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# INHIBITION OF CHLOROPLAST COUPLING FACTOR BY NAPHTHYLGLYOXAL

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**The trypsin-activated $Ca^{2+}$-ATPase of spinach chloroplast membranes was completely inhibited by treatment with naphthylglyoxal, a fluorescent compound that should bind covalently to arginine residues. The inhibition followed apparent first-order kinetics. The apparent order of reaction with respect to inhibitor concentration gave values near unity, suggesting that inactivation is a consequence of modifying one arginine residue per active site. Partial protection against naphthylglyoxal was afforded by ADP and ATP, with either less or no protection by other nucleotide bases. At inhibition levels less than complete, the $K_m$ for ATP was not affected but the $V_{max}$ of the enzyme was diminished. The light-dependent exchange of tightly bound nucleotides on the membrane-bound enzyme was not inhibited by naphthylglyoxal treatment, indicating significant retention of the conformational response of the enzyme to the membrane high-energy state. Using [$^3$H]naphthylglyoxal, the extent of inhibition was a linear function of the amount of naphthylglyoxal bound up to 60% inhibition. The curves extrapolated to 2 mol naphthylglyoxal bound, associated with complete inhibition of ATPase. The radioactive naphthylglyoxal was distributed equally between $\alpha$- and $\beta$-subunits.**

## Introduction

The mechanism for ATP formation, or hydrolysis, by membrane-bound ATPase of chloroplasts ($CF_1$) is undoubtedly complex. While many advances have been made towards the understanding of structure and function in these activities [1], basically the mechanism is still not known. One approach has been the use of covalent chemical modifiers of the enzyme. These have indicated the occurrence of a light-induced conformational change [2], and have suggested essential roles of sulfhydryl [3–5], tyrosyl [6], lysyl [7,8] or carboxyl [9,10] groups in its catalytic activities.

Arginine residues have been shown to play a role in the adenine nucleotide-binding site of several enzymes (e.g., see Refs 11–14). Moreover, X-ray crystallography of adenylate kinase revealed several arginine residues surrounding ATP bound to the catalytic site [15]. Chemical modification studies using dicarbonyl compounds such as phenylglyoxal or butanedione have suggested the existence of essential arginine residues in $CF_1$ from chloroplasts [12,14], *Rhodospirillum rubrum* chromatophores [13] and mitochondrial [11] and thermophilic bacterial [16] ATPases.

It was shown earlier [14] that phenylglyoxal behaves as an energy-transfer inhibitor of $CF_1$ on chloroplasts. Phenylglyoxal is a covalent modifier of arginine residues [17,18] and peptides contain-

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $CF_1$, chloroplast coupling factor 1; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 4-morpholinepropanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; Chl, chlorophyll.

ing the modified arginine of several proteins have been isolated.

In this study we introduce a fluorescent analogue of phenylglyoxal, namely naphthylglyoxal, as a covalent modifier of $CF_1$ on chloroplast membranes. Its reactivity with arginine is basically similar to that of phenylglyoxal, and it is able to inhibit thylakoid membrane ATPase completely. However, in contrast to phenylglyoxal, it interferes with establishment of the high-energy state of the membranes in the light, and seems to attack different residues on $CF_1$ than does phenylglyoxal.

## Materials and Methods

Leaves from either market or greenhouse-grown spinach were homogenized and chloroplasts isolated as described earlier [13].

Naphthylglyoxal was synthesized from acetonaphthone by the method of Godyrev and Postovskii [19] and recrystallized three times from hot water. The recrystallized hydrate had a melting point between 100 and 110°C. As judged by NMR spectroscopy the purity of the product was better than 95%. It was dissolved in dimethyl sulfoxide for application to chloroplasts. Control experiments showed that dimethyl sulfoxide up to 5% had no effect on the enzymatic activities of chloroplasts or of $CF_1$ isolated from them. Naphthylglyoxal concentrations were measured by means of the Girard T reagent at pH 2.9 [20]. For preparation of radioactive naphthylglyoxal, acetonaphthone was custom-tritiated by the Amersham Corp. using catalytic exchange in aqueous media. The [$^3$H[acetonaphthone was then converted to [$^3$H]naphthylglyoxal as above, with maximum specific activity of 43 mCi/mmol.

To react membrane-bound $CF_1$ with naphthylglyoxal, the incubation mixture contained chloroplasts with 0.3–1.0 mg Chl/ml, 50 mM Hepes-NaOH (pH 8.0), 0.1 M sorbitol and varying amounts of naphthylglyoxal as indicated. The incubations were performed at 20°C for lengths of time as indicated in the particular experiment. The reaction was terminated by dilution with 10 vol. of 50 mM Tricine-NaOH (pH 8.0), 0.1 M sorbitol, 5 mM arginine, 2 mM dithiothreitol. The chloroplasts were sedimented by centrifuging at $3000 \times g$ for 10 min, resuspended and washed once with 10 mM Tricine-NaOH, (pH 8.0), 0.1 M sorbitol. The $CF_1$ enzyme was labeled with [$^3$H]naphthylglyoxal at 11.3 mCi/mmol by the same procedure, and extracted and purified as described earlier [21].

