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SULFHYDRYL GROUPS IN PHOTOSYNTHETIC ENERGY CONSERVATION

VI. SUBUNIT DISTRIBUTION OF SULFHYDRYL GROUPS AND DISULFIDE BONDS IN CHLOROPLAST COUPLING FACTOR AND ATPase ACTIVITY

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Summary

The subunit distribution of sulfhydryl groups and disulfide bonds of spinach chloroplasts coupling factor I has been determined.

Native coupling factor I with a latent ATPase activity has eight sulfhydryl groups distributed 4 : 2 : 0 : 0 : 2 in the α, β, γ, δ and ϵ subunits, respectively.

Heat treatment of coupling factor I, in addition to the activation of its ATPase activity, induces a dithiol-disulfide interchange between the γ and the α subunit, changing the sulfhydryl groups' distribution to 2 : 2 : 2 : 0 : 2.

Reduction of disulfide bonds of coupling factor I by dithioerythritol during heat treatment gives a subunit distribution of 4 : 4 : 4 : 0 : 2, suggesting that native coupling factor I has three disulfide bonds, two in the γ subunit and one in one of the β subunits.

The results suggest an asymmetric redox state of some of the subunits of coupling factor I and an asymmetric positioning of some of them in the molecular structure of coupling factor I.

Introduction

Solubilized CF_1 has latent ATPase activity that can be activated by trypsin, heat or dithioerythritol treatment [1—5].

Abbreviations: CF_1, chloroplast coupling factor I; F_1, mitochondrial coupling factor I; Tricine, *N*-tris-(hydroxymethyl)methylglycine; SDS, sodium dodecyl sulfate.

Heat activation of the ATPase activity of CF_1 in the absence or presence of sulfhydryl compounds was carefully studied by Farron and Racker [5]. They suggested that activation was associated with a disulfide interchange of CF_1. They found that CF_1 has 12 half-cystines/mol, eight as free sulfhydryl groups and four in two disulfide bonds [5]. The number of free sulfhydryl groups did not change from native to heat-activated CF_1. Five different subunits have been found and purified from CF_1 [6—9]. Amino acid analysis of purified subunits indicated two, three, six and one half-cystine per subunit for the α, β, γ and ϵ subunits, respectively, giving a total of 18 half-cystines/mol when the stoichiometry is taken into account [1,7,8]. However, Binder et al. [9] have recently found 14 half-cystines/mol of CF_1 distributed two in α, two in β, three in γ, one in δ and one in ϵ subunit. They proposed a subunit stoichiometry of 2 : 2 : 1 : 1 : 2, in agreement with previous estimates [1,10,11].

The ϵ subunit of CF_1 has been purified and shown to act as the ATPase inhibitor [7]. It is sensitive to trypsin and activation of the latent ATPase activity is considered to be a consequence of removing the ϵ subunit from the catalytic site [7,11].

We have recently shown that the ATPase activity of soluble CF_1 can be inhibited by several sulfhydryl reagents only when they are present during heat activation [12]. The modification of CF_1 induced by one of them, *o*-iodosobenzoate, was traced down to the oxidation of two vicinal dithiols, one in a β subunit and the other in the γ subunit [13]. We also found that heat activation in the presence of dithioerythritol reduced all the disulfide bonds of CF_1.

In this paper we report the subunit localization of the free sulfhydryl groups and disulfide bonds of native and heat-activated CF_1 and the nature of the sulfhydryl group rearrangement associated with heat activation of the ATPase activity.

Materials and Methods

CF_1 was purified from spinach chloroplast according to the method described in Ref. 13. Preparations with a specific Ca^{2+}-ATPase activity of 14—20 μmol P_i released per min per mg after heat activation of the enzyme were used. Aliquots of enzyme stored at 4°C in 2 M $(NH_4)_2SO_4$, were centrifuged for 10 min at 10000 × *g* and dissolved in 0.5—1 ml 40 mM Tricine-NaOH (pH 8)/2 mM EDTA. The enzyme was desalted by centrifugation in Sephadex G-50 columns [14] equilibrated with the same solution. Heat activation of CF_1 was carried out and ATPase activity was measured as described earlier [12].

Protein was determined according to the method of Lowry et al. [15] or spectrophotometrically using an extinction coefficient of 0.476 ml/mg per cm [16]. A molecular weight of 325000 [17,18] was used to determine the molar concentrations of CF_1.

