Biochimica et Biophysica Acta, 682 (1982) 135–144
Elsevier Biomedical Press

BBA 41172

THE INTERACTION OF PHENYLGLYOXAL WITH SOLUBLE AND MEMBRANE-BOUND CHLOROPLAST COUPLING FACTOR 1

ALEJANDRO M. VIALE, CARLOS S. ANDREO and RUBÉN H. VALLEJOS *

CEFOBI, Suipacha 531, 2000 Rosario (Argentina)

(Received April 29th, 1982)

Key words: Chemical modification; ATPase; Phenylglyoxal; Coupling factor; (Spinach chloroplast)

The rate of inhibition of cyclic photophosphorylation in chloroplast thylakoids by the arginine reagent phenylglyoxal was enhanced in the light, i.e., under conditions where membrane energization occurred. Uncouplers, but not energy-transfer inhibitors, prevented the effect of light. Chemical modification of chloroplast thylakoids by phenylglyoxal under dark or in light conditions affected differently the light-induced exchange of tightly bound ADP. In both cases the exchange was less inhibited than photophosphorylation. Complete inhibition of ATPase activity of soluble CF_1 was correlated with the incorporation of 8 mol [^{14}C]phenylglyoxal per mol enzyme. About 50% of the incorporated radioactivity was lost at different rates depending on the buffer present and suggesting a change in the stoichiometry of the adduct from 2:1 to 1:1. Inhibition of ATPase and photophosphorylating activities of chloroplasts by modification with [^{14}C]phenylglyoxal in the dark was associated with the incorporation of 1 and 2 mol reagent per mol membrane-bound CF_1, respectively. In the light the rate of incorporation was enhanced and both reactions were inactivated when 2 mol [^{14}C]phenylglyoxal/CF_1 were bound. In all the labelling experiments the radioactivity was mainly recovered from the α- and β-subunits.

Introduction

The synthesis of ATP in chloroplasts is catalyzed by an ATPase complex capable of utilizing energy derived from light-induced electron transport. It consists of a hydrophobic component embedded in the thylakoid membrane and a hydrophilic one, the coupling factor 1 (CF_1) that contains the catalytic site for synthesis and hydrolysis of ATP.

Chemical modification of chloroplasts with the arginine reagents 2,3-butanedione or phenylglyoxal resulted in inhibition of photophosphorylation, ATP-P_i exchange and Mg^{2+}-ATPase activities [1–5]. These modifiers behaved as energy-transfer inhibitors [1]. The ATPase activity of soluble CF_1 was completely inhibited by the reagents, suggesting that the essential arginines are located on the coupling factor molecule [2,3]. Adenine nucleotides afforded protection to chloroplasts and CF_1 against inactivation, suggesting that the essential arginine residues may be involved in substrate binding [2,3].

Chemical modification of the chloroplast ATPase by inorganic sulfate [6], sulfhydryl reagents [7–10] or permanganate [11] was shown to be light dependent. Light also induces an exchange of tightly bound adenine nucleotides on membrane-bound CF_1 with medium nucleotides [12–14]. These effects of light are associated with conformational changes of CF_1 that occur during

* To whom correspondence should be addressed

Abbreviations: Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PPO, 2,5-diphenyloxazole.

0005-2728/82/0000-0000/$02.75

membrane energization as originally shown by Ryrie and Jagendorf [15,16].

In the present work, we have studied the effects of phenylglyoxal on chloroplast thylakoids when acting in the dark and under conditions of membrane energization. We have also studied the relationship between inactivation of photophosphorylation, ATPase activities and [^{14}C]phenylglyoxal incorporation into CF_1.

Materials and Methods

Chloroplasts were obtained as described previously [17] from market spinach leaves in a medium composed of 25 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 5 mM $MgCl_2$ and 10 mM NaCl. They were washed once in 10 mM NaCl, and finally resuspended in 0.05 M borate buffer (pH 8.0) and 1 mM $MgCl_2$ at a chlorophyll concentration of 2 mg/ml.

CF_1 was isolated from market spinach as described earlier [18]. Polyacrylamide disc gel electrophoresis according to the method of Davis [19] showed that the protein was more than 95% pure. ATPase activities of preparations used in the experiments reported in this paper varied, after trypsin activation, between 20 and 25 μmol ATP/min per mg.

Cyclic photophosphorylation was measured as previously described [17]. The unreacted $^{32}P_i$ was removed as a phosphomolybdate-triethylamine complex as described earlier [20]. Light- and dithioerythritol-activated ATPase was measured as described previously [21].

The light-induced [^{14}C]ADP exchange of control and phenylglyoxal-modified chloroplast thylakoids was measured according to the method of Strotmann et al. [22] at an adenine nucleotide concentration of 10 μM. The radioactivity bound to the washed membranes was measured by liquid scintillation counting in 10 ml of scintillation fluid containing 6 g PPO and 333 ml Triton X-100 per l toluene.

ATPase activity of CF_1 activated by heat [23] or by trypsin [24] was measured in 1 ml reaction mixture containing 50 mM Tris-HCl (pH 8.5), 5 mM ATP, 5 mM $CaCl_2$ and CF_1 (3–5 μg). After 5 min at 37°C the reaction was stopped with 1 ml of ice-cold 8% trichloroacetic acid, and the P_i liberated from the ATP was measured [25].

Protein was determined according to the method of Lowry et al. [26] or by the Coomassie G dye-binding method [27] using as standard bovine serum albumin ($E^{1\%}_{279} = 6.67$) or a standard CF_1 solution whose concentration was determined spectrophotometrically [23].

When CF_1 was modified with [^{14}C]phenylglyoxal, the nonbound reagent was separated from the protein by a filtration-centrifugation column [28] equilibrated with 50 mM Tricine-NaOH (pH 8.0), 2 mM EDTA, 1 mM ATP. The eluates were assayed for protein, ATPase activity and radioactivity. Incorporation of [^{14}C]phenylglyoxal into membrane-bound CF_1 was carried out in a medium containing 50 mM borate buffer (pH 8.0), 5 mM $MgCl_2$, 10 mM [^{14}C]phenylglyoxal (58 dpm/nmol) and thylakoids (0.4 mg Chl/ml). Aliquots containing 1–2 mg Chl were diluted at the times stated in 10 vol. of 25 mM Tricine-NaOH (pH 8.0), 1 mM $MgCl_2$, 50 mM NaCl and centrifuged. Thylakoids were resuspended in the same medium and cyclic photophosphorylation, and light- and dithioerythritol-triggered ATPase measured. In another aliquot, CF_1 was extracted by the chloroform method [29]. The concentration of solubilized CF_1 was determined by the immunoelectrodiffusion method [30]. Then, CF_1 was quantitatively precipitated by a specific antibody. The immunoprecipitates were left overnight at 4°C, collected by centrifugation and washed twice in 20 mM P_i (pH 7.0), 0.1 M NaCl and 0.5% Triton X-100. The pellets were finally dissolved in 0.2 ml of 2% SDS, 3 ml of 0.5% PPO and 10% naphthalene in 1,4-dioxane were added and the radioactivity measured by scintillation counting.

SDS-polyacrylamide gel electrophoresis was carried out in a slab gel (180 × 120 × 1 mm), using the gel and buffer systems described by Neville and Glossmann [31]. This system gave a good resolution between α- , β- and γ-subunits with the minor ones comigrating with the front. The δ- and ϵ-subunits were separated from small amounts of peptides of low molecular weight in a second gel containing 15% acrylamide and 0.3% bisacrylamide. After staining and destaining [31], the subunit bands were sliced and each treated overnight with 0.3 ml of NCS tissue solubilizer (Amersham). 3 ml of 0.6% PPO, 33% Triton X-100 in toluene

were added, and the radioactivity measured by liquid scintillation counting. Radioactivity measurements were made in a Beckman LS 8100 liquid scintillation counter.

Phenylglyoxal solutions were prepared freshly for each experiment in 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.0), or 0.05 M borate buffer (pH 8.0), and the pH readjusted to 8.0 with 1 M NaOH. The concentration of the solutions was measured spectrophotometrically assuming $\epsilon_{253} = 12600\ M^{-1} \cdot cm^{-1}$ in water [32].

[2-^{14}C]Phenylglyoxal was obtained from the Comisión Nacional de Energía Atómica (Argentina) or the Commissariat a L'Energie Atomique (France). Both nonlabelled and [^{14}C]phenylglyoxal were determined to be more than 95% pure by ascendent paper chromatogaphy of the compounds and their adducts with trimethylaminoacetohydrazine chloride (a specific reagent for α-dicarbonyl compounds [33]) in the solvent system benzene/methanol/acetic acid (45:8:4, v/v).

[8-^{14}C]ADP (52 mCi/mmol) was obtained from New England Nuclear. Nucleotides, phenazine methosulfate, Sephadex, Tricine, trypsin, soybean trypsin inhibitor and phenylglyoxal were purchased from Sigma (U.S.A.). All other chemicals were analytical grade from BDH Chemicals (U.K.).

Results

Modification of chloroplast thylakoids with phenylglyoxal

Incubation of chloroplast thylakoids with phenylglyoxal in the light and in the presence of phenazine methosulfate resulted in an enhancement in the rate of inactivation of photophosphorylation when compared with the inhibition obtained in the dark under similar conditions (Fig. 1). When plotted against time of incubation and at a fixed phenylglyoxal concentration, the inhibition in the dark or in the light followed apparent first-order kinetics (Fig. 1). The apparent k (defined as $\ln 2/t_{0.5}$) for light inactivation ($k'_{light} = 0.99\ min^{-1}$) was 1.5-times larger than that observed for dark inactivation ($k'_{dark} = 0.39\ min^{-1}$). Light and an added electron carrier such as phenazine methosulfate were required for the enhancement observed in inhibition, as shown in Table I. When uncouplers (such as NH_4Cl or

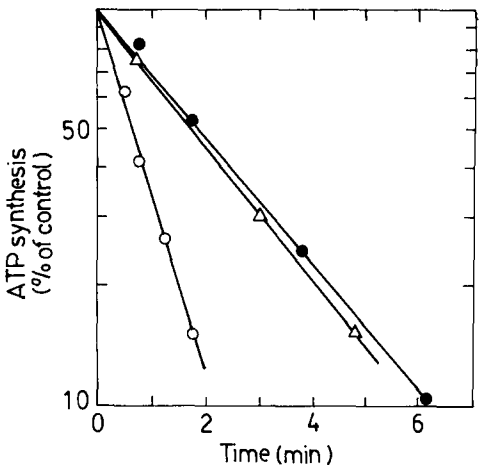

Fig. 1. Inhibition of cyclic photophosphorylation by phenylglyoxal: Effect of light. The reaction mixture contained, in a final volume of 1 ml, 0.05 M borate buffer (pH 8.0), 5 mM $MgCl_2$, 50 μM phenazine methosulfate, 50 mM phenylglyoxal and chloroplast thylakoids (0.2 mg Chl/ml). It was incubated at 25°C in the dark (●——●), light (10^6 erg/cm per s) (○——○), or light plus 5 μM FCCP (△——△). At the times indicated aliquots corresponding to 5 μg Chl were withdrawn and assayed for cyclic photophosphorylation as described in Materials and Methods. Controls varied between 780 and 815 μmol ATP/h per mg Chl.

FCCP) were present during preincubation, the inhibition obtained in the light was almost the same as that obtained in the dark (Fig. 1 and Table I). Thus, uncouplers prevented the effect of light, indicating that an energized membrane condition was necessary for the acceleration of the rate of inhibition. Energy-transfer inhibitors of the type that blocks the catalytic activity of CF_1 (such as Dio-9 or phlorizin) did not alter significantly the effect of light on the modification (Table I).

Phenylglyoxal inhibition of ADP exchange

Adenine nucleotide exchange in thylakoids has been shown to consist of two different equilibrium states, one in the light and one in the dark following illumination [22]. Pretreatment of chloroplast thylakoids with phenylglyoxal in the dark or in the light affected differently the extent of ADP exchange, as shown in Table II.

For the dark-modified chloroplasts, the extent of inhibition for the exchange of [^{14}C]ADP in the light and in the dark (following illumination) was

TABLE I

PHENYLGLYOXAL MODIFICATION OF CHLOROPLAST THYLAKOIDS IN THE DARK AND IN THE LIGHT

Chloroplast thylakoids were incubated for 2 min at 25°C in the dark or in the light in the following medium: 0.05 M borate buffer (pH 8.0), 5 mM $MgCl_2$, 50 mM phenylglyoxal and chloroplasts (0.2 mg Chl/ml) with the additions stated in the table. Where indicated, phenazine methosulfate (PMS) was at a concentration of 50 μM, NH_4Cl 5 mM, FCCP 5 μM, phlorizin 1 mM, and Dio-9 10 μg/ml. After modification, aliquots containing 5 μg Chl were assayed for cyclic photophosphorylation. PGO-Chl, chloroplast modified with phenylglyoxal. Values in parentheses indicate % of controls.

Additions during preincubation	Cyclic photophosphorylation (μmol ATP/mg Chl per h)			
	Dark		Light	
	Control	PGO-Chl	Control	PGO-Chl
None	801	536 (67)	643	389 (61)
PMS	790	525 (66)	623	235 (38)
PMS, NH_4Cl	567	366 (65)	515	360 (70)
PMS, FCCP	585	293 (50)	514	262 (51)
PMS, phlorizin	431	276 (64)	349	112 (32)
PMS, Dio-9	745	417 (56)	559	173 (31)

similar. For the light-modified chloroplasts the extent of inhibition was higher, and the dark [^{14}C]ADP exchange was more depressed than the exchange in the light as shown in Table II. Cyclic photophosphorylation was similarly inhibited in both cases.

The kinetics of binding of [^{14}C]ADP to phenylglyoxal-modified thylakoids are shown in Fig. 2. In chloroplasts modified in the dark a slight inhibi-

TABLE II

EFFECT OF PHENYLGLYOXAL ON THE LIGHT-INDUCED [^{14}C]ADP EXCHANGE IN CHLOROPLAST THYLAKOIDS

Modification of thylakoids with 50 mM phenylglyoxal (PGO) was carried out for 8 min in the dark or 2 min in the light in a reaction mixture containing, in a final volume of 2 ml, 0.05 M borate buffer (pH 8.0), 5 mM $MgCl_2$, 50 μM phenazine methosulfate and chloroplasts (0.2 mg Chl/ml). The modification was stopped and the adenine nucleotide exchange was measured as described in Materials and Methods. The light exchange was measured after 1 min of illumination, and the light-induced dark binding after 1 min in the dark following preillumination. Values in parentheses indicate % inhibition.

