/North-Holland, New York
5. McCarty, R. E., and Racker, E. (1968) J. Biol. Chem. 243, 129–137
6. Vammutas, V. K., and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667
7. Farron, F., and Racker, E. (1970) Biochemistry 9, 3829–3836
8. Moroney, J. V., Lopresti, L., McMenemy, B., Hamnes, G. G., and McCarty, R. E. (1983) FEBS Lett. 158, 58–62
9. McCarty, R. E., Pittman, P. R., and Tsuchiya, Y. (1972) J. Biol. Chem. 247, 3048–3051

2 J. V. Moroney, unpublished observation.

Fig. 7. Model for sulfhydryl oxidation-reduction in the γ subunit. A possible correlation between sulfhydryl residues and activation of the latent ATPase by heat or dithiothreitol. SH, free sulfhydryls; S-S, disulfides; IBZ, iodosobenzoate; DTT, dithiothreitol. For details, see "Discussion."
Activation of the ATPase of \( CF_1 \).

10. McCarty, R. E., and Fagan, J. (1973) Biochemistry 12, 1503-1507

11. Cantley, L. C., and Hammes, G. G. (1976) Biochemistry 15, 9-14

12. Nalin, C. M., Béliveau, R., and McCarty, R. E. (1983) J. Biol. Chem. 258, 3576-3581

13. Arana, J. L., and Vallejos, R. H. (1982) J. Biol. Chem. 257, 1125-1127

14. Mills, J. D., and Hind, G. (1979) Biochim. Biophys. Acta 547, 455-462

15. Vallejos, R. H., Arana, J. L., and Ravizzini, R. A. (1983) J. Biol. Chem. 258, 7317-7321

16. McCarty, R. E., and Racker, E. (1967) J. Biol. Chem. 242, 3435-3439

17. Binder, A., Jagendorf, A., and Ngo, E. (1978) J. Biol. Chem. 253, 3094-3100

18. Penefsky, H. S. (1977) J. Biol. Chem. 252, 2891-2899

19. Chua, N.-H. (1980) Methods Enzymol. 69, 434-446

20. Taussky, H., and Shorr, E. (1953) J. Biol. Chem. 202, 675-685

21. Bruist, M. F., and Hammes, G. G. (1981) Biochemistry 20, 6298-6305

22. Peterson, G. L. (1977) Anal. Biochem. 83, 346-365

23. Moroney, J. V., and McCarty, R. E. (1982) J. Biol. Chem. 257, 5910-5914

24. Zurawaski, G., Bottomley, W., and Whitfield, P. R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6260-6264

25. Ravizzini, R. A., Andreeo, C. S., and Vallejos, R. H. (1980) Biochim. Biophys. Acta 591, 135-141

26. Cerione, R. A., McCarty, R. E., and Hammes, G. G. (1982) Biochemistry 22, 769-776

27. Farron, F. (1970) Biochemistry 9, 3823-3928

28. Moroney, J. V., Fullmer, C. F., and McCarty, R. E. (1984) J. Biol. Chem. 259, 7281-7285

29. Ketcham, S. R., Davenport, J. W., Warncke, K., and McCarty, R. E. (1984) J. Biol. Chem. 259, 7286-7293

30. Deno, H., Shinozaki, K., and Sugiura, M. (1983) Nucleic Acids Res. 11, 2185-2191

31. Kanazawa, H., Kayano, T., Mabuchi, K., and Futai, M. (1981) Biochim. Biophys. Res. Commun. 103, 604-612

32. Nelson, N. (1976) Biochim. Biophys. Acta 456, 314-338

33. Gay, N. J., and Walker, J. E. (1981) Nucleic Acids Res. 9, 2187-2194

34. Andreeo, C. S., Patrie, W. J., and McCarty, R. E. (1982) J. Biol. Chem. 257, 9968-9975