To examine the effects of naphthylglyoxal on the light-induced adenine nucleotide exchange, the following sequence was used: (a) $CF_1$ was preloaded with [$^3$H]ADP, (b) the membranes were treated with naphthylglyoxal, (c) the labeled-inhibited and labeled-control enzymes were extracted and reconstituted with fresh EDTA-stripped membranes, and (d) release of the label was observed on illumination of these reconstituted thylakoid membranes. Prelabeling of $CF_1$ with [$^3$H]ADP was carried out according to the method of either Shavit and Strotman [22] or Strotman and Bickel-Sankdotter [23]. Chloroplasts containing 10 mg Chl were illuminated as described [22,23] with 2.1 $\mu$M [$^3$H]ADP at 280 $\mu$Ci/$\mu$mol. After the reaction was terminated the chloroplasts were washed three times with 25 mM Tricine-NaOH (pH 8.0), 50 mM NaCl. They were then treated with naphthylglyoxal (or not, in the controls) and $CF_1$ extracted as described above. Control $CF_1$ and naphthylglyoxal-treated $CF_1$ labeled this way had specific radioactivities of 243 and 265 dpm/$\mu$g protein, respectively; and $Ca^{2+}$-ATPase activities of 22 and 3.1 $\mu$mol/mg protein per min, respectively, indicating 86% inhibition. Reconstitution to fresh, EDTA-uncoupled membranes was by the method of Telfer et al. [24]. About 0.1 mg of either control or inhibited $CF_1$ was bound per mg Chl of the uncoupled membranes. The reaction mixture for light-induced [$^3$H]ADP release from these membranes contained 25 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM methyl viologen, 0.25 mM ADP and 40 $\mu$g/ml Chl. After illumination for the amount of time shown the suspension was immediately centrifuged at $15000 \times g$ for 2 min, and washed four times with a medium containing 25 mM Tricine-NaOH (pH 7.8), 50 mM NaCl. The radioactivity of the chloroplast pellet (20 $\mu$g Chl) was determined after resuspension in 0.3 ml of 25 mM Tricine-NaOH (pH 7.8), using 3 ml of 33% (v/v) Triton-toluene cocktail for liquid scintillation counting.

ATPase of chloroplasts and soluble $CF_1$ was assayed as described earlier [21]. In all cases the

released $P_i$ was determined colorimetrically by the method of Lebel et al. [26]. Soluble proteins were measured by the procedure of Lowry et al. [27] following co-precipitation with deoxycholate and trichloroacetic acid [28].

## Results

In working with naphthylglyoxal, the nature of the buffer is critical. Using the Girard T reagent to assay for free glyoxal [20], naphthylglyoxal was found to react rapidly with Tris, Tricine or glycylglycine buffers: i.e., primary or secondary amines with $pK_a$ values near 8.2. Thus, they could not be used in the present studies.

Borate, the essential buffer for butanedione binding to arginine [29], has a complex interaction with naphthylglyoxal. We found that 5 mM borate accelerates the reaction of the glyoxal (10 mM) with the model compound, *N*-acetylarginine (10 mM) about 35% at pH 7.0 and 53% at pH 8.0. In addition, borate has the capacity to form a complex with naphthylglyoxal as indicated by a major change in the ultraviolet absorption spectrum when the two are mixed at pH 9.0 (Fig. 1). Smaller changes occur at pH 8.0, so probably the complex is not a predominant form under the conditions used in most of these experiments. In spite of the more rapid reaction between naphthylglyoxal and a model compound due to adding borate, the reaction of naphthylglyoxal with $CF_1$ was very considerably slowed down when borate was used as the buffer at 50 mM and pH 8.0. The half-time for inhibiting ATPase by naphthylglyoxal went from 4.4 min in the control with Hepes buffer, to 23 min in the presence of borate. Borate may possibly compete with naphthylglyoxal by binding to an anionic substrate site on $CF_1$. Thus, for the experiments reported here, *N*-ethylmorpholine, Mops or Hepes buffers were used.

Phenylglyoxal was also reported to interact with borate [30]. For inactivation of $CF_1$ by phenylglyoxal [14] borate buffer gave slower rates than *N*-ethylmorpholine, and unlike the results with naphthylglyoxal, Hepes buffer protected completely.

The inhibition of trypsin-activated $Ca^{2+}$-ATPase on the thylakoid membranes by naphthylglyoxal in Hepes buffer followed pseudo-first-order kinetics (Fig. 2). The reaction order with respect to naphthylglyoxal was 1.03, suggesting that inhibition occurred due to binding of 1 mol of the reagent [31] to $CF_1$, either on the active site or in some position where it interferes with catalysis. The same reaction order was reported for inhibition of membrane-bound $CF_1$ by phenylglyoxal in *N*-ethylmorpholine [14] or borate [12] buffers.