Pretreatment of chloroplasts	[^{14}C]ADP exchange (mol/mg Chl)		Photophosphorylation (% inhibition)
	Light	Dark	
Dark	0.28	0.62	
Dark + PGO	0.20 (29)	0.45 (28)	91
Light	0.29	0.61	
Light + PGO	0.17 (41)	0.20 (67)	82

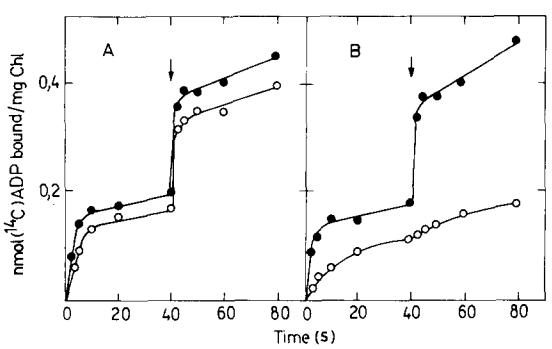

Fig. 2. Kinetics of [^{14}C]ADP binding to phenylglyoxal-modified chloroplast thylakoids. Chloroplasts (0.2 mg Chl/ml) were modified with 50 mM phenylglyoxal for 2 min in the light or 6 min in the dark in the reaction mixture described in the legend to Fig. 1, in a final volume of 5 ml. The reaction was stopped by dilution in 10 vol. of ice-cold medium containing 25 mM Tricine-NaOH (pH 8.0), 1 mM $MgCl_2$, 50 mM NaCl and 5 μM ADP, and the modified chloroplasts were washed twice in the same medium without ADP. The kinetic of [^{14}C]ADP incorporation for the dark- (A) or the light- (B) modified chloroplasts were measured as described in Materials and Methods. Arrow indicates light off. (●——●) Control chloroplasts; (○——○) phenylglyoxal-modified chloroplasts.

tion in the extent of binding in the light and in the dark was observed (Fig. 2A). When chloroplasts were modified in the light, the extent of binding of ADP in the light was partially inhibited, and the rapid phase of binding that occurred in the dark was almost completely inhibited by the reagent (Fig. 2B).

Incorporation of [^{14}C] phenylglyoxal into soluble CF_1

It was previously suggested [2] that the inhibition of ATPase activity of CF_1 by phenylglyoxal is a consequence of the modification of one arginine residue per active site. Since the inhibition of the activity was prevented in the presence of ATP, it was suggested that the essential arginine residue is located in the catalytic site [2].

Fig. 3 shows that the inhibition of the ATPase activity of native CF_1 or heat-activated ATPase was concomitant with the incorporation of [^{14}C]phenylglyoxal into the protein. The incorporation followed a biphasic pattern. However, the degree of inactivation was directly proportional to the extent of incorporation during the initial 60–70% loss of enzyme activity. Extrapolation of the linear portion of the curve to total inactivation gives a value of about 8 mol [^{14}C]phenylglyoxal/mol CF_1. When ATP or GTP were present during CF_1 modification incorporation of reagent into CF_1 and inactivation were diminished. For instance, protection of the ATPase by 20 mM GTP was associated with a reduction of the incorporation from 8 to 6 mol [^{14}C]phenylglyoxal/mol CF_1. It is unlikely that GTP afforded more protection than ATP, since half-times of inactivation, determined under experimental conditions identical to those of Fig. 3, were increased 3- and 2-fold, respectively, by 10 mM nucleotide. Moreover, GMP was as affective as GTP while AMP afforded less protection than ATP [2]. Frigeri et al. [34] found that GDP protected the mitochondrial ATPase against phenylglyoxal and considered that the protective effect was improbable due to an interaction of the reagent with GDP. However, it is known that glyoxal derivatives react with guanine [35]. Specifically, phenylglyoxal was recently reported to form an adduct with GMP involving the primary amino group and the N_1 from the guanine [36,37]. Therefore, we suspect that since guanosine nucleotides may react with phenylglyoxal, we and others [34] may have observed an unknown combination of both effects, interaction with the reagent and protection.

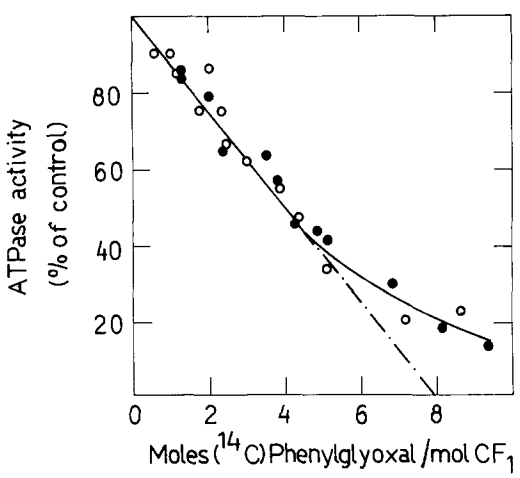

Fig. 3. Correlation between ATPase inactivation and [^{14}C]phenylglyoxal incorporation into soluble CF_1. Native (○) or heat-activated (●) CF_1 was incubated at 25°C in a reaction medium containing 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.0), 1 mM EDTA, 1 mM [^{14}C]phenylglyoxal and CF_1 (2 mg/ml). ATPase activity and radioactivity incorporated into CF_1 were measured as described in the text. Native CF_1 was activated by trypsin after modification.

When the native or heat-activated enzyme was modified with [^{14}C]phenylglyoxal, part of the radioactivity was recovered from and about equally distributed in the α- and β-subunits after SDS-polyacrylamide gel electrophoresis. The radioactivity recovered from the minor subunits did not differ significantly from the gel background. A partial explanation of the low recovery of radioactivity may be a change in the stoichiometry of the reaction of phenylglyoxal with the guanidinium group of arginine. It has been reported that the stoichiometry is 2:1 [41], although a 1:1 ratio has also been reported [39,40].