Radiochemical titration of sulfhydryl groups was performed by incubation of 1 mg CF_1 with 2 mM *N*-[*ethyl*-2-^{3}H]maleimide (15 μCi/mol) for 1 h at 37°C in a medium (1 ml) containing 40 mM Tricine-NaOH (pH 8) and 2 mM EDTA. Then SDS was added (1%, final concentration) and the mixture was incubated for a further 2 h period. Finally the excess of *N*-[*ethyl*-2-^{3}H]maleimide was eliminated by two consecutive centrifugations in Sephadex G-50 fine columns

[14] equilibrated with 40 mM Tricine-NaOH (pH 8)/2 mM EDTA and 1% dodecyl sulfate. The final concentration of *N*-[*ethyl*-2-^{3}H]maleimide in solution was calculated using a molar extinction coefficient of 620 at 305 nm [19].

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (0.1%) was performed as described by Weber and Osborn [20]. Gels that were 9 cm long and 10% or 12% in polyacrylamide were run for 8 or 16 h, respectively, at 8 mA/tube. After electrophoresis, the gel were cut into 1 mm sections according to Matsumura and Noda [21]. Each section was cut in half, one of which was stained with Coomasie brilliant blue R at 50°C for 2 h and destained by several washings with 7.5% acetic acid/5% CH_3OH. The fixed dye was extracted by incubation of the gels with 1 ml 25% pyridine overnight at room temperature [9]. The absorbance at 605 nm of the pyridine extract was measured and used to localize the subunits. The other half of each gel slice was dissolved in 0.3 ml H_2O_2 (40, v/v) in sealed scintillation vials for 5 h at 50°C. After cooling, 5 ml of scintillation fluid (5 g 2,5-diphenyloxazole and 100 g naphthalene in 1 l 1,4-dioxane) was added and the radioactivity of *N*-[*ethyl*-2-^{3}H]maleimide was counted in a Beckman LS 233 liquid scintillation counter. The efficiency determined with an internal standard was 24%. Samples of protein (10—20 μg) similar to those applied to the gel were counted in the same conditions and the mol of *N*-[*ethyl*-2-^{3}H]maleimide incorporated/mol CF_1 were calculated. Distribution of *N*-[*ethyl*-2-^{3}H]maleimide into subunit bands was calculated from the percentage of total radioactivity applied on to the gel. Recovery of radioactivity applied to the gel was 95—100%.

Tricine, dithioerythritol, *N*-ethylmaleimide and ATP were obtained from Sigma Chemical Co.; *N*-[*ethyl*-2-^{3}H]maleimide (specific radioactivity 160 Ci/mol) was obtained from New England Nuclear; 2,5-diphenyloxazole and naphthalene from Beckman and 1,4-dioxane from Baker Chemical Co. All other reagents were of analytical grade.

Results

The subunit distribution of sulfhydryl groups of CF_1 was determined by *N*-[*ethyl*-2-^{3}H]maleimide incorporation into CF_1 subunits separated by SDS-polyacrylamide gel electrophoresis in 10% and 12% gels. The latter were used to resolve α and β subunits. Table I shows the results found with native CF_1 and CF_1 heat-activated in the absence or presence of dithioerythritol.

The number of free sulfhydryl groups of native CF_1 remained eight/mol CF_1 after heat activation of its ATPase activity (Table I) as was shown previously [5]. When heat activation was carried out in the presence of dithioerythritol, a higher ATPase activity was obtained, the disulfide bonds were reduced, and 12.9 sulfhydryl groups/mol of CF_1 were found (Table I, third line; see also Ref. 13).

Using iodo[1-^{14}C]acetamide, Farron and Racker [5] found that the total number of sulfhydryl groups of CF_1 was also about 13. The average number of half-cystines per CF_1 determined in isolated subunits of CF_1 by Binder et al. [9] was also 13. The latter authors favor a theoretical total of 14 per CF_1.

TABLE I

SUBUNIT DISTRIBUTION OF FREE SULFHYDRYL GROUPS IN CF_1

Values shown are means ± S.D. from four experiments for each treatment of CF_1. 10% and 12% gels were run for each experiment. The values for α and β subunits were calculated from the radioactivity incorporated into α and β bands in 12% gels.