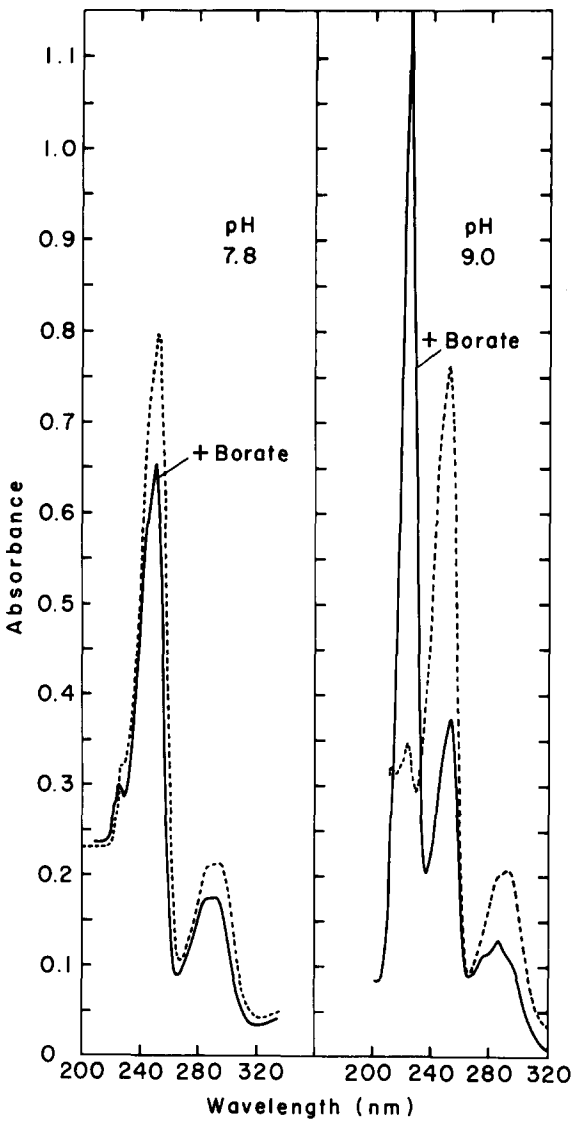


Fig. 1. Spectra of 50 μM naphthylglyoxal at pH 7.8 and 9.0, with and without the addition of 5 mM borate. The buffer was 0.1 M $NaHCO_3/Na_2CO_3$. The normal naphthylglyoxal peaks at 252 and 285 nm are decreased by adding borate, and a new peak appears at 224 nm.

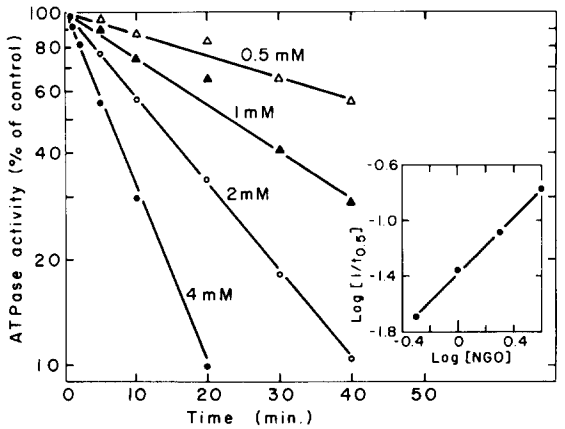


Fig. 2. Inactivation of chloroplast ATPase by various concentrations of naphthylglyoxal. Chloroplasts (0.3 mg Chl/ml) were modified by 0.5 (△), 1.0 (▲), 2.0 (○) and 4.0 (●) mM naphthylglyoxal as described in Materials and Methods, and aliquots were removed at the indicated times. They were immediately diluted, centrifuged, and the chlorplasts washed with 0.1 M sorbitol, 10 mM Tricine (pH 8.0). Control chloroplasts were incubated under the same conditions but without naphthylglyoxal. The control rate of trypsin-activated $Ca^{2+}$-ATPase was 458 μmol/mg Chl per h. NGO, naphthylglyoxal.

TABLE I

SUBSTRATE PROTECTION AGAINST NAPHTHYLGLYOXAL

The chloroplasts were preincubated (0.3 mg Chl/ml) in Hepes-NaOH (pH 7.8) buffer for 10 min before naphthylglyoxal was added to 1.0 mM. Aliquots were removed at intervals up to 30 min, diluted and washed, and trypsin-activated $Ca^{2+}$-ATPase measured. From the curve showing activity decay vs. time, the half-times for inactivation were calculated in each case. The control ATPase rate was 591 μmol/mg Chl per h. CDP and CTP at 10 mM had no effect on inactivation rates.

| Addition during Preincubation | $t_{0.5}$ for ATPase loss (min) | % increase in $t_{0.5}$ |
|---|---|---|
| None | 17 | |
| 1 mM ADP | 23.4 | 38 |
| 10 mM ADP | 29.4 | 73 |
| 1 mM ATP | 23.5 | 38 |
| 10 mM ATP | 30 | 76 |
| 10 mM IDP | 22.5 | 32 |
| 10 mM ITP | 25 | 48 |
| 10 mM $P_i$ | 25.5 | 50 |
| 10 mM $MgCl_2$ | 16 | 0 |
| 10 mM $MgCl_2$ + 10 mM $PP_i$ | 18 | 6 |

However, 2 mol of naphthylglyoxal were found to react with each gaunidino group in model compounds or proteins (Takabe, T. and Jagendorf, A.T., unpublished results), hence it is the rate-determining step of the modification reaction which must be first order with respect to naphthylglyoxal. Similar considerations apply to the reaction of phenylglyoxal [11].

The presence of ADP or ATP during incubation of chloroplasts with naphthylglyoxal afforded some protection against inactivation (Table I). IDP and $P_i$ were less effective, and CDP and CTP gave no protection at all. GDP and GTP gave effective protection; however, they were found to react with naphthylglyoxal as rapidly as arginine does. Therefore, the data with these two nucleotides are not included in Table I. There was no effect of including $Mg^{2+}$ with the adenine nucleotides (data not shown). $PP_i$ gave a very small but reproducible amount of protection, but ony in the presence of $MgCl_2$.

