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DIFFERENTIAL INHIBITION OF P_i-ATP EXCHANGE IN RELATION TO ATP SYNTHESIS AND HYDROLYSIS BY MODIFICATION OF CHLOROPLAST THYLAKOID MEMBRANES WITH GLUTARALDEHYDE

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Limited modification of thylakoid membranes with glutaraldehyde inhibits the P_i-ATP exchange reaction much more than ATP synthesis or hydrolysis. More extensive modification of the membranes results in the inhibition of all activities of the ATP synthetase, but does not affect electron transport. Limited modification also does not have much effect on the tight binding of [^{3}H]ADP or the ΔpH supported by ATP hydrolysis. The modification affects the catalytic process itself and not the activation of the latent enzyme. Cross-linking between thylakoid polypeptides is observed only after extensive treatment with glutaraldehyde, while limited modification does not result in cross-linking between polypeptides. The differential inhibition of the P_i-ATP exchange relative to ATP hydrolysis can be explained by the decrease in only one of the kinetic rate constants involved in these reactions. However, the relative insensitivity of photophosphorylation to the modification suggests that different enzyme conformations may participate in phosphorylation (light) and ATP hydrolysis or P_i-ATP exchange (dark).

Introduction

The chloroplast ATP synthetase catalyzes the synthesis of ATP supported by energy supplied (in the form of $\Delta\mu_{H^+}$) by light-dependent electron transport. In isolated thylakoid membranes, the enzyme catalyzes ATP synthesis but has to be preactivated in order to carry out P_i-ATP exchange or ATP hydrolysis (see Ref. 1 for a review). The participation of different enzyme conformations or altered catalytic site(s) in the catalysis of ATP- and ADP-utilizing reactions has been suggested [1–5]. Chemical modification of the isolated and membrane-bound CF_1 has been used to study its mechanism of action. Modification of the soluble enzyme was studied extensively (for a review, see Ref. 6). However, these studies do not allow a direct comparison between the different reactions, since most of them are catalyzed by the membrane-bound enzyme only. Specific modification of various amino acid residues on the membrane-bound ATP synthetase occurs mainly on the larger subunits [6–9], and leads to inactivation of the enzyme. The differential inhibition of the partial reactions of photophosphorylation was explained as due to modification of different arginine residues on the ATP synthetase [8–10].

Several reagents were shown to react more extensively in the light than in the dark [9,11–13], suggesting that the equilibrium between alternate conformational states of the enzyme may be changed upon energization. Modification of sulfhydryl groups on the γ-subunit [13], which depends on energization, also suggests changes in the conformational state of the enzyme. Interaction of

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CF_1, chloroplast coupling factor one (the hydrophilic portion of the ATP synthetase).

fragmented antibodies against CF_1 with the thylakoid membranes results in the differential inhibition of P_i-ATP exchange, while photophosphorylation [3] or ATP hydrolysis [14] by the membrane-bound enzyme is only slightly affected.

Glutaraldehyde is a useful cross-linking reagent for studies of enzyme structure and function, even though its detailed mechanism of action is not clear [15]. Interaction of thylakoid membranes with relatively high glutaraldehyde concentrations has been shown to affect electron transport and photophosphorylation [16], and to decrease the number of free amino groups in thylakoid membranes [17].

In this communication, we compare the effect of glutaraldehyde modification of thylakoids, using very low reagent concentrations and short exposure times, on several reactions catalyzed by the ATPase complex in the membrane. Extensive modification results in the inactivation of photophosphorylation, ATP hydrolysis, P_i-ATP exchange and exchange of tightly bound nucleotides. Less extensive modification, on the other hand, inactivates mainly the P_i-ATP exchange reaction with much less effect on the other activities. The differential inactivation of P_i-ATP exchange in relation to ATP hydrolysis does not require the participation of more than one type of catalytic site, because a change in only one rate constant can accommodate these results. However, the lower sensitivity of photophosphorylation suggests the participation of different conformational states of the enzyme in these reactions. A preliminary report on this work was presented elsewhere [14].

Materials and Methods

Chloroplast thylakoids were prepared from fresh market lettuce leaves [18], washed with a cold solution of 50 mM NaCl, 2 mM Tricine-NaOH (pH 8.0) (washing medium), suspended in this medium to a chlorophyll concentration of 1.2–1.5 mg/ml [19] and used immediately.

Glutaraldehyde, unlabeled nucleotides, dithiothreitol, $NaBH_4$ and molecular weight markers for electrophoresis were purchased from Sigma Chemical Co. Carrier-free $^{32}P_i$ was obtained from the Nuclear Research Center-Negev, Israel. [γ-^{32}P]ATP was synthesized and purified as described previously [20]. [2-^{3}H]ADP, [^{14}C]methylamine, [U-^{14}C]sorbitol and ^3H_2O were purchased from Amersham, U.K. Silicon oils, Versilube F(50) and SF 96(50), were a gift from Dr. R.E. McCarty (Cornell University, Ithaca, NY). All other reagents were of analytical grade.