Treatment of CF_1	ATPase activity (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	mol *N*-[*ethyl*-2-^{3}H]-maleimide/mol CF_1	Sulfhydryl groups				
			Subunit distribution (mol *N*[*-ethyl*-2-^{3}H]maleimide/mol CF_1)				
			α	β	γ	δ	ϵ
None	2.9 ± 0.4	7.7 ± 0.4	4.2 ± 0.4	2.1 ± 0.3	0.4 ± 0.4	0.1 ± 0.2	1.0 ± 0.7
Heat activation	15.4 ± 2.4	7.9 ± 0.3	1.9 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	0	1.8 ± 0.3
Heat activation in the presence of 5 mM dithioerythritol	27.1 ± 5.5	12.9 ± 0.3	4.1 ± 0.2	3.9 ± 0.6	3.5 ± 0.9	0.1 ± 0.1	1.5 ± 0.8

These results and ours (see below) suggest the presence of three rather than two [5] disulfide bonds.

The number of sulfhydryl groups found per α, β, γ, δ and ϵ subunits of native CF_1 were 4, 2, 0, 0 and 2, respectively, after rounding off the figures of Table I (first line).

Heat activation of CF_1 resulted in a decrease in the free sulfydryl groups of α subunits from 4.2 to 1.9 and in an increase in the γ subunit from 0.4 to 2.1 while minor changes or none at all were observed for the β and ϵ subunits (Table I). These results suggest that two vicinal sulfhydryl groups of one of the two α subunits were oxidized to a disulfide bond by the γ subunit during heat activation (the disulfide bonds of CF_1 are all intrapeptidic; Ref. 13).

After heat activation of CF_1 in the presence of dithioerythritol the sulfhydryl groups of the β and γ subunits increased to four from two and none, respectively, in native CF_1 (Table I). Thus, in native CF_1 there are three disulfide bonds, one in a β subunit and two in the γ subunit. On the other hand the sulfhydryl groups of α, β and γ subunits from CF_1 heat-activated in the absence of dithioerythritol increased from two to four when dithioerythritol was present suggesting that the three disulfide bonds of heat-activated CF_1 are localized one in each one of α, β and γ subunits. These results together with the increase from eight to 13 sulfhydryl groups/mol of CF_1 after the treatment with dithioerythritol discussed above suggest that CF_1 has a total number of 14 sulfhydryl groups/mol, in agreement with the calculations of Binder et al. [9].

The results shown in Table I are means from four different experiments for each treatment of CF_1. Results obtained from other experiments carried out under different experimental conditions or for other purposes (e.g., localization of disulfide bonds formed by *o*-iodosobenzoate; Ref. 13) were in agreement with these. For instance, in six experiments where no resolution was achieved between α and β subunits, the incorporation of *N*-[*ethyl*-2-^{3}H]maleimide into $\alpha + \beta$ subunits corresponded with the sum of radioactivity incorporated into each of them shown in Table I.

Sulfhydryl groups were never found in the δ subunit, in agreement with Nelson [1] and McCarty [2], but at variance with Binder et al. [9].

Discussion

Some of the results presented in this paper and in a previous one [13] are summarized in Fig. 1. We found that: (1) the distribution of sulfhydryl groups in the α, β, γ, δ, and ϵ subunits is 4 : 2 : 0 : 0 : 2 in native CF_1 (Fig. 1A) and 2 : 2 : 2 : 0 : 2 in heat-activated CF_1 (Fig. 1B). (2) There are three intrapeptidic disulfide bonds in native CF_1, one located in a β subunit and two in the γ subunit. (3) Heat activation of CF_1 is associated with the formation of one disulfide bond in the α subunit at the expense of one disulfide bond in the γ subunit.