We observed that the rates of inactivation of ATPase and incorporation of [^{14}C]phenylglyoxal into CF_1 were similar in *N*-ethylmorpholine or in borate buffers (Fig. 4). However, when excess reagent was removed the rate of loss of [^{14}C]phenylglyoxal from the labelled enzyme was markedely different in both systems. From the enzyme modified in *N*-ethylmorpholine buffer (Fig. 4A) 50% of the radioactivity was lost in about 30 h, whereas in the protein modified in borate buffer, 40–50% of

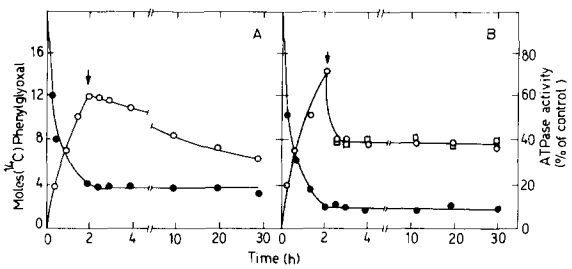

Fig. 4. Relationship between ATPase inactivation and [^{14}C]phenylglyoxal incorporation into CF_1. Loss of radioactivity after gel filtration in different buffers. CF_1 (heat activated) was incubated at 25°C in (A), 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.0), 1 mM EDTA, 1 mM [^{14}C]phenylglyoxal and 2 mg/ml enzyme, and (B) 0.05 M borate buffer (pH 8.0), 1 mM EDTA, 10 mM [^{14}C]phenylglyoxal and 2 mg/ml CF_1. After 2 h the excess labelled reagent was separated from the protein with a filtration-centrifugation column equilibrated with the same buffer used during modification. At the times indicated 100-μl aliquots were passed through a second filtration-centrifugation column, and in the eluates, protein, ATPase activity and radioactivity were measured. ATPase activity (●——●); mol [^{14}C]phenylglyoxal/mol CF_1 (○——○), mol [^{14}C]phenylglyoxal/mol CF_1 in the presence of 1% SDS (□——□).

the bound [^{14}C]phenylglyoxal was lost in less than 30 min (Fig. 4B). The latter pattern was not altered by addition of SDS added immediately after removing excess reagent. It is interesting to note that the remaining bound [^{14}C]phenylglyoxal was stable for the time tested, and no recovery of ATPase activity was observed in any of the buffers used.

Incorporation of [^{14}C]phenylglyoxal into membrane-bound CF_1

Fig. 5 shows the kinetics of incorporation of [^{14}C]phenylglyoxal into CF_1 when chloroplasts were incubated with the reagent in the dark or in the light. Both curves are biphasic and the initial rate of incorporation in the light was twice the rate in the dark, in good agreement with the difference in the rate of inactivation (Fig. 1). At the given times, aliquots were taken for assays of their ATPase and phosphorylating activities, and for purification of CF_1 and determination of the amount of [^{14}C]phenylglyoxal incorporated. There was a linear relationship between the inactivation of the light- and dithioerythritol-triggered ATPase

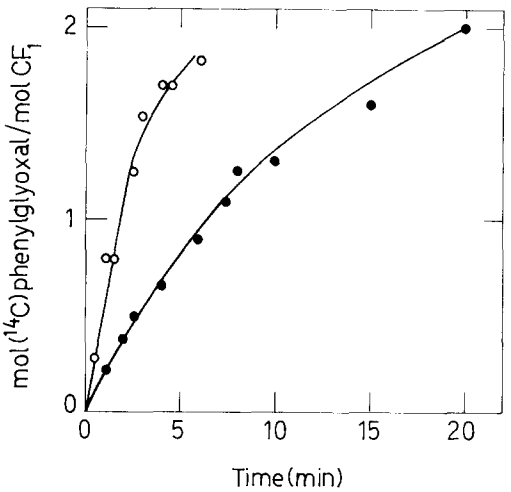

Fig. 5. Time course of [^{14}C]phenylglyoxal incorporation into membrane-bound CF_1 of thylakoids modified in the dark (●——●) or in the light (○——○).

and incorporation of [^{14}C]phenylglyoxal into CF_1 that extrapolates to about 1.3 mol/mol CF_1 (Fig. 6). Photophosphorylation was still not affected when the ATPase was 50% inhibited and 0.6–0.7 mol [^{14}C]phenylglyoxal was incorporated per mol CF_1. Complete inhibition of photophosphorylation seems to correlate with incorporation of 2 mol [^{14}C]phenylglyoxal/mol CF_1.

When modification of chloroplasts was carried out in the light, which enhanced the rate of inactivation (Fig. 1), the correlation between [^{14}C]phenylglyoxal incorporated into CF_1 and inhibition of ATPase and phosphorylating activities was linear for both reactions, and comparable in extent and extrapolated to 2 mol [^{14}C]phenylglyoxal/mol CF_1 (Fig. 7). Incorporation of 0.7 mol [^{14}C]phenylglyoxal/mol CF_1 was associated with 40% inhibition of photophosphorylation when modification was carried in the light, in contrast with the lack of effect of that level of incorporation when modification was in the dark.

The ATPase activity elicited by trypsin treatment in CF_1 extracted from chloroplasts modified either in the dark or in the light was inhibited similarly to the light- and dithioerythritol-triggered ATPase of the same chloroplasts (data not shown).

