

## Thiol modulation of CF<sub>0</sub>–CF<sub>1</sub> stimulates acid/base-dependent phosphorylation of ADP by broken pea chloroplasts

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### 1. INTRODUCTION

There is now considerable evidence that the reversible protonmotive ATPase of chloroplasts (CF<sub>0</sub>–CF<sub>1</sub>) is a highly regulated enzyme both in vivo [1,2] and in vitro [3–11]. Studies with broken chloroplasts (that lack an outer envelope and stroma) have shown that CF<sub>0</sub>–CF<sub>1</sub> in dark-adapted chloroplasts is catalytically inactive, but undergoes activation when a difference in the electrochemical potential of protons ( $\Delta\bar{\mu}H^+$ ) is generated across the thylakoid [3–10]. This activation is accompanied by the release of 1 mol bound ADP/mol CF<sub>1</sub>, by which means the activation process has been extensively studied [5–10]. Deactivation of CF<sub>0</sub>–CF<sub>1</sub> occurs upon the collapse of  $\Delta\bar{\mu}H^+$  and is associated with the rebinding of ADP [8–10]. Activation of CF<sub>0</sub>–CF<sub>1</sub> may simply reflect a dual pH optimum requirement of CF<sub>1</sub>, whereby the stromal, or *N* pole, of CF<sub>1</sub> has an optimum around pH 8 but the intrathylakoid, or *P* pole, has an optimum around pH 5 [12]. These differential pH conditions would normally only exist when  $\Delta\bar{\mu}H^+$  was present across the thylakoid. Deactivation would occur when the appropriate pH conditions ceased to exist at either

the *P* or *N* poles of CF<sub>1</sub>, and its rate would be influenced by the presence of bound nucleotides. We call this type of regulation of CF<sub>0</sub>–CF<sub>1</sub> 'pH (de)activation'.

In addition to pH activation, CF<sub>0</sub>–CF<sub>1</sub> activity is also modulated by reduced dithiols [3, 13–17]. In the absence of reduced dithiols, CF<sub>0</sub>–CF<sub>1</sub> is in a demodulated state and is observed to catalyse net synthesis of ATP under the appropriate conditions, but little ATPase or ATP=P<sub>i</sub> exchange activity is observed. In contrast, the presence of reduced dithiols modulates the pH-activated CF<sub>0</sub>–CF<sub>1</sub> [3] and ATP hydrolysis [3,13] and P<sub>i</sub>⇌ATP exchange [14] are readily observed.

Work from this laboratory has shown that thiol modulation of CF<sub>0</sub>–CF<sub>1</sub> is a physiological process that, in vivo, is probably catalysed by the thioredoxin system [11,16]. Illumination of intact chloroplasts [11,16–18] (or leaves [1,2]) results in both the pH activation and the thiol modulation of CF<sub>0</sub>–CF<sub>1</sub> and both these processes are readily reversed in the dark [17], providing that the chloroplasts remain intact. In an attempt to understand why CF<sub>0</sub>–CF<sub>1</sub> is subject to thiol modulation in vivo, we have considered whether the process improves the efficiency of photophosphorylation. As a first step, we have studied acid/base-dependent phosphorylation [19] in order to eliminate the influence of electron transport reactions. We show here that, contrary to an early report [15], thiol modulation of CF<sub>0</sub>–CF<sub>1</sub> increases the yield of ATP synthesis by chloroplasts subjected to an acid/base transition. The stimulation is mainly observed under conditions of limiting  $\Delta pH$ , suggesting that, in demodulated chloroplasts, the  $\Delta pH$  required for

*Abbreviations:* CF<sub>0</sub>–CF<sub>1</sub>, reversible protonmotive ATPase of chloroplasts; MES, 2-(*N*-morpholino)ethanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Tricine, *N*-tris(hydroxymethyl)methyl glycine; pH<sub>N</sub>, pH<sub>P</sub> bulk pH of the extra- and intrathylakoid aqueous phases;  $\Delta\bar{\mu}H^+$ ,  $\Delta pH$ , difference in electrochemical potential of protons or pH between the aqueous phases separated by the thylakoid membrane;  $\Delta\bar{\mu}H^+_{act}$ , minimum  $\Delta\bar{\mu}H^+$  required for activation of CF<sub>0</sub>–CF<sub>1</sub>

activation of  $CF_0$ - $CF_1$  is larger than that required thermodynamically for the synthesis of ATP.

## 2. MATERIALS AND METHODS

Intact chloroplasts were isolated from *Pisum sativum* [11] (variety Meteor) as previously described except that the grinding medium contained 0.36 M sorbitol, 5 mM  $MgCl_2$ , 50 mM KCl, 5 mM ascorbate, 5 mM MES (pH 6.5). The organelles were lysed [11], washed twice by recentrifugation, and finally resuspended in a small volume of grinding medium at 2–4 mg chlorophyll/ml. Chloroplasts were thiol modulated by mixing (typically) 200  $\mu$ l stock suspension with 0.8 ml of a medium containing 15 mM tricine, 5 mM  $MgCl_2$ , 12 mM dithiothreitol, 100  $\mu$ M methyl viologen, 1600 U catalase (type C40, Sigma Chemical Co.) and 2.5  $\mu$ M diadenosine pentaphosphate (to inhibit adenylate kinase). The chloroplasts were illuminated for 6 min at 80 W/m<sup>2</sup> (300 W projector filtered through Corning 3–97 glass filter) then stored on ice and used within 30 min. Demodulated chