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ON THE MECHANISM OF ACTIVATION OF THE ATPase IN CHLOROPLASTS

TILLY BAKKER-GRUNWALD and KAREL VAN DAM

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)

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SUMMARY

1. The Mg^{2+}-ATPase induced in chloroplasts by light and trypsin is subjected to the same energetic feedback control as is the light-dependent dithioerythritol-induced Mg^{2+}-ATPase.

2. The modification by dithioerythritol or by trypsin of the membrane-bound chloroplast coupling factor is not readily reversible. After treatment in the light with dithioerythritol and subsequent washing, a stable chloroplast preparation is obtained which potentially can be energized by ATP.

3. The decay of the ATPase-active conformation in the dark in the absence of ATP is caused by a non-energy linked, Mg^{2+}-stimulated degradation; it can be reversed by a short light trigger.

4. Addition of ATP to dithioerythritol-treated chloroplasts in the dark initiates, under appropriate conditions, a rapid autocatalytic membrane conformation towards the ATPase-active form.

INTRODUCTION

In a previous paper [1], we characterized the energetic aspects of the light-induced Mg-dithioerythritol-dependent ATPase in spinach chloroplasts. It is possible, however, as Lynn and Straub [2] found some years ago, to induce an ATPase reaction by other means, namely by treatment of the chloroplasts with trypsin. Like the dithioerythritol-dependent ATPase, this trypsin-dependent ATPase was shown to be a manifestation of chloroplast coupling factor (CF_1) activity [2, 3]. Furthermore, like dithioerythritol action, trypsin action is promoted by light and the resulting ATPase is Mg-dependent.

Because of these similarities, it became of interest to investigate whether the trypsin-induced ATPase activity is subjected to the same kind of energetic feedback control as the dithioerythritol ATPase [1].

Abbreviations: CF_1, chloroplast coupling factor; S_{13}, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; TPCK-trypsin, trypsin treated with L-(1-tosylamido-2-phenyl)ethylchloro-methyl ketone, a specific chymotrypsin inhibitor.

From the experiments by Lynn and Straub [2] it is already clear that the trypsin-induced modifications in chloroplasts giving rise to ATPase activity are irreversible: trypsin treatment yields a stable chloroplast preparation that retains its ability to hydrolyse ATP after a triggering light pulse (cf. also ref. 3).

There is evidence in the literature, however, that the modification of membrane-bound CF_1 by dithiol compounds is also not readily reversible: CF_1 extracted from dithioerythritol- or dithiothreitol-treated chloroplasts exhibits Ca^{2+}-ATPase activity [4, 5], which means that some dithiol-induced modification persists during the (dark) extraction steps.

An important consequence is that a chloroplast preparation, after treatment with dithioerythritol in the light, centrifugation and washing to remove the dithioerythritol, should still be energizable by ATP.

Finally, by comparison of the properties of trypsin-induced ATPase and dithioerythritol-induced ATPase we hoped to obtain some information about the mechanism of activation of both ATPase systems.

METHODS AND MATERIALS

'Type-C' [6] spinach chloroplasts were prepared as described before [1], and suspended in a medium containing 100 mM KCl, 5 mM $MgCl_2$ and 5 mM sodium tricine (pH 8.0).

Dithioerythritol-treated chloroplasts were prepared by illuminating a type-C chloroplast suspension (50 μg chlorophyll/ml) for 5 min in the presence of 5 mM dithioerythritol, turning the light off, spinning the chloroplasts down and washing them once with the suspension medium.

Total chlorophyll was determined according to the method of Whatley and Arnon [7].

The reaction vessel used was designed by Mr J. W. T. Fiolet, and, as described before [1], offered the possibility of simultaneously monitoring fluorescence and medium pH.

The reaction mixture contained, at 20 °C, in a final volume of 2.5 ml, 250 μmoles KCl, 12.5 μmoles $MgCl_2$, 12.5 μmoles sodium tricine (pH 8.0), 25 nmoles pyocyanine and 50 μg chlorophyll. ATP (final concentration 1 mM) was present in the reaction mixture, unless stated otherwise.