To assess the effect of naphthylglyoxal on kinetic parameters, membranes were treated with

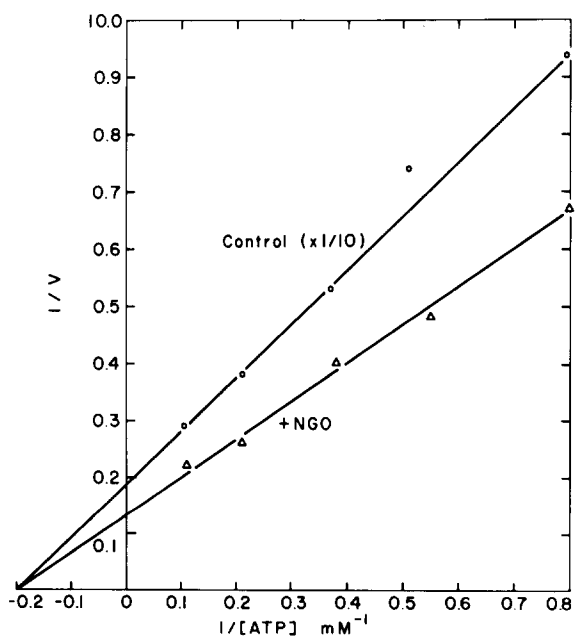


Fig. 3. Kinetic analysis of the effects of naphthylglyoxal inhibition on $Ca^{2+}$-ATPase activity. Control, and naphthylglyoxal (NGO) treated chloroplasts were treated with chloroform to liberate $CF_1$. The trypsin-activated $Ca^{2+}$-ATPase was assayed as described in Materials and Methods.

the inhibitor, then $CF_1$ was solubilized by chloroform extraction [32]. The $K_m$ values with respect to ATP were unchanged by naphthylglyoxal treatment, with a value of 5.1 mM for both control and inhibited enzyme (Fig. 3). However, the $V_{max}$ was substantially decreased (86% inhibition in the experiment shown). These results are consistent with a complete inactivation of $CF_1$ when naphthylglyoxal binds.

Naphthylglyoxal was found to inhibit the light- and dithiothreitol-activated $Mg^{2+}$-ATPase of thylakoid membranes, and also photophosphorylation. The kinetics of inhibition of these reactions was compared to that of the trypsin-activated $Ca^{2+}$-ATPase (Fig. 4); the $Mg^{2+}$-ATPase was inhibited much more rapidly than the other two. As it has been suggested [33] that there might be different active sites for the two ATPases, or for ATPase and ATP synthesis, inhibition of the $Mg^{2+}$-ATPase was studied in more detail. The kinetics of activation of $Mg^{2+}$-ATPase by light did not change, following naphthylglyoxal treatment to inhibit 50% of this activity (data not

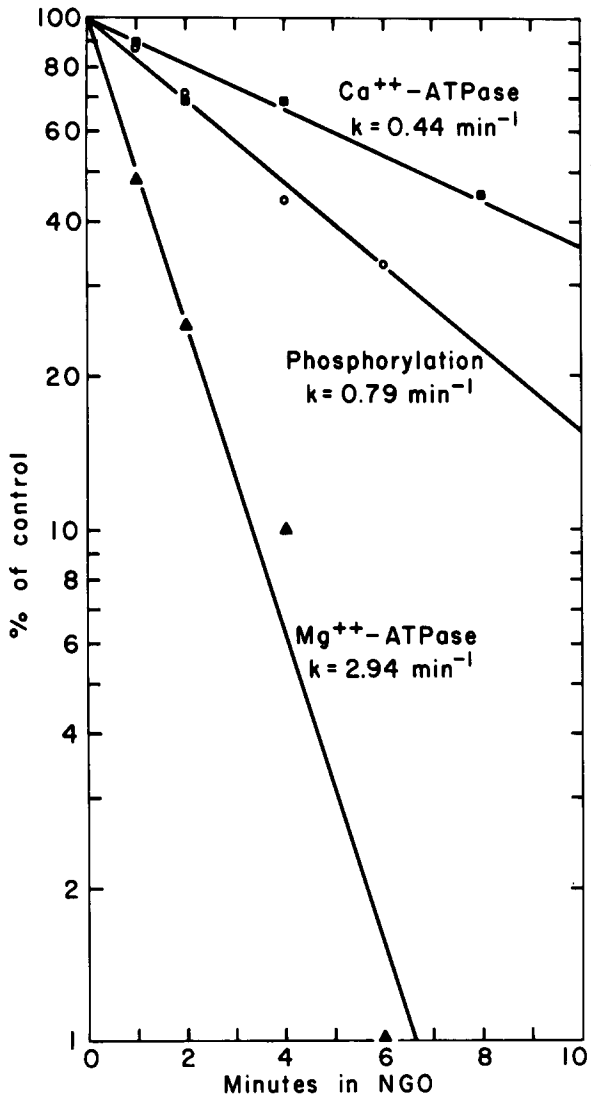


Fig. 4. The kinetics of inactivation of chloroplast phosphorylation ability and ATPases. Chloroplasts were incubated with 2 mM naphthylglyoxal (NGO) for the length of time indicated; aliquots were removed, diluted and washed, then the trypsin-activated $Ca^{2+}$-ATPase, light- and dithiothreitol-activated $Mg^{2+}$-ATPase and photophosphorylation with phenazine methosulfate as redox dye were assayed.