Modification with glutaraldehyde

A freshly diluted glutaraldehyde solution was added to a mixture containing 50 mM Tricine-NaOH (pH 8.0), 20 mM NaCl, 10 mM $MgCl_2$, 2.5 mM dithiothreitol and thylakoid membranes containing 100–140 μg Chl/ml. The reaction was carried out in the dark at 20°C and terminated by the addition of $NaBH_4$ (dissolved just before use in cold Tricine-NaOH, 10 mM (pH 8.0)) to a final concentration of 10 mM. Modified thylakoids were kept on ice until use. $NaBH_4$ added before glutaraldehyde or alone caused 10–30% inhibition.

Activity assays

P_i-ATP exchange and ATP hydrolysis were assayed simultaneously under identical conditions. Thylakoid membranes were activated by illumination in the presence of dithiothreitol as described earlier [18], except that the Tricine concentration was 50 mM. The substrate mixture (5 mM P_i and 5 mM ATP) was added 5 s after activation. This mixture also contained $^{32}P_i$ ($2–5 \cdot 10^6$ cpm) or [γ-^{32}P]ATP ($0.3–1 \cdot 10^6$ cpm) for the assay of P_i-ATP exchange or ATP hydrolysis, respectively. $^{32}P_i$ released or incorporated was determined as described previously [18]. Since the $^{32}P_i$ released from labeled ATP in the presence of unlabeled P_i represents the sum of net ATP hydrolysis (defined as ADP released) and concomitant P_i-ATP exchange, rates of ATP hydrolysis were calculated by subtraction of the $^{32}P_i$ incorporated in the P_i-ATP exchange reaction from the total $^{32}P_i$ released. Cyclic photophosphorylation was assayed under similar conditions except that 1 mM ADP replaced ATP. Activated and nonactivated thylakoids were used as indicated. Unless otherwise indicated, samples were illuminated with saturating white light ($0.23\ J \cdot cm^{-2} \cdot s^{-1}$) for 15–30 s.

For assays of electron transport, modified thylakoids were washed twice to remove dithiothreitol and $NaBH_4$, to avoid chemical reduction of ferricyanide. Photophosphorylation and ferricyanide reduction were assayed as described in Ref.

21. The pH gradient formed during ATP hydrolysis was determined according to the method of Pick and McCarty [22]. Glutaraldehyde modified thylakoids were activated as above. After 10 s in the dark to allow decay of the light-induced ΔpH, P_i and ATP were added. The reaction mixture was then layered on a silicon (Versilube F(50)/SF96(50) (6 : 1, w/w)) layer and centrifuged in the dark. The internal volume of the thylakoids was determined under the same conditions [22], and was not changed by the modification.

[^{3}H]ADP binding in the light or dark was determined as described earlier [18]. [^{3}H]ADP (5 μM) was added before or after illumination, and samples were quenched in the light or dark by addition of 5 mM unlabeled ADP and 6 μM FCCP. To measure release of tightly bound ADP, [^{3}H]ADP was prebound to unmodified thylakoids as described above but without quenching the reaction. The pellets were washed twice in the dark with washing medium and modified with glutaraldehyde as described above. Release of bound [^{3}H]ADP was determined after illumination for 1 min with saturating white light and quenched in the light as described for [^{3}H]ADP binding. The membranes were then centrifuged for 5 min at 12000 $\times$ g and the radioactivity in the supernatant determined.

Cross-linking studies

Thylakoid membranes were washed three times with the washing medium to remove ribulose-1,5-diphosphate carboxylase [23] and then reacted with glutaraldehyde. Modified membranes (containing 20–30 μg Chl) were transfered to microcentrifuge tubes and centrifuged for 5 min at 12000 $\times$ g. The supernatant was gently sucked off and the pellets were resuspended in 40 μl distilled water and 10 μl of a solution containing 50 mM sodium phosphate (pH 7.0), 5% SDS and 25% β-mercaptoethanol, before heating at 90°C for 10 min. Electrophoresis was done according to the method of Weber and Osborn [24] using a 5% polyacrylamide slab gel. The gel was stained with Coomassie brilliant blue R [24], photographed, and transparencies were scanned at 500 nm; peaks were cut out from the recorder scan and weighed to determine the relative amounts of protein in each band.