ATPase activity was monitored as a change in medium pH. ΔpH was estimated from the quenching of 9-aminoacridine fluorescence [8], as in the previous paper [1]; the concentration of 9-aminoacridine was 4 μM. Routinely, at the end of each run a saturating amount of uncoupler was added to obtain the 100 % fluorescence level [1].

9-Aminoacridine was obtained from British Drug Houses, trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK-trypsin) from Worthington and soybean trypsin inhibitor from Sigma; stock solutions contained 5 mg TPCK-trypsin/ml in 2 mM HCl, and 8 mg trypsin inhibitor/ml in 10 mM sodium tricine (pH 8.0), respectively. All other chemicals were analytical grade.

5-Chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S_{13}) was kindly donated by Dr P. C. Hamm, Monsanto Comp., St. Louis, Mo. (U.S.A.).

RESULTS

Trypsin ATPase

Fig. 1 shows some typical characteristics of the trypsin-induced ATPase. As already reported by Lynn and Straub [2], the trypsin activation is light-dependent. In our hands, the induced ATPase was never completely stable but decayed in the dark, concomitant with a reappearance of 9-aminoacridine fluorescence. It could be stabilized by phosphate or arsenate (dotted lines in Fig. 1). The degree of stabilization increased with the concentration of those effectors, saturation being reached at about 2 mM. By a second illumination cycle, all of the original ATPase activity could be restored.

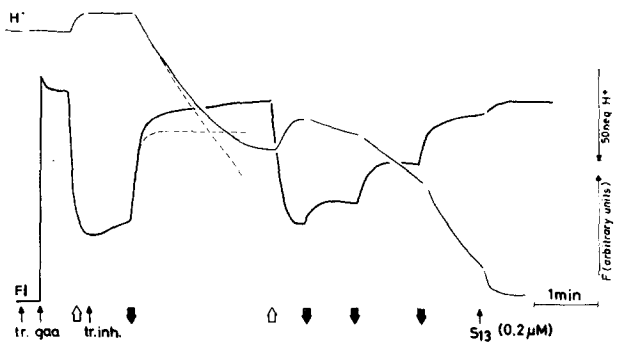

Fig. 1. Characteristics of trypsin-induced ATPase. The reaction mixture was as described under Methods and Materials. 9aa, 9-aminoacridine; tr., trypsin (30 μg); tr. inh., trypsin inhibitor (80 μg). After a dark ATPase period, a new full-intensity light pulse was given, followed by a stepwise reduction of light intensity. Light intensities corresponded to 17 mW/cm^2, 7 mW/cm^2, 3 mW/cm^2 and 0 mW/cm^2. Finally, 0.2 μM S_{13} was added. - - -, 1 mM P_i or AS_i was added to the reaction mixture.

Fig. 1 illustrates that it is also possible to stabilize the trypsin ATPase by some background illumination, and, antagonistically, to destabilize it with uncoupler. Apparently, then, the trypsin ATPase is subjected to the same energetic feedback control as the dithioerythritol ATPase [1]. After triggering, the maintenance of a certain energy level is needed in order to ensure continuation of ATPase activity. (In fact, the experiments shown by Lynn and Straub [2] can also be interpreted in the same way.) Moreover, the stability properties of the trypsin ATPase could be completely mimicked by a somewhat uncoupled dithioerythritol-ATPase system: stabilization by background light [5], destabilization by uncoupler [1] and also the stabilization by phosphate or arsenate (Fig. 2). This implies that the trypsin treatment is functionally equivalent to the dithioerythritol treatment except for some uncoupling side effects in the former treatment.

In Fig. 3, trypsin ATPase activity is shown as a function of the illumination time at different trypsin concentrations. Clearly, the duration of the light pulse needed for a given degree of activation can be reduced by increasing the trypsin concentration. Trypsin hardly has any effect when added at the moment the light is turned off or afterwards (not shown); this indicates that the energized state required for trypsin action decays within the order of seconds.