Our results (Ref. 13 and this paper) point to several asymmetries in the CF_1 molecule (Fig. 1): (1) Only two sulfhydryl groups, one in β and one in ϵ, are accessible to reaction with *N*-ethylmaleimide in native CF_1 [13]. Thus the positioning of the two β and two ϵ subunits in the molecular architecture of CF_1 is asymmetric. (2) The redox states of the two half-cystine groups of the

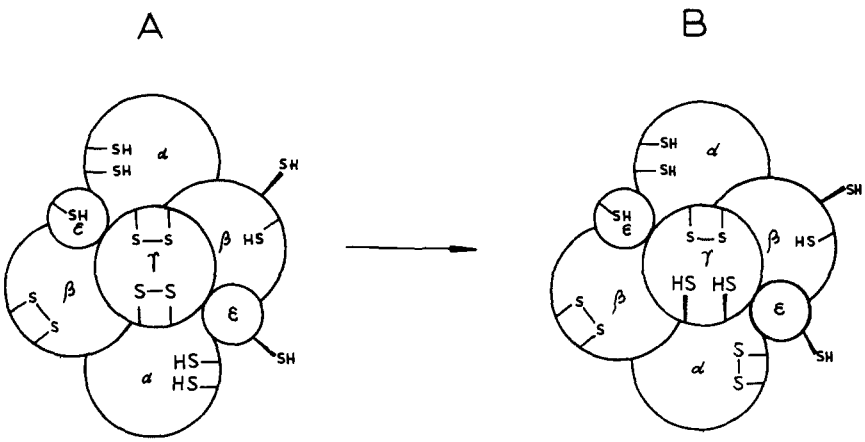

Fig. 1. Asymmetric distribution and rearrangement of sulfhydryl groups in CF_1. (A) Native CF_1; (B) heat-activated CF_1. The accessible sulfhydryl groups are represented with a larger bar. The groups involved in the thiol-disulfide interchange between the α and γ subunits are presented with larger letters. The δ subunit which has no sulfhydryl groups is not shown for simplicity.

two β subunits are different: in one subunit they are reduced and in the other they form a disulfide bond. (3) Heat activation of CF_1 results in the formation of a disulfide bond in one of the α subunits by disulfide interchange with the γ subunit. Thus the two α subunits are then in a different redox state.

The redox state of CF_1 subunits should be kept in mind when attempting reconstitution from purified subunits, since if purification is carried out under reducing conditions not all the subunits may be in the right redox condition for reconstitution.

Senior [22,23] has located the sulfhydryl groups and disulfide bonds of beef heart mitochondrial coupling factor I (F_1). Similar to CF_1, F_1 has eight free sulfhydryl groups, two of them freely accessible to reagents. Increase in the ATPase activity of F_1 and CF_1 by heating is associated with the disappearance of the accessible groups of F_1 [22] and the appearance of two more in CF_1 [5]. At variance with CF_1 [13], one of the disulfide bonds of F_1 [23] is interpeptidic.

Our results support the suggestion of Farron and Racker [5] that heat activation of CF_1 was related to rearrangement of disulfide bonds and are not against the proposal of Nelson et al. [7,11] that ATPase activation results from the displacement of the ϵ subunits from CF_1 since both phenomena may occur during heat activation. It is interesting to note that an antiserum against the γ subunit of CF_1 as well as dithiothreitol interfered with the inhibition of heat-activated CF_1 by the purified ϵ subunit [6]. Since the γ subunit of CF_1 is involved in the disulfide interchange and the anti-γ prevents the effect of the CF_1 inhibitor, it is possible that the inhibitor acts by inducing a rearrangement of disulfide bonds back to the state of native CF_1.

It has recently been suggested [24,25] that sulfhydryl groups may play a role in the transport of protons to the active site of ATP synthesis. Considering the different redox state of the two β subunits and that most of the evidence available suggests that the catalytic site(s) is on the β subunits, it would be

interesting to know if this asymmetric redox state plays any role in the catalytic activity of CF_1. For instance, considering the alternating site cooperativity suggested for ATP synthesis [26] it is possible that the alternation of the β subunits between two conformational states is associated with a shift of the disulfide bond between them.

The intramolecular thiol-disulfide interchange between α and γ subunits has been observed in in vitro experiments with soluble and purified CF_1 and requires heating of CF_1. However, it may also happen under 'in vivo' conditions where the disulfide exchange may depend on illumination of the chloroplasts. This suggestion is supported by the fact that solubilized CF_1 has no free sulfydryl group in its γ subunit, but modification of chloroplasts in the light by *o*-iodosobenzoate showed that under these condition there is a vicinal dithiol in the γ subunit [13,17]. Thus, this vicinal dithiol is either formed during illumination of the chloroplasts or is oxidized to a disulfide when CF_1 is solubilized. The former seems more likely and may proceed by a disulfide interchange similar to that described in this paper.

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