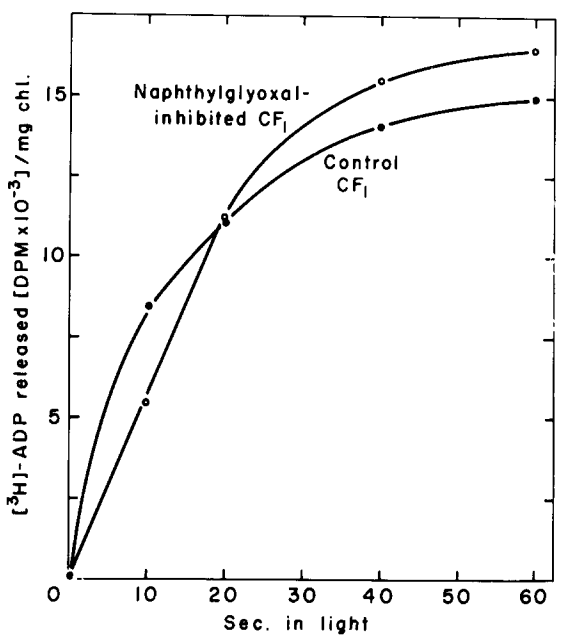


Fig. 5. Light-induced exchange of tightly bound [$^3$H]ADP from $CF_1$ reconstituted with fresh EDTA-uncoupled membranes. Modification by 4 mM naphthylglyoxal was carried out after previous incorporation of [$^3$H]ADP in the light. The labeled control or naphthylglyoxal-inhibited $CF_1$ species was solubilized, reconstituted with EDTA-uncoupled membranes and illuminated a second time as described in Materials and Methods. Radioactivity shown is that remaining with the chloroplast membranes following different times of exposure to light. The $Ca^{2+}$-ATPase rates were 22 $\mu$mol/min per mg protein for the control enzyme and 3.1 for the naphthylglyoxal-treated enzyme.

TABLE II

NAPHTHYLGLYOXAL INHIBITION OF $CF_1$-ATPase

Chloroplasts were incubated with or without 4 mM naphthylglyoxal for 5 min as shown, then diluted, centrifuged and washed. Activation of ATPases on the membranes, solubilization of $CF_1$ and measurement of ATPase are as described in Materials and Methods. The $Mg^{2+}$-ATPase was assayed after heat activation, in 40 mM Tricine, and 60 mM maleate buffer at pH 8.0. Where indicated, $Ca^{2+}$ was 8 mM and $Mg^{2+}$, 2 mM; the ATP concentration was 10 mM. Similar results were obtained when Tris was substituted for Tricine.

| Cation | 4 mM naphthyl-glyoxal | Membranes | | Soluble | |
|---|---|---|---|---|---|
| | | Rate [a] | Inhibition (%) | Rate [b] | Inhibition (%) |
| $Ca^{2+}$ (trypsin) | – | 272 | | 9.9 | |
| | + | 126 | 53 | 2.6 | 73 |
| $Mg^{2+}$ (light + dithiothreitol) | – | 204 | | 6.4 | |
| | + | 18 | 91 | 1.8 | 72 |

[a] $\mu$mol ATP/mg Chl per h.
[b] $\mu$mol ATP/mg protein per min.

shown), so the greater effectiveness of naphthylglyoxal on the $Mg^{2+}$-ATPase is probably not due to specific inhibition of its activation. This also indicates that the high-energy state of the membrane was not collapsed completely under these conditions. Solubilized $CF_1$ will exhibit $Mg^{2+}$-dependent ATPase provided the $Mg^{2+}$ concentration is not too high [34,35] and this is especially true if organic acid buffers are used [36]. Even though the $Mg^{2+}$- and $Ca^{2+}$-ATPases of the membrane-bound enzyme showed a strong difference in inhibition by naphthylglyoxal, the same enzyme after solubilization showed equal inhibition whether $Ca^{2+}$ or $Mg^{2+}$ was the cation used (Table II).

Naphthylglyoxal had multiple effects on chloro-

TABLE III

EFFECT OF NAPHTHYLGLYOXAL ON PHOTOPHOSPHORYLATION, ATPase, ELECTRON TRANSPORT AND PROTON UPTAKE

Chloroplasts were treated with 4 mM naphthylglyoxal in the dark for 10 min diluted and washed, prior to assay for activities shown. Phenazine methosulfate at 200 $\mu$M was the redox cofactor added for photophosphorylation. Electron transport was assayed for $O_2$ uptake using a Clark-type electrode in 50 mM Tricine at pH 8.0, 0.1 M sorbitol, 25 mM NaCl, 2.5 mM $NaN_3$ with 60 $\mu$M dichlorophenolindophenol reduced by 2.5 mM ascorbate as the electron source, and 50 $\mu$M methyl viologen as auto-oxidizable electron acceptor. When present, $NH_4Cl$ was used at 2 mM together with 2 $\mu$M gramicidin. Proton uptake was monitored as the pH change using pyocyanine as redox dye, and quantitated by titration with HCl and NaOH.

| Activity | Control | Naphthylglyoxal-treated | Inhibition (%) |
|---|---|---|---|
| Photophosphorylation | 1151 [a] | 59 | 95 |
| $Ca^{2+}$-ATPase | 509 [a] | 111 | 78 |
| Electron transport | 215 [b] | 289 | – |
| Electron transport, $+NH_4Cl$ + gramicidin | 511 [b] | 248 | 51 |
| Proton uptake | 0.21 [c] | 0.01 | 95 |

[a] $\mu$mol $P_i$ esterified or released/mg Chl per h.
[b] $\mu$mol $O_2$ consumed/mg Chl per h.
[c] $\mu$equiv. $H^+$ taken up/mg Chl in the light.