Table III shows the subunit localization of [^{14}C]phenylglyoxal incorporated into membrane-

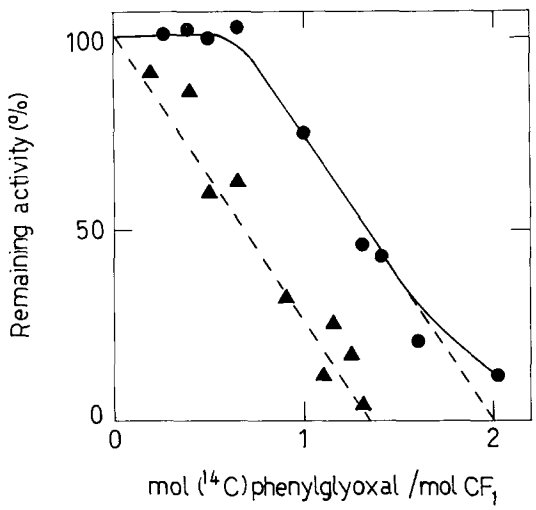

Fig. 6. Correlation between inactivation of Mg^{2+}-ATPase (▲——▲) or photophosphorylation (●——●) and incorporation of [^{14}C]phenylglyoxal into membrane-bound CF_1 of chloroplasts modified in the dark.

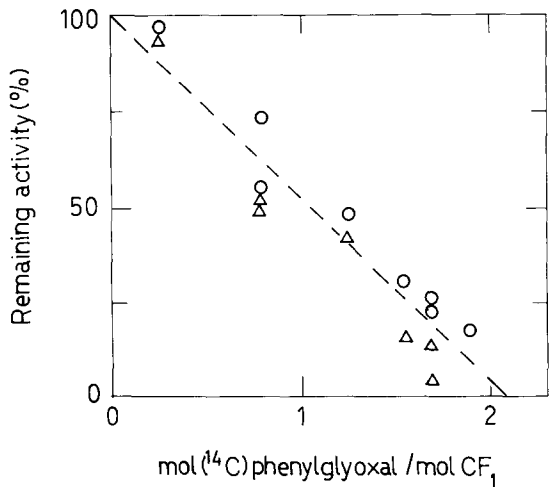

Fig. 7. Correlation between inactivation of Mg^{2+}-ATPase (▲——▲) or photophosphorylation (○——○) and incorporation of [^{14}C]phenylglyoxal into membrane-bound CF_1 of chloroplasts modified in the light.

bound CF_1 when chloroplasts were modified in the dark or in the light under experimental conditions similar to those used in Figs. 6 and 7. Three periods of incubation were chosen to obtain about 50% inhibition of ATPase and photophosphorylation in chloroplasts modified in the dark and in the light. The corresponding incorporation of reagent was, as expected from Figs. 6 and 7, 0.5 mol [^{14}C]phenylglyoxal/mol CF_1 for the ATPase (dark modification) and about 1 for photophosphorylation (either dark or light modification). Slightly more than 80% of the recovered radioactivity was

TABLE III

DISTRIBUTION OF [^{14}C]PHENYLGLYOXAL IN THE SUBUNITS OF CF_1 PURIFIED FROM CHLOROPLASTS MODIFIED IN THE DARK OR IN THE LIGHT

Chloroplast thylakoids were modified with 1 mM [^{14}C]phenylglyoxal (containing 11600 dpm/nmol) for 10 or 30 min in the dark, or for 10 min in the light, and either Mg^{2+}-ATPase or photophosphorylation activity was measured under each experimental condition. Remaining activities are shown in the first row. Then, the incorporation of [^{14}C]phenylglyoxal into membrane-bound CF_1 and its subunit distribution were determined as described in the text. The recovery of radioactivity from the stained bands was between 62 and 77%

	Mg^{2+}-ATPase (dark, 10 min)	Photophosphorylation	
		Dark, 30 min	Light, 10 min
Remaining activity (%)	53	49	51
mol [^{14}C]phenylglyoxal/mol CF_1	0.5	1.2	0.8
Radioactivity distribution (dpm)			
α-Subunit	207	349	412
β-Subunit	328	567	467
γ-Subunit	34	60	68
δ-Subunit	24	26	27
ε-Subunit	32	58	45
Front	48	53	63

found nearly equally distributed in the α- and β-subunits. In the dark experiments 50% of the recovered radioactivity was found in the β-subunit and 31% in the α-subunit while in the light the distribution was 43 and 38%, respectively.

Discussion

Modification of chloroplasts in the light instead of in the dark resulted in (a) an enhancement of the rate of inactivation of photophosphorylation and (b) a large increase in the extent of inhibition of ADP exchange. The first phenomenon clearly requires energization of the thylakoid membrane, since uncouplers (Table I and Fig. 1) prevented it. Thus, it is similar to light-dependent chemical modification of chloroplasts by other reagents [6–11]. The effect of light may be mediated by a conformational change of CF_1 [15,16] that either increases the reactivity of the essential arginine [2,3] towards phenylglyoxal or exposses new residues to the reagent. A similar effect of light was described in the derivate-formation reaction of arginines in *Rhodospirillum rubrum* chromatophores [42]. Schmid et al. [1] found no greater inhibition of photophosphorylation by arginine reagents in the light. Since the actual experiments were not reported, the difference with respect to our results may be due to partial uncoupling of their chloroplast preparation or lack of a catalyst in their reaction medium.