Results

Inactivation of photophosphorylation

Treatment of thylakoid membranes with glutaraldehyde leads to a progressive, irreversible loss of their various enzymic activities. The kinetics of the inactivation depend on the glutaraldehyde concentration. In this work, we used glutaraldehyde concentrations about 100-times lower than those usually used for thylakoid fixation and in studies on the inhibition of electron transport [16,17]. The kinetics of the inactivation of photophosphorylation (Fig. 1) cannot be fitted by a single-exponential decay curve. The inactivation by glutaraldehyde is clearly a complex process and therefore is not a priori expected to be pseudo-first order with respect to the modifier. The biphasic time course of the inactivation, where an enhanced rate follows an initial slow phase, is more apparent if photophosphorylation with modified thylakoids is assayed at low ligh intensities. This suggests that the step affected during the initial phase of the modifi-

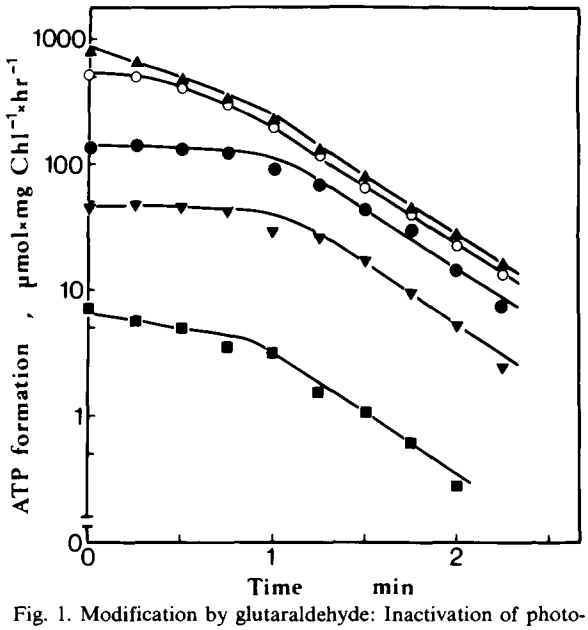


Fig. 1. Modification by glutaraldehyde: Inactivation of photophosphorylation. Thylakoids were modified with 0.05% glutaraldehyde for the indicated times as described in Materials and Methods. Photophosphorylation was assayed for the indicated times (in s) and light intensities (J·cm^{-2}·s^{-1}), respectively: (▲) 30, 0.23; (○) 45, 0.15: (●) 60, 0.072: (▼) 120, 0.041; (■) 240, 0.021.

TABLE I

MODIFICATION BY GLUTARALDEHYDE: CONDITIONS AFFECTING THE RATE OF THE SECOND PHASE OF INACTIVATION OF PHOTOPHOSPHORYLATION

Thylakoids were modified with 0.05% glutaraldehyde as described in Materials and Methods ('complete') or with the indicated changes. Photophosphorylation with modified thylakoids was assayed under identical conditions. The half-time for the second phase was calculated from a semilogarithmic plot.

Modification conditions	$t_{1/2}$ (s)
Dark, complete (+ 10 mM Mg^{2+}, +2.5 mM dithiothreitol)	17
Dark, $-Mg^{2+}$	38
Dark, −dithiothreitol	33
Dark, $-Mg^{2+}$, −dithiothreitol	43
Dark, + 1 mM ADP	20
Dark, + 1 mM ATP	21
Light, complete	14
Light, −dithiothreitol	38

cation is more limiting at high light intensities. More extensive modification has the same effect on the rate of photophosphorylation, independent of light intensity.

The effect of various compounds present during modification on the second (fast) phase of inactivation, is shown in Table I. Mg^{2+} and dithiothreitol (up to 5 mM) enhance the rate of inactivation while ADP and ATP, at 1 mM, have only a minor effect. It makes no difference whether the modification with glutaraldehyde is done in the dark or during illumination.

The basal rate of electron-transport activity (H_2O to ferricyanide) is not affected in glutaraldehyde-modified thylakoids, but the electron-transport activity coupled to ATP formation is inhibited. The sensitivity of the phosphorylation reaction with ferricyanide as electron acceptor was similar to that with phenazine methosulfate (not shown).

Inactivation of the ATP-utilizing reactions

Since the glutaraldehyde modification appears to affect mainly the ATP synthetase, we tested the effect of the modification on the P_i-ATP exchange and ATP-hydrolysis reactions. These reactions, in contrast to phosphorylation, are assayed in the dark under identical conditions. The P_i-ATP exchange is more susceptible to inhibition by glutaraldehyde than are ATP hydrolysis and photophosphorylation (Table II), since shorter exposure times and lower glutaraldehyde concentrations (limited modification) are required to inactivate the exchange reaction. Preactivation of modified thylakoids before assaying photophos-

TABLE II

INACTIVATION OF P_i-ATP EXCHANGE, ATP HYDROLYSIS AND ATP FORMATION BY GLUTARALDEHYDE MODIFICATION

Modification and assays were as described in Materials and Methods. Photophosphorylation was assayed with ferricyanide. Values in parenthesis are percent of control. Values of P_i-ATP exchange, ATP hydrolysis and photophosphorylation are expressed as μmol/mg Chl per h.