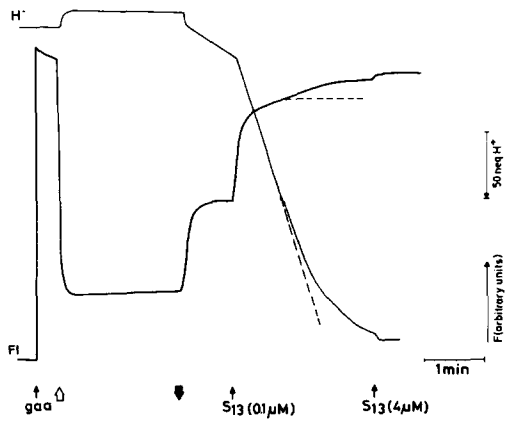

Fig. 2. Stabilization of an uncoupled dithioerythritol ATPase by P_i. The reaction mixture was as described under Methods and Materials, with 5 mM dithioerythritol added. - - -, 1 mM P_i or As_i was added to the reaction mixture.

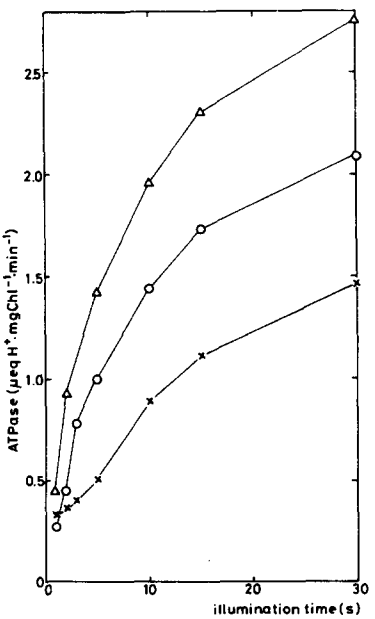

Fig. 3. Trypsin ATPase as a function of illumination time at different trypsin concentrations. Trypsin was added in the dark, and the mixture (with 1 mM P_i added) was illuminated for the indicated periods of time. ×–×, 30 μg trypsin; ○–○, 75 μg trypsin; △–△, 150 μg trypsin.

Dithioerythritol-treated chloroplasts

As mentioned in the Introduction, we expected that chloroplasts treated in the light with dithioerythritol would retain their ability to hydrolyse ATP after centrifugation and washing to remove the dithioerythritol. As can be seen in Fig. 4, these dithioerythritol-treated chloroplasts in fact exhibited ATPase activity, but, like

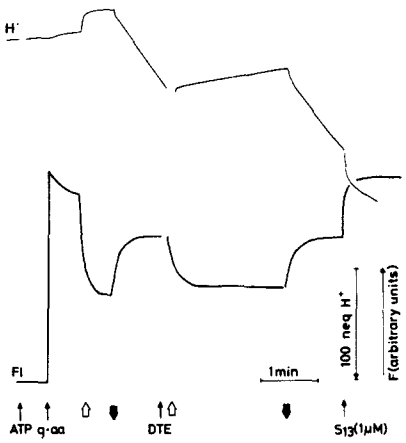

Fig. 4. Characteristics of ATPase activity in dithioerythritol-treated chloroplasts (DTE). The reaction mixture was as described under Methods and Materials. [DTE] = 5 mM.

trypsin-treated chloroplasts, only after a triggering light pulse. The magnitude of both the ATPase activity and the quenching level associated with it was, after optimal light triggering, equal to that exhibited in a conventional experiment where dithioerythritol, after the light treatment, was not removed by centrifugation [1]. Continued illumination of this chloroplast preparation in the presence of dithioerythritol gave hardly any further stimulation (Fig. 4). The dithioerythritol-treated chloroplast preparation was stable for at least some hours, but showed a decline in ATPase activity after a longer period of time.

In Fig. 5, the ATPase activity is plotted as a function of light-triggering time at saturating light intensity. Whilst the dithioerythritol treatment itself takes some minutes [1], the triggering step involved here takes place within a much shorter time scale, more comparable to the activation of trypsin ATPase at high trypsin concentration (Fig. 3).