The second phenomenon, i.e., the larger inhibition of ADP exchange in chloroplasts modified with phenylglyoxal in the light, may be explained as follows: the light-induced exchange of adenine nucleotides in chloroplast thylakoids has been suggested to include three different conformational states of CF_1 [14]: (I) a stable, nonexchangeable form with tightly bound nucleotides; (II) an unstable form containing loosely bound nucleotides and (III) a metastable depleted form. These three forms coexist in equilibrium in the light.

If arginine residues are involved in the binding of tightly bound adenine nucleotides, the modification with phenylglyoxal in the dark should modify only those residues not protected by the tight bound nucleotides. Then, the exchange of ADP in the light and in the rapid phase in the dark following preillumination would be slightly modified in extent and rate (Fig. 2A).

When modification is carried out in the light, the metastable form of the equilibrium (state III) would be modified by the reagent altering the equilibrium irreversibly. Under such conditions, we would expect a large inhibition both in the rate and extent of the ADP binding following deenergization, and a minor inhibition of the light exchange (partially protected by the remaining ADP), as shown in Fig. 2B,

Thus, the inhibition of ADP exchange by phenylglyoxal suggests that arginine residues, present in the tight-binding adenine nucleotide sites on membrane-bound CF_1, become accessible to the reagent due to a light-induced conformational change of the coupling factor.

However, these residues seem to be different from those involved in photophosphorylation, since the latter are accessible to the reagent in the dark.

Our results suggest (Fig. 4) that even in the presence of borate, the stoichiometry between phenylglyoxal and arginine residues of CF_1 under our experimental conditions was initially 2:1 as shown by Takahashi [38] and then changed to 1:1 with the rate of loss of bound radioactivity depending on the buffer present. This suggestion was supported by the following observations: (1) the loss of ATPase activity was correlated with the mol phenylglyoxal incorporated/mol CF_1 in *N*-ethylmorpholine or borate buffers (Fig. 4A and B), when the rapid centrifugation columns were employed to separate the excess of reagent; (2) in both cases, the loss of bound radioactivity was about 50% and no ATPase activity was recovered (3) the presence of SDS, a denaturing agent, did not change the pattern of loss of bound radioactivity, thus suggesting that the reported derivative stoichiometry of 2:1 was changing to 1:1, rather than the loss being a dissociation of phenylglyoxal from less reactive arginines in the folded protein.

The rate of dissociation of [^{14}C]phenylglyoxal from the protein was enhanced in borate (Fig. 4) rather than in other buffers tested, thus indicating that the complex between the 1:1 diol derivative and borate (suggested by Werber et al. [41]) accelerates the rate of dissociation of the second molecule of phenylglyoxal from the adduct Arg(phenylglyoxal)$_2$. The use of slow static columns may be

the cause of the stoichiometry of 1:1 found in borate [41].

We have assumed a stoichiometry of 1:1 for the adduct phenylglyoxal-arginine in experiments concerning the membrane-bound CF_1, as they were carried out in borate buffer and we routinely left the immunoprecipitates overnight to assure completely recovery of CF_1 and reversion of the adduct of 2:1 stoichiometry to 1:1. Therefore, the incorporation of 1 or 2 mol [^{14}C]phenylglyoxal/mol CF_1 shown in Figs. 6 and 7 would correspond to the modification of one or two arginine residues/mol CF_1, respectively.

The correlation between incorporation of [^{14}C]phenylglyoxal into membrane-bound CF_1 in the dark and inhibition of synthesis and hydrolysis of ATP in chloroplasts (Fig. 6) suggests that the first mol reagent bound per CF_1 affected the latter rather than the former and that photophosphorylation may be inhibited by modification of only one arginine residue per mol enzyme. On the other hand, when incorporation was carried out in the light (Figs. 5 and 7) the rate was enhanced and both reactions were similarly associated with modification of two arginines per membrane-bound CF_1.

In the experiments involving modification of soluble CF_1, the incorporation of 8 mol [^{14}C]phenylglyoxal per mol CF_1, that resulted from extrapolation to 100% inhibition (Fig. 3), would correspond to derivative formation of four residues of arginine, since the stoichiometry of 2:1 applies because the modification was carried out in *N*-ethylmorpholine buffer and the incorporated reagent was measured immediately after gel filtration (Fig. 4A). Since there are 154 arginines per mol CF_1 [18], it is not possible to confirm the stoichiometry by amino acid analysis.

More arginine residues were modified in soluble CF_1 than in membrane-bound CF_1 for 100% inativation of the ATPase activity. This difference may reflect the allotropic nature of the enzyme that may have more residues accessible to the reagent in the soluble form. This extra labelling could be unspecific.

The subunit distribution in membrane-bound CF_1 of the incorporated [^{14}C]phenylglyoxal, mainly in the α- and β-subunits (Table III), is intriguing. It may be related to the suggestion that the catalytic site lies between α- and β-subunits [43]. Recently, De Benedetti et al. [44] reported a similar distribution between α- and β-subunits of radioactive naphthylglyoxal which also inhibited ATP synthesis and hydrolysis in spinach chloroplasts.