Incubation with glutaraldehyde		P_i-ATP exchange	ATP hydrolysis	Phosphorylation
Time (s)	Concentration (%, w/v)			
–	–	7.9	73.1	94.2
90	0.01	1.2 (15)	54.4 (74)	81.8 (87)
150	0.05	0	2.2 (3)	5.2 (5)

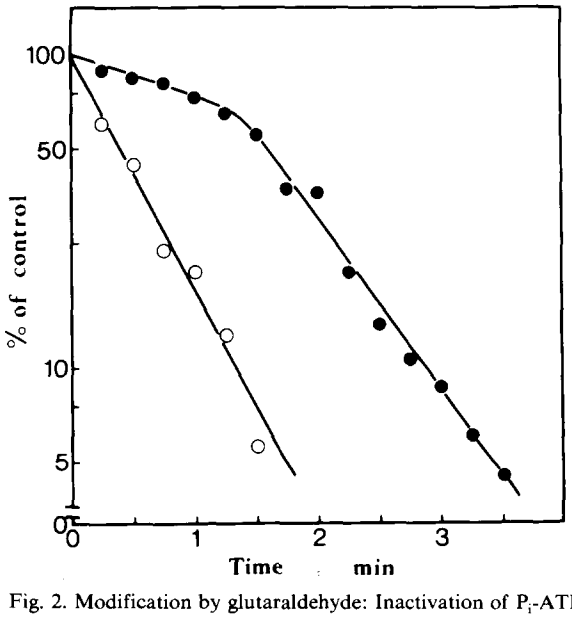


Fig. 2. Modification by glutaraldehyde: Inactivation of P_i-ATP exchange and ATP hydrolysis. Thylakoids were modified with 0.01% glutaraldehyde. Modified thylakoids were activated and assayed as described in Materials and Methods. (○) P_i-ATP exchange, (●) ATP hydrolysis.

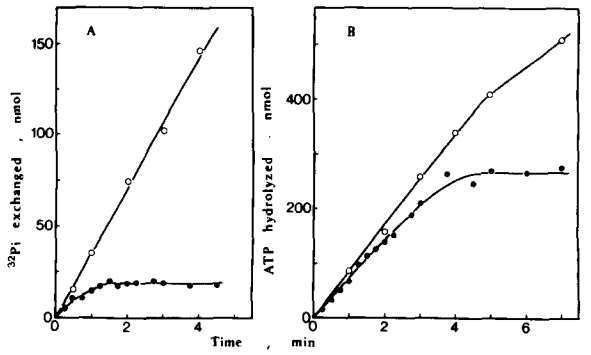


Fig. 3. Inactivation of P_i-ATP exchange and ATP hydrolysis by glutaraldehyde added to activated thylakoids. Activation was done as described in Materials and Methods. 1 mM ATP and 5 mM P_i (containing [γ-^{32}P]ATP or $^{32}P_i$) were added alone (○) or together with 0.015% glutaraldehyde (●), 5 s after activation. Aliquots were removed and quenched with trichloroacetic acid as indicated. (A) P_i-ATP exchange. (B) ATP hydrolysis. Reaction volume was 1 ml, and thylakoids, equivalent to 127 μg Chl.

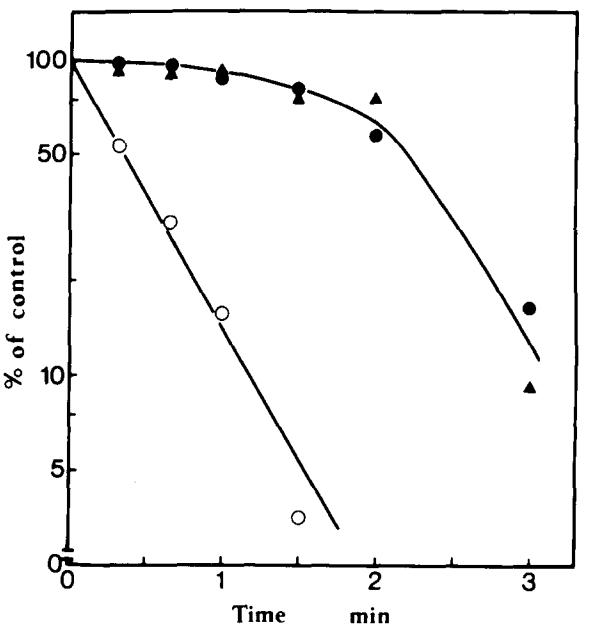


Fig. 4. Modification with glutaraldehyde: Effect on ΔpH supported by ATP hydrolysis. Thylakoids (containing 277 μg Chl/ml) were modified for the indicated time with 0.025% glutaraldehyde. Modification and activity assays were done as described in Materials and Methods. (▲) H^+ concentration ratio (in/out); rates of ATP hydrolysis (●) or P_i-ATP exchange (○). ΔpH values of 1.82 and 2.28 were found for ATPase induced or light-driven H^+ translocation, respectively.