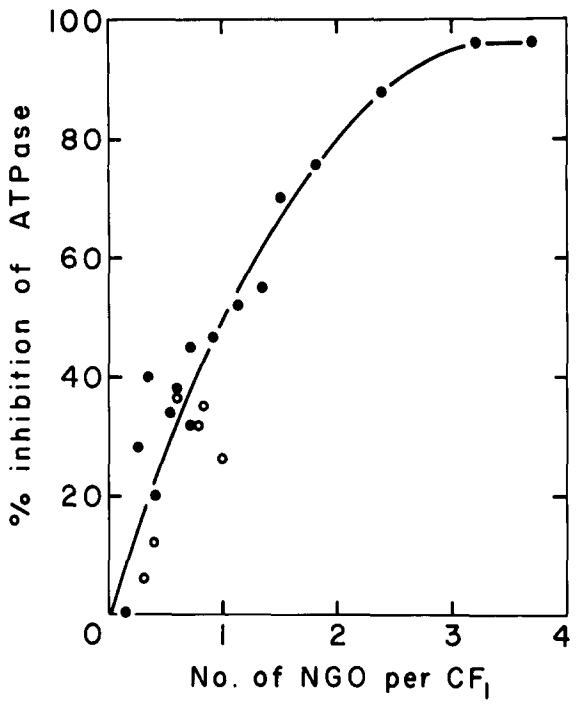


Fig. 6. Correlation between extent of inhibition, and incorporation of [$^3$H]naphthylglyoxal into $CF_1$. Modification of the chloroplasts at 1 mg Chl/ml was carried out using 2 mM [$^3$H]naphthylglyoxal in 50 mM Hepes-NaOH (pH 7.8), 0.1 M sorbitol in the absence (●) or presence (○) of 10 mM borate as shown. Individual points are derived from several different experiments. NGO, naphthylglyoxal.

plast reactions (Table III). Proton uptake mediated by phenazine methosulfate decreased parallel to the inhibition of photophosphorylation. While there was almost no effect (only a slight stimulation) on basal electron transport, uncoupled electron transport through Photosystem I was inhibited to a considerable extent. This complex of reactions suggests that extra inhibition of the membrane-bound $Mg^{2+}$-ATPase may be related to naphthylglyoxal interference with interactions between $CF_1$ and the membrane, rather than to a difference in the catalytic sites.

Since naphthylglyoxal both uncoupled chloroplast membranes and tended to inhibit their electron transport, determination of the effect of naphthylglyoxal on adenylate exchange of $CF_1$ had to be done in a multiple-step experiment. First, the ADP on the tight binding sites of membrane-bound $CF_1$ was exchanged for [$^3$H]ADP in the presence of light and phenazine methosulfate [22,23]. Next, the chloroplasts were centrifuged, resuspended, and treated with 4 mM naphthylglyoxal for 15 min in the dark. Control experiments showed that this treatment did not cause release of the bound, radioactive ADP. Third, the $CF_1$ was extracted from these membranes using EDTA, then rebound [24] to fresh uncoupled membranes. These were then illuminated wih phenazine methosulfate and unlabeled ADP. The kinetics of exchange of the bound ADP were found to be almost identical, comparing control $CF_1$ with naphthylglyoxal-treated $CF_1$ whose ATPase had been inhibited 86% (Fig. 5).

To determine how much naphthylglyoxal was incorporated into $CF_1$ during inactivation of its APTase, [$^3$H]naphthylglyoxal was used, and the labelled $CF_1$ isolated and purified. Incorporation was found to be linear with inhibition up to 60% (Fig. 6). Extrapolation to 100% inactivation indicates 2 mol [$^3$H]naphthylglyoxal incorporated. The same relationship was found when the labeling was done in the presence of 10 mM borate (some of the points in Fig. 6).

TABLE IV

EFFECT OF PREVIOUS MODIFICATION WITH PHENYLGLYOXAL ON INCORPORATION OF [$^3$H]NAPHTHYLGLYOXAL INTO $CF_1$

Chloroplasts (1 mg Chl/ml) were incubated with 20 mM phenylglyoxal in 0.1 M *N*-ethylmorpholine-HCl (pH 7.8), 0.1 M sorbitol, 5 mM NaCl at 20°C for 20 min. The reaction was stopped by dilution with 6 vol. of 0.4 M sorbitol, 10 mM NaCl, and chloroplasts were centrifuged, then washed twice with this medium. Initial ATPase activities of the control and treated chloroplasts were 14 and 2.8 μmol/mg protein per min, respectively. The two types of chloroplasts were then incubated with 1 mM [$^3$H]naphthylglyoxal at 20°C at 1 mg Chl/ml, aliquots were removed at the time shown and $CF_1$ was extracted and purified as described in Materials and Methods.

| Incubation time (min) | Mol naphthylglyoxal/mol $CF_1$ | |
|---|---|---|
| | Control | Phenylglyoxal-treated |
| 20 | 0.18 | 0.20 |
| 40 | 0.38 | 0.54 |
| 60 | 0.70 | 0.60 |
| 80 | 1.20 | 1.12 |