As in the conventional procedure [1, 9] the ATPase activity of dithioerythritol-

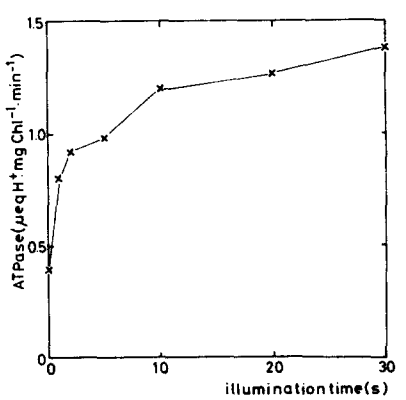

Fig. 5. ATPase by dithioerythritol-treated chloroplasts as a function of light-triggering time. Conditions were as described under Methods and Materials.

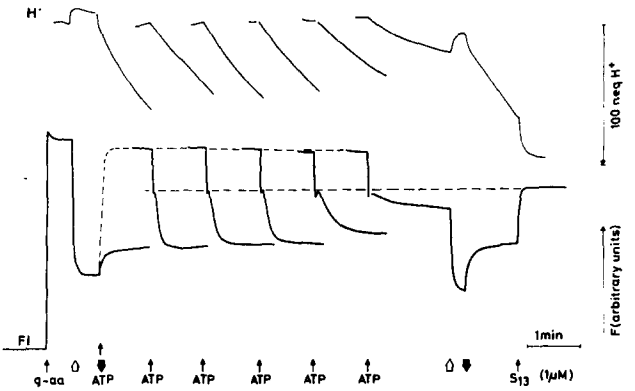

Fig. 6. Dark decay of light-triggered ATPase in dithioerythritol-treated chloroplasts. Reaction mixture was as described under Methods and Materials. After a 30 s light trigger, the light was turned off, and ATP added after 0, 1, 2, 3, 4 and 5 min, respectively. The dotted lines give the 100 % fluorescence levels for the mixture in the absence (upper line) and presence (lower line) of ATP. In the last curve, the original ATPase activity is restored by a second light trigger.

treated chloroplasts declined in the dark in the absence of ATP. Traces are shown in Fig. 6. Immediately after addition of ATP an initial rapid decrease in fluorescence is seen in the fluorescence traces; this is caused by direct interaction between 9-aminoacridine and the adenine moiety (the same effect is displayed by ADP and AMP). The final level of quenching is reached more slowly the longer the period before adding the ATP. In the first four curves this final level is virtually constant, but then a sudden breakdown occurs. For all curves, the original ATPase activity could be restored by a second light trigger (shown for the last curve in Fig. 6); this is analogous to the reactivation of trypsin ATPase (Fig. 1).

The fact that under these conditions light activation is very rapid indicates that the dark decay is not a complete reversal of dithioerythritol action. In Fig. 7, the influence of uncoupler on the dark decay is shown: uncoupler accelerated the decay, but only when it was present at the moment ATP was added (and then irrespective of whether it was already present during the whole preceding dark period). Its influence was abolished if it was removed with bovine serum albumin just before the addition of ATP.

Without uncoupler, ΔpH calculated from fluorescence quenching maintained by ATPase was virtually constant till a sudden breakdown after 4 min (cf. fluorescence traces in Fig. 6); in the presence of the uncoupler the decay of ΔpH started immediately.

Thus, the dark decay itself is clearly not linked to the energy state of the chloroplasts. The possibility was considered, then, that an aspecific enzymic degradation was involved, for instance a phosphatase splitting off a phosphate group essential for ATPase activity. Since phosphatases are known to be Mg^{2+}-dependent, we studied the effect of omitting Mg^{2+} during the dark stage. As can be seen in Fig. 8, this indeed dramatically slowed down the dark decay. In Fig. 8 are also plotted the final levels of ΔpH reached during ATPase: as in Fig. 7, a sudden breakdown occurs after 4 min dark decay in the presence of Mg^{2+}.