phorylation, which mimics the reaction conditions for P_i-ATP exchange and ATP hydrolysis, does not increase the sensitivity of this reaction. Moreover, inhibition of P_i-ATP exchange on modified thylakoids occurs rapidly and does not involve two reaction phases, as do ATP hydrolysis and phosphorylation (Fig. 2). Thus, by controlling the time of interaction and the glutaraldehyde concentration, one can modify thylakoids to an extent whereby they lose most of their P_i-ATP exchange activity but retain a great part of their phosphorylation and ATPase activities. Addition of glutaraldehyde, together with ATP and P_i, to previously activated thylakoids (Fig. 3) inhibits these reactions in the same manner. This implies that modification by glutaraldehyde affects the catalytic process itself and not the activation of the enzyme. Since the P_i-ATP exchange requires the establishment of a pH gradient across the membrane, we checked whether the greater sensitivity of the exchange reaction may be due to uncoupled ATP hydrolysis in the modified thylakoids. Uncoupling of ATP hydrolysis from ΔpH should mainly affect the P_i-ATP exchange and not ATP hydrolysis. On the other hand, light-dependent ATP formation may be less sensitive to the modification because illuminated modified membranes may still be capable of maintaining a sufficient $\Delta\mu_{H^+}$. As shown in Fig. 4, this does not appear to be the case, since limited glutaraldehyde modification, which results in the almost complete loss of P_i-ATP exchange activity, only slightly affects the ATPase activity and the pH gradient.

Changes in kinetic parameters for ATP hydrolysis and P_i-ATP exchange in modified thylakoids

Glutaraldehyde modification increases the apparent K_m for ATP in the ATPase reaction (Fig. 5A). Apparent K_m values of the 48 ± 13 and 83 ± 12 μM were calculated for control and modified thylakoids, respectively. These values were obtained after modification with glutaraldehyde so as to inhibit most of the P_i-ATP exchange and only slightly affect the maximal rate of ATP hydrolysis (see Fig. 2). A similar increase in the apparent K_m for ATP occurred even when ATP hydrolysis was assayed in the absence of P_i (not shown). On the other hand, the K_i for ADP as a competitive inhibitor of ATP during ATP hydrolysis was not significantly changed (Fig. 5B). From the slope of

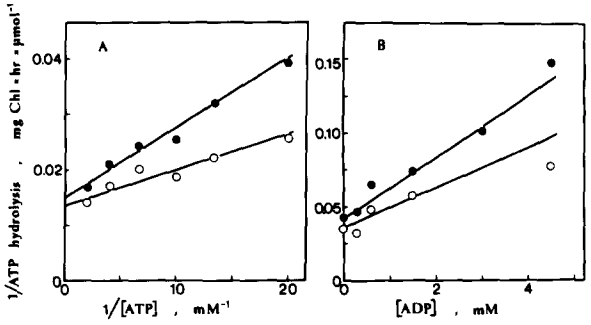


Fig. 5. Modification by glutaraldehyde: Effect on the kinetic parameters for ATP hydrolysis. Thylakoids were modified with 0.01% glutaraldehyde for 90 s, and then activated and assayed as described in Materials and Methods. (A) Lineweaver-Burk plot. Nonlinear regression fit was done according to the method of Duggleby [25]. (B) Dixon plot for ADP as a competitive inhibitor of ATP in ATP hydrolysis. (○ and ●) Control and modified thylakoids, respectively. Each point is the difference between P_i released from ATP and P_i-ATP exchange. The residual exchange activity in modified thylakoids was less than 15%.

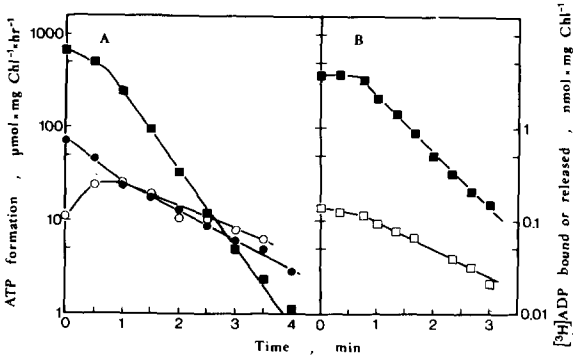


Fig. 6. Modification by glutaraldehyde: Effect on the exchange of tightly bound [^{3}H]ADP. (A) Thylakoids were modified as in Fig. 1 for the times indicated. Photophosphorylation (■), [^{3}H]ADP binding in the postillumination dark period (●) or [^{3}H]ADP binding during illumination (○) were assayed as described in Materials and Methods. (B) Thylakoids containing tightly bound [^{3}H]ADP were prepared as described in Materials and Methods, and then modified for the indicated times with 0.05% glutaraldehyde. Photophosphorylation (■) and light-induced release of tightly bound [^{3}H]ADP (□) were assayed as described in Materials and Methods.