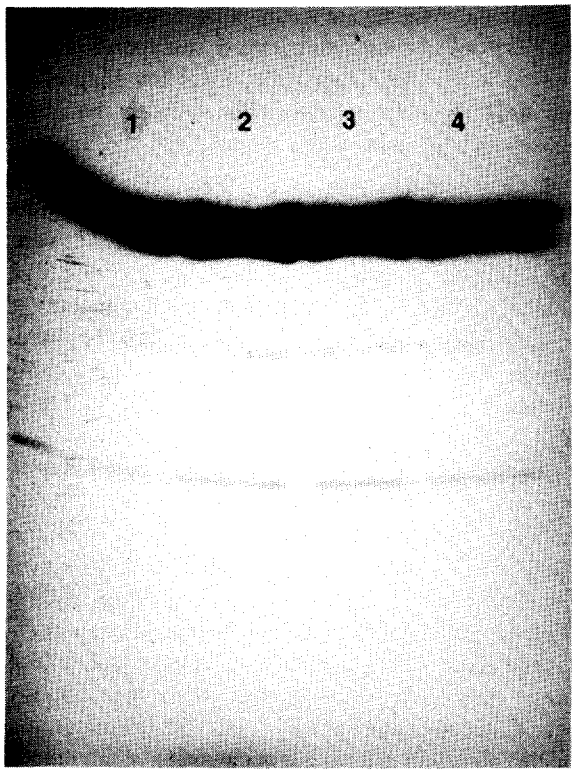


Fig. 7. Distribution of label associated with $CF_1$ subunits on polyacrylamide gel electrophoresis. $CF_1$ labeled with [$^3$H]naphthylglyoxal was denatured with 1% SDS then electropphoresed on a polyacrylamide vertical gel (3.6% acrylamide, 0.4% diallyltartardiamide for the stacking gel with 50 mM Tris-phosphate at pH 6.8; 7.8% acrylamide, 0.2% *N,N'*-bismethyleneacrylamide in 100 mM Tris-phosphate at pH 7.2 for running gel) for 7 h at 4°C. Electrophoresis was from top (cathode) to bottom (anode). The gel was stained briefly with Coomassie blue R, and destained with 7% acetic acid at room temperature. The gel was then soaked in 'Enhance' (New England Nuclear Corp.), dried, and arranged for fluorography with pre-fogged Kodak XAR-5 film at −60°C for 2 months. The position of the radioactive bands corresponds to that of α-, β-, γ- and ε-subunits in the stained gel. The four lanes are from four different $CF_1$ preparations, with ATPase inhibited 38% (lane 1), 43% (lane 2), 52% (lane 3) and 61% (lane 4), with 0.53, 0.63, 1.89 and 2.11 mmol naphthylglyoxal/mol $CF_1$, respectively. Equal amounts of radioactivity were loaded on the gel in each case; it is apparent that the distribution was similar at all points in the inhibition curve.

When soluble $CF_1$ was treated with [$^3$H]naphthylglyoxal, considerably more radioactivity was incorporated per mol. From 4 to 6 mol naphthylglyoxal were bound, associated with complete inhibition of the enzyme.

Electrophoresis of naphthylglyoxal-modified $CF_1$ with dodecyl sulfate showed the presence of the normal five subunits of $CF_1$. When radioactive naphthylglyoxal was used, the radioactivity that was associated with the protein was distributed equally between the α- and β-subunits (Fig. 7) with only small amounts on the γ-, δ- or ε-subunits. The distribution pattern was unchanged at four different points in the inhibition time course.

Previous modification of $CF_1$ on the membranes with phenylglyoxal, enough to inhibit ATPase 80%, had no effect on the incorporation of [$^3$H]naphthylglyoxal in a subsequent step (Table IV). Furthermore, when [$^{14}$C]phenylglyoxal was used to inhibit $CF_1$ on the thylakoid membranes with about 2 mol incorporated per mol $CF_1$, no displacement of the $^{14}$C label occurred due to subsequent treatment with 4 mM naphthylglyoxal (data not shown). These results suggest that there are two different types of functional arginine groups in $CF_1$, one sensitive to phenylglyoxal and the other to naphthylglyoxal.

## Discussion

Naphthylglyoxal is an effective covalent inhibitor for chloroplast reactions. The trypsin-activated $Ca^{2+}$-dependent ATPase, and light- and dithiothreitol-activated $Mg^{2+}$-dependent ATPase are both inhibited completely following pseudo-first-order kinetics (Figs. 2 and 4). In addition, it both uncouples chloroplasts, and inhibits uncoupled electron flow through Photosystem I (Table III, row 4). This is in contrast to phenylglyoxal [14] which was reported to inhibit ATPase and phosphorylation, but not to uncouple chloroplasts. While phenylglyoxal inhibited Photosystem II electron transport it had no effect on Photosystem I. It is probable that naphthylglyoxal is able to penetrate more readily into hydrophobic regions of membranes or polypeptides than phenylglyoxal, and may attack the $CF_0$ component of ATPase and electron-transport enzymes as well as $CF_1$.