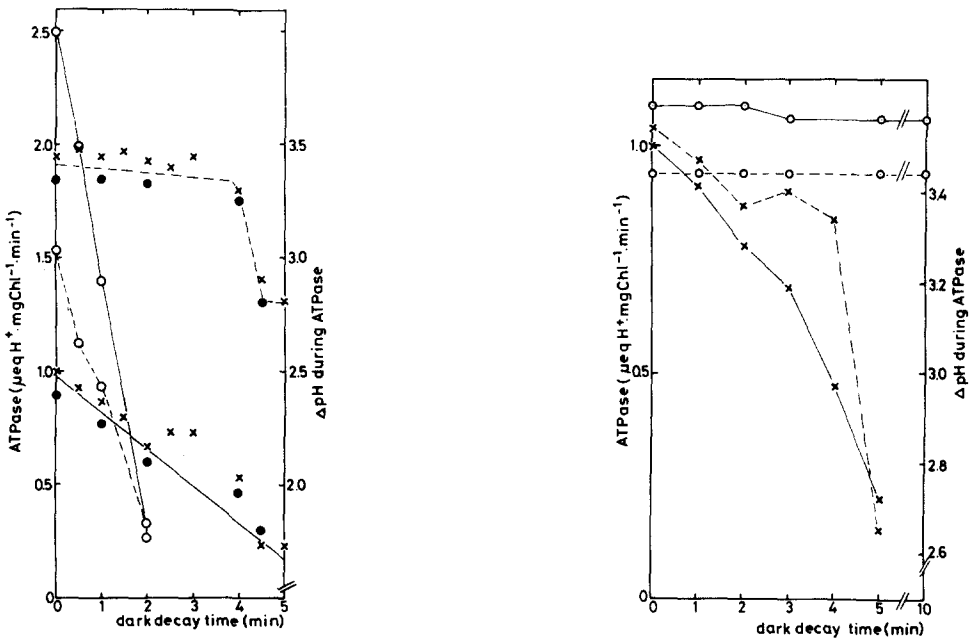

Fig. 7. Dark decay of light-triggered ATPase in dithioerythritol-treated chloroplasts; influence of uncoupler. The reaction conditions were as described in the legend of Fig. 6. ×–×, reaction mixture without additions; ○–○, reaction mixture with 0.4 μM S_{13} added at the moment the light was turned off; ●–●, reaction mixture with 0.4 μM S_{13} added the moment the light was turned off, and 0.1 mg bovine serum albumin added 5 s before ATP. —, ATPase; - - -, final ΔpH during ATPase, calculated as in ref. 1.

Fig. 8. Dark decay of light-triggered ATPase in dithioerythritol-treated chloroplasts; influence of omitting Mg^{2+}. Dithioerythritol-treated chloroplasts were washed in a medium containing 100 mM KCl, 5 mM sodium tricine and 0.5 mM sodium EDTA, pH 8.2. ×–×, reaction conditions as described in the legend of Fig. 6. ○–○, the dark decay took place in the above medium, supplemented with 10 μM pyocyanine. After the indicated periods of time a mixture of ATP and Mg^{2+} was added to final concentrations of 1 mM and 5 mM, respectively. (This caused an acidification of the mixture from pH 8.2 to pH 8.0.) —, ATPase; - - -, final ΔpH reached during ATPase, calculated as in ref. 1.

DISCUSSION

On the basis of the data presented above we propose a model for the activation of chloroplast ATPase induced by either trypsin or dithioerythritol. As a minimum hypothesis, the model contains the following elements which will be discussed in turn (Fig. 9). Upon energization by light, a conformational change takes place exposing the trypsin- and dithioerythritol-sensitive sites (Step I). Those agents then modify the ATPase in an irreversible (trypsin) or at least not readily reversible (dithioerythritol) way (Step II). From studies with isolated CF_1 it seems likely that they destroy or interfere with the action of the so-called inhibitor, an inhibiting subunit of the ATPase complex.