the Dixon plot and using the K_m values mentioned above, K_i values for ADP of 180 and 165 μM were calculated in control and modified thylakoids, respectively. Modification with glutaraldehyde also increases the apparent K_m values for ATP and P_i in the P_i-ATP exchange reaction together with the decrease in the V_{max} of this reaction (not shown).

Effect on the exchange of tightly bound nucleotides

Limited modification of thylakoid membranes, so as to inactivate most of the P_i-ATP exchange activity, has little effect on the binding of ADP to the tight nucleotide-binding site(s) of the ATP synthetase. However, this modification increases the steady-state level of binding in the light (Table III). After more extensive modification, the binding in the dark and in the light, as well as light-induced release of tightly bound ADP, are strongly inhibited (Fig. 6). The increase in the level of bound ADP in the light seems to parallel the initial slow phase of reaction with glutaraldehyde, and the subsequent decrease coincides with the more rapid phase of inactivation.

TABLE III

LIMITED MODIFICATION WITH GLUTARALDEHYDE: EFFECT ON THE TIGHT BINDING OF [^{3}H]ADP

Thylakoids were modified for 2 min with 0.01% glutaraldehyde and activities were assayed as described in Materials and Methods. Binding was quenched after 1 min. Values of P_i-ATP exchange and ATP hydrolysis are expressed as μmol/mg Chl per h.

Thylakoid preparation	[^{3}H]ADP bound (nmol/mg Chl)		P_i-ATP exchange	ATP hydrolysis
	Dark	Light		
Control	0.309	0.088	12.0	26.7
Modified	0.281	0.124	3.2	21.7

Cross-linking of thylakoid polypeptides

Extensive modification of thylakoids with glutaraldehyde so as to inhibit all activities also results in a change in the pattern of thylakoid polypeptides, as revealed by polyacrylamide electrophoresis in presence of SDS (Fig. 7). The levels of the α- and β-subunits of CF_1 (approx. 55000 molecular weight protein band), a band of 35000 molecular weight (probably the γ-subunit) and another band containing unresolved low molecular weight polypeptides (less than or equal to 20000) are reduced significantly. Three new polypeptide bands with apparent molecular weights of 44000, 97000 and 120000 appear, which results from

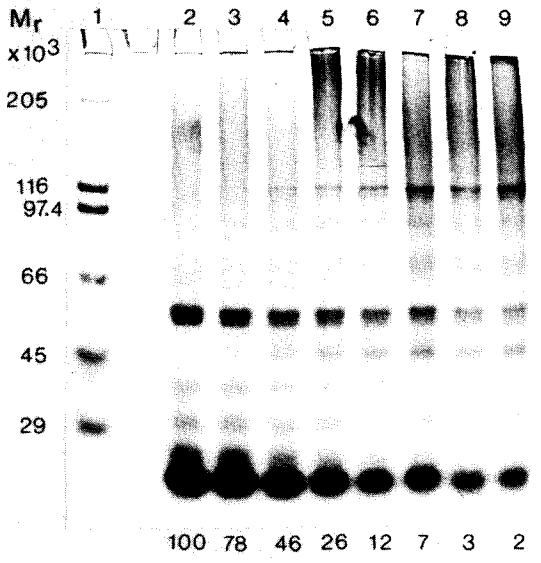


Fig. 7. Cross-linking of thylakoid membranes by glutaraldehyde. Thylakoids were modified with 0.05% glutaraldehyde as described in Materials and Methods. Samples were taken out every 30 s (lanes 2–9), modification was terminated with $NaBH_4$, and the samples were assayed for photophosphorylation and P_i-ATP exchange. Lane 1, molecular weight standards. Residual phosphorylation rates (% of control) of the modified thylakoids are indicated at the bottom of the gel. P_i-ATP exchange activity was completely inhibited after modification for 30 s (lane 3).

cross-linking between lower molecular weight polypeptides. The 120000 molecular weight band may be a combination between the α- and β-subunits, the 97000 band may result from cross-linking of the γ- and α- or β-subunits, while the 44000 band might result from cross-linking of the γ-subunit and/or other lower molecular weight polypeptides (e.g., in the 20000 band). The modification also produces a material of rather high molecular weight which remains on the top of the gel.