When a covalent inhibitor interferes with enzymatic activity, it might do so either by binding at or close to the catalytic site, or by preventing the necessary conformational changes involved in catalysis. The latter appearred to be the case, for instance for inhibition of $CF_1$-ATPase by 7-nitro-

1,2,3-benzooxadiazolyl-4-amino chloride [37]. In the case of naphthylglyoxal, modification did not alter the $K_m$ value for ATP (Fig. 3), and partially inhibitory concentrations had no effect on the kinetics of activation of $Ca^{2+}$-ATPase by trypsin, or on the kinetics of activation of $Mg^{2+}$-ATPase by light (data not shown). Perhaps more importantly, it did not interfere significantly with the light-dependent exchange of tightly bound adenine nucleotides (Fig. 5), a reaction generally thought to result from conformational changes of the membrane-bound enzyme [1]. These data are all most consistent with an attack by naphthylglyoxal at or near the active site, to interfere with catalysis directly rather than indirectly.

Many of the previous modifications of $CF_1$ (e.g., see Refs. 3, 7, 25) either required, or were accelerated by, the light-induced high-energy state of the chloroplasts. This is not the case for naphthylglyoxal, possibly for two reasons. In the first place, it is an effective and presumably irreversible uncoupler (i.e., loss of proton uptake, Table III), so it is not possible to maintain the high-energy state during any prolonged incubation period. Secondly, the targets for naphthylglyoxal are either exposed in the dark, or are in hydrophobic regions that naphthylglyoxal is able to reach because of its own solubility properties. Nevertheless, the partial protection of $CF_1$ against naphthylglyoxal by adenine nucleotides or phosphate (Table I) suggests that the binding site of naphthylglyoxal is either close to, or is influenced by, the catalytic site.

Compared to phenylglyoxal, naphthylglyoxal has the drawback of lesser specificity, since it is an uncoupler and it inhibits electron transport as well as inhibiting energy transfer. On the other hand, its reaction rate with the ATPase is about twice that of phenylglyoxal, using Hepes buffer for both reagents. An unexpected finding is that the two apparent arginine-specific reagents must react with different functional groups on $CF_1$, since prior reaction with one does not prevent addition of the second, and addition of the second does not displace the first. Thus, a further investigation of their respective sites for attack will provide different information about the structure and function of $CF_1$. And finally, the fluorescence properties of naphthylglyoxal should prove useful for biophysical investigations of the enzyme at a later date.

The reactivity of naphthylglyoxal with primary and secondary amino groups, as well as with guanidino groups, means that the specificity of its attack has to be examined carefully. However, the most reactive amino groups are those with $pK_a$ values near 8.2. On proteins these would be primarily the N-terminal amino groups. $CF_1$ has its N-terminal amino acids blocked on all subunits, so this reactivity is not of immediate concern. A difference in specificity is also found between soluble and membrane-bound $CF_1$ with four to five naphthylglyoxal moieties bound by treatment of the soluble enzyme and only two with the membrane-bound one. Thus, in the present work only thylakoid-bound $CF_1$ was treated with naphthylglyoxal.

Complete inhibition of $CF_1$-ATPase was associated with the binding of 2 mol naphthylglyoxal (Fig. 6). However, the labeled compound was found to be equally distributed between the $\alpha$- and $\beta$-subunits (Fig. 7); hence, there must have been one on the $\alpha$- and one on the $\beta$-subunit. Since there are two $\alpha$- and two $\beta$-subunits per mol, at complete inhibition only one of each is modified. It is possible that the two $\alpha$- and the two $\beta$-subunits are not identical in their ability to react with naphthylglyoxal under the conditions used. Alternatively, they may be the same, but once one residue of naphthylglyoxal is attached to one of the two subunits it prevents the binding of a second naphthylglyoxal to the equivalent site on the other subunit. In any case, it is not necessary to modify both $\alpha$- or both $\beta$-subunits to achieve complete inhibition.

Further, if on $CF_1$ as on simpler proteins and model compounds two naphthylglyoxal molecules react with each arginine, then on each inhibited enzyme only one out of the four possible binding sites is actually modified. Our inhibited preparation would then consist of two equal populations, one with only one $\alpha$-subunit modified and the other with only one $\beta$-subunit modified.

A virtually identical labeling pattern was reported for the photoaffinity label, 3′-*O*-[3-[*N*-azido-2-nitrophenyl)amino]propionyl]ADP [38] with 1 mol bound per mol $CF_1$ and the label equally distributed between the $\alpha$- and the $\beta$-subunits. With 8-azido-ATP or -ADP [39], and with

pyridoxal phosphate [8] 2 mol of modifier were bound covalently per mol $CF_1$ at 100% inhibition, and again the label was equally on $\alpha$- and $\beta$-subunits. Only with 7-nitro-1,2,3-benzooxadiazolyl-4-amino chloride [6] and DCCD [9] could the inhibitory covalent modifier be found entirely on the $\beta$-subunit. In the former case, inhibition was later ascribed to a structural rearrangement of the protein, rather than necessarily to binding to the active site. For DCCD, however, specific protection by effective divalent cations [9,10] suggests the active site is found, at least in part, on the $\beta$-subunit. The evidence with photoaffinity label analogues of adenine nucleotides, and the work reported here, suggest the possibility that some fraction of the active site, or at least the pocket containing the active site, is located at the interface between $\alpha$- and $\beta$-chains.

Further work is in progress to determine the amino acid(s) to which naphthylglyoxal binds, and to attempt isolation of a peptide bearing naphthylglyoxal from proteolytic digests of labeled $CF_1$.

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