A conformational change similar to the one discussed here was proposed earlier by McCarty and Fagan [11] in order to explain the light dependency of *N*-ethylmaleimide binding to the coupling factor; also the light-dependent ^3H-exchange

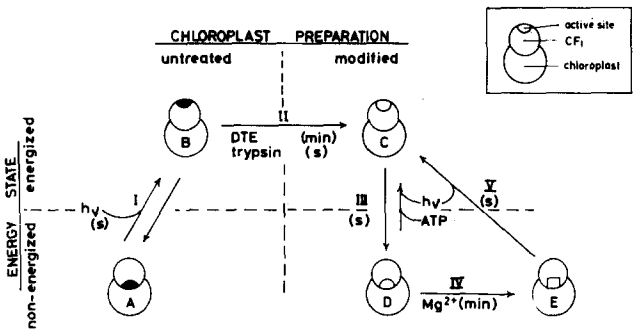

Fig. 9. Model for activation of chloroplast ATPase.

with the coupling factor found by Ryrie and Jagendorf [12] might be relevant in this respect.

The data shown in Fig. 3 for saturating trypsin concentration indicate that the half-time of Step I lies in the order of seconds; as trypsin has virtually no effect when added the moment the light is turned off, Step I should be reversible within the same time scale. That a true equilibrium is involved also follows from the fact that even in the dark dithioerythritol and trypsin exert their modifying action, only much more slowly [4, 2].

The high-energy form C is the only one exhibiting ATPase activity, as its ATPase site is not only depleted of inhibitor, but also accessible to ATP. This conformation can be maintained by the energy supplied by Mg^{2+}-ATPase activity itself. The modified chloroplasts are involved in the same energy-dependent conformational equilibrium as the untreated ones. In the dark, without ATP, this equilibrium (III) will be mainly towards the side of inactive form D. This form is subject to a non-energy linked Mg^{2+}-catalyzed degradation process (Fig. 8). The resulting inactive form E may only be converted back into the active form C by a short light trigger (Step V). The bulk of the dithioerythritol-treated chloroplasts are thought to be in form E.

Addition of ATP to the dark system may, by mediation of the fraction of ATPase molecules remaining in active form C, start an autocatalytic energization process. Direct experimental evidence for such a process is given in the fluorescence traces of Fig. 6. It should be realized that the increasing delay is observed in the most sensitive part of the fluorescence scale [8]; this may explain why a similar lag has not been observed in the pH traces themselves.

Thus, the 'dark decay' is a complex phenomenon, namely the decrease in time in the fraction of active molecules C finally regained by the ATP-induced autocatalytic activation process. Whether, and to what degree, this process takes place depends on the net energy gain obtained by the initial ATPase activity, and this is determined both by the initial magnitude of fraction C (in its turn determined by the position of equilibrium III and the progress of degradation IV), and the degree of coupling of the chloroplasts. A compound influencing the dark decay may affect any of these parameters as well as their mutual interaction; this means that no absolute value can be attached to K_m values determined for the effect of ADP and P_i [9] on the dark decay. A criterion deciding whether a compound exerts its action on the level of

equilibrium III or on that of degradation IV is that in the former case it only needs to be present the moment ATP is added (as was found for uncoupler, Fig. 7), whilst in the latter case it has to be present during the whole dark period (like Mg^{2+}, Fig. 8).

Dithioerythritol-treated chloroplasts should prove useful in studying the possibility of driving energy-requiring processes by ATPase activity (for instance, reversal of electron transport [14]): they offer a much purer system than the conventional one, in which dithioerythritol is still present as a potentially disturbing contaminant. In this paper we showed that the energetic aspects of dithioerythritol-induced ATPase and trypsin-induced ATPase are very similar. Therefore, 'trypsin-treated chloroplasts' as prepared by Lynn and Straub [2] may in principle be analogous to dithioerythritol-treated chloroplasts. However, because of the uncoupling side effects of trypsin, we would prefer to use the dithioerythritol-treated chloroplasts for routine experiments.

ACKNOWLEDGEMENTS

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