Limited modification of the thylakoids, which results mainly in the inhibition of P_i-ATP exchange (Table II), does not change the polypeptide pattern and composition. As shown in lane 3 in Fig. 7, even treatment with glutaraldehyde so as to inhibit completely the P_i-ATP exchange and about 20% of photophosphorylation does not result in significant cross-linking. It is possible that, under these conditions, either glutaraldehyde does not react as a bifunctional reagent or intrasubunit cross-linking occurs.

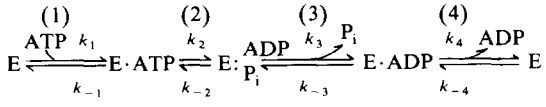
Discussion

Treatment of thylakoid membranes with glutaraldehyde inhibits all of the reactions catalyzed by the membrane-bound ATP synthetase. Glutaraldehyde tends to exist as an oligomer in solution, and probably reacts with proteins as a complex multivalent reagent, capable of simultaneously forming stable α,β-unsaturated imine bonds with two or more primary amine groups on the protein surface [15]. Glutaraldehyde was also shown to react with sulfhydryl groups and, to a lesser extent, with tyrosine and histidine residues [26]. The inactivation of the ATP synthetase by glutaraldehyde is not prevented by the presence of substrates (Table I), which suggests that glutaraldehyde most probably does not react with groups in or near the catalytic site(s). Since the degree of inactivation is not affected by energization, it follows that changes in the conformation of the enzyme do not expose groups on the enzyme that are inaccessible to the modifier in the dark [9,11–13]. Mg^{2+} enhances the rate of inactivation by glutaraldehyde (Table I), similar to its effect on the modification of chloroplast [7] and mitochondrial [27] ATPases by pyridoxal phosphate, which also reacts with protein lysine residues.

The greater sensitivity of the P_i-ATP exchange reaction (Fig. 2), compared with the other reactions catalyzed by the ATP synthetase, could be attributed to uncoupling of ATP hydrolysis by the glutaraldehyde modification. However, limited modification that partially inhibits ATP hydrolysis has little effect on the ΔpH (Fig. 4). Binding of ADP to the tight nucleotide-binding site(s) on the ATP synthetase may be involved in the regulation of ATP-utilizing reactions [18,20]. However, it is not likely that modification affects this type of site(s), since binding of [^{3}H]ADP to thylakoids in the dark was only slightly inhibited by the limited glutaraldehyde treatment (Table III). Moreover, tight binding of ADP inhibits both the P_i-ATP and ATP-hydrolysis reactions in parallel [18,20]. Limited modification does not affect the activation of the latent enzyme (Fig. 3), but inhibits the catalytic process itself.

Covalent modification of an active site should inhibit all of the activities inherent to this site and affect mainly the maximal rate of reaction but not the K_m values. Consequently, a modification which results in the differential inhibition of one of the activities catalyzed by an enzyme can be explained by assuming that different and independent sites on the enzyme are involved in these reactions. Alternatively, if a single site is reponsible for these activities, the modification of a group(s) at another site may prevent a conformational change required for only one of the reactions. In the first case, one might expect that the K_m for the substrate at the unmodified site will remain unchanged. In the latter case, however, changes in one or more of the kinetic constants may affect both the maximal velocities and also the apparent K_m values for the substrates in these reactions. We have observed that limited glutaraldehyde modification affects the apprent K_m values for substrates in both the ATP-hydrolysis and P_i-ATP exchange reactions. In addition, the V_{max} of the exchange reaction is more inhibited than that of the hydrolytic process.

Assuming that hydrolysis and exchange involve the same catalytic site, the following mechanism may apply:



This ordered Uni-Bi mechanism for ATP hydrolysis assumes that P_i is released before ADP and is in agreement with the ordered Bi-Uni reaction proposed for photophosphorylation [28]. The P_i-ATP exchange reaction occurs concomitant with net ATP hydrolysis and involves steps 1–3. Binding of ATP from the medium finally yields E · ADP. This complex can either dissociate via step 4 resulting in net ATP hydrolysis, or bind labeled P_i from the medium and reform ATP in a reversal of the steps involved in hydrolysis. The steady-state expressions, derived according to Cleland [29], are as follows:

For ATP hydrolysis;

$$\frac{v}{E_t} = k_1k_2k_3k_4[ATP]/k_4(k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3) + k_1(k_2k_3 + k_2k_4 + k_{-2}k_4 + k_3k_4)[ATP] + k_{-1}k_{-2}k_{-3}[P_i] + k_1k_{-3}(k_2 + k_{-2})[ATP][P_i] \quad (1)$$

For P_i-ATP exchange;

$$\frac{v}{E_t} = \frac{k_1k_{-1}k_2k_{-2}k_3k_{-3}[ATP][P_i]}{(k_{-1}k_3 + k_2k_3 + k_{-1}k_{-2})\Delta} \quad (2)$$

where Δ is the denominator of Eqn. 1.