However, it is also possible that inactivation may be related only to the modification of the β-subunit where the catalytic site is generally accepted to be, and that the incorporation of labelled reagent into the other subunits may be unspecific. Further work is in progress in order to clarify this possibility.

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Cientificas y Técnicas (Argentina). R.H.V., C.S.A. and A.M.V. are members of the Investigator Career.

References

1 Schmid, R., Jagendorf, A.T. and Hulkower, S. (1977) Biochim. Biophys. Acta 462, 177–186
2 Vallejos, R.H., Viale, A.M. and Andreo, C.S. (1977) FEBS Lett. 84, 304–308
3 Andreo, C.S. and Vallejos, R.H. (1977) FEBS Lett. 78, 207–210
4 Vallejos, R.H., Andreo, C.S., Ravizzini, R.A. and Viale, A.M. (1980) in Proceedings of the 5th International Congress on Photosynthesis Sept. 7-13, Halkidiki, Greece. (Akoyunoglou, G., ed.), Vol. 2, pp. 707–717, Balaban International Science Services, Philadelphia
5 Vallejos, R.H. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 129–139, Elsevier/North-Holland, New York
6 Ryrie, I. and Jagendorf, A.T. (1971) J. Biol. Chem. 246, 582–588
7 McCarty, R.E., Pittman, P. and Tsuchiya, Y. (1972) J. Biol. Chem. 247, 3048–3051
8 Andreo, C.S. and Vallejos, R.H. (1976) Biochim. Biophys. Acta 423, 590–601
9 Vallejos, R.H. and Andreo, C.S. (1976) FEBS Lett. 61, 95–99
10 Weiss, M. and McCarty, R.E. (1977) J. Biol. Chem. 252, 8007–8012
11 Datta, D., Ryrie, I. and Jagendorf, A.T. (1974) J. Biol. Chem. 249, 4404–4411
12 Harris, D. and Slater, E. (1975) Biochim. Biophys. Acta 387, 335–348
13 Magnusson, R. and McCarty, R.E. (1976) J. Biol. Chem. 251, 7417–7422
14 Strotmann, H. and Bickel-Sandkotter, S. (1977) Biochim. Biophys. Acta 460, 126–135

15 Ryrie, I.J. and Jagendorf, A.T. (1971) J. Biol. Chem. 246, 3771–3774
16 Ryrie, I.J. and Jagendorf, A.T. (1972) J.Biol. Chem. 247, 4453–4459
17 Vallejos, R.H. (1973) Biochim. Biophys. Acta 292, 193–196
18 Binder, A., Jagendorf, A.T. and Ngo, E. (1978) J. Biol. Chem. 253, 3094–3100
19 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
30 Sugino, Y. and Miyoshi, Y. (1964) J. Biol. Chem. 239, 2360–2364
21 Carmeli, C. and Avron, M. (1972) Methods Enzymol. 24, 92–96
22 Strotmann, H., Bickel-Sandkotter, S. and Shoshan, V. (1979) FEBS Lett. 101, 316–320
23 Farron, F. and Racker, E. (1970) Biochemistry 9, 3829–3836
24 Vambutas, E. and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667
25 Taussky, H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
27 Sedmak, J.J. and Grossberg, S.E. (1977) Anal. Biochem. 79, 544–552
28 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
29 Younis, H.M., Winget, G.D. and Racker, E. (1977) J. Biol. Chem. 252, 1814–1818
30 Laurell, C.B. (1966) Anal. Biochem. 15, 45–52
31 Neville, D.M. and Glossmann, H. (1974) Methods Enzymol. 32, 92–102
32 Kohlbrener, W.E.and Cross, R.L. (1978) J. Biol. Chem. 253, 7609–7611
33 Mitchell, R.E. and Birboim, H.C. (1977) Anal. Chem. 81, 47–56
34 Frigeri, L., Galante, Y.M. and Hatefi, Y. (1978) J. Biol. Chem. 253, 8935–8940
35 Stachelin, M. (1959) Biochim. Biophys. Acta 31, 448–454
36 Vanin, E.F., Burkhard, S.J. and Kaiser, I.I. (1981) FEBS Lett. 124, 89–92
37 Vanin, E.F., Burkhard, S.J. and Kaiser, I.I. (1979) Abstr. 11th Int. Congr. Biochem. No. 02-2-H67
38 Takahasi, K. (1968) J. Biol. Chem. 243, 6171–6179
39 Riordan, J.F. (1979) Mol. Cell. Biochem. 26, 71–92
40 Borders, C.L. and Riordan, J.F. (1975) Biochemistry 14, 4699–4707
41 Werber, M.M., Moldovan, M. and Sokilovsky, M. (1975) Eur. J. Biochem. 53, 207–216
42 Vallejos, R.H., Lescano, W.I.M. and Lucero, H.A. (1978) Arch. Biochem. Biophys. 190, 578–584
43 Bruist, M.F. and Hammes, G.G. (1981) Biochemistry 20, 6298–6305
44 De Benedetti, E., Jagendorf, A.T. and Schmid, R. (1979) Abstracts 11th Int. Congr. Biochem., Toronto, Canada, p. 448