By dividing Eqn. 2 by Eqn. 1 we obtain the ratio between the rates of the two activities, which is independent of the ATP concentration:

$$\frac{v_{exchange}}{v_{hydrolysis}} = \frac{K_i^{ATP}[P_i]}{K_m^{ATP}K_i^{P_i}} \quad (3)$$

where:

$$K_m^{ATP} = \frac{k_4(k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)}{k_1(k_2k_3 + k_2k_4 + k_{-2}k_4 + k_3k_4)}$$

$$K_i^{ATP} = \frac{k_{-1}k_{-2}}{k_1(k_2 + k_{-2})}$$

and

$$K_i^{P_i} = \frac{k_2k_3 + k_2k_4 + k_{-2}k_4 + k_3k_4}{k_{-3}(k_2 + k_{-2})}$$

A significant decrease in the V_{max} of the P_i-ATP exchange reaction without a marked effect on the V_{max} of ATP hydrolysis may be obtained if we assume that glytaraldehyde modifies groups outside the catalytic site which indirectly affect the catalysis of both reactions. In this case, the differential inhibition of these activities can be explained by a decrease in the rate constant of one or more of the steps in the conversion of E · ADP and medium P_i to E + ATP. The rate constant(s) affected must be found in the numerator of Eqn. 2 but not in that of Eqn. 1. The decrease in the rate constant also has to affect the apparent K_m values for ATP and P_i (Fig. 5). Consequently, it is most likely that glutaraldehyde modification changes the rate constant, k_{-2}. Changing other rate constants, i.e., k_{-1} or k_{-3}, might have the same effect, i.e., reduce the V_{max} of the exchange more and the V_{max} of the hydrolytic reaction less, but will not result in a corresponding change in the Michaelis constants for P_i and ATP, respectively (Eqns. 1–3). A change in k_{-2} would also not affect the K_i for ADP in ATP hydrolysis (Fig. 5). Thus, to explain

the higher sensitivity of the P_i-ATP exchange in comparison to that of ATP hydrolysis (Fig. 2), and the observed change in the K_m values for substrates, we propose that the modification affects mainly the rate constant k_{-2}.

If the kinetic mechanism given above also represents phosphorylation of ADP during ATP synthesis, glutaraldehyde should inhibit phosphorylation and P_i-ATP exchange to the same extent, because k_{-2} would be in the numerator of the equation for ATP synthesis. However, similar degrees of inactivation were not observed, even when phosphorylation was assayed at low light intensities yielding reaction rates similar to those of the P_i-ATP exchange (Fig. 1). Moreover, preillumination of thylakoids, in order to mimic the conditions of the reactions utilizing ATP, did not result in a greater susceptibility of ATP synthesis to glutaraldehyde modification. However, the different assay conditions (mainly the extent of energy input) do not allow a comparable kinetic analysis. This apparent discrepancy can be circumvented by assuming that the rate constants for some steps are energy dependent, and that under phosphorylating conditions (with abundant energy supplied by light) these rate constants are different from those when limited energy is supplied by ATP hydrolysis in the dark. The affinity for substrates [30] and for inhibitors [31] is known to depend on the energization state of the membrane. Differential inhibition of ATP hydrolysis compared to ATP synthesis in submitochondrial particles, reconstituted with F_1 that was pretreated with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole [32], could also be explained by different levels of energization [33].

Limited glutaraldehyde modification of thylakoids does not result in extensive cross-linking of proteins (Fig. 7), but thylakoids modified to an extent that inhibits all the reactions show significant cross-linking. Thus P_i-ATP exchange inhibition may result from modifications of groups on protein subunits that are not necessarily cross-linked (Fig. 7). This situation is similar to that obtained with antibodies against CF_1 [3], which inhibit all the reactions catalyzed by the ATP synthetase and form an insoluble matrix via cross-linking, while monovalent antibodies (Fab), not being able to form cross-links, mainly inhibit P_i-ATP exchange [3,14]. Thus, we suggest that different conformational states of the enzyme participate in phosphorylation and in the hydrolysis or exchange reactions. These conformations show a similar specificity for their substrates in the photophosphorylation and the hydrolytic reactions [31]. In order to explain the greater nucleotide specificity of the P_i-ATP exchange reaction, if we assume one type of catalytic site, we have to invoke again a change in one or more rate constants (becoming slower) for the steps in which the E · NDP complex rebinds medium P_i and reforms E + NTP (N, nucleoside). A corollary of this study is that the P_i-ATP exchange represents a true exchange reaction and is not the sum of ATP hydrolysis and resynthesis involving medium ADP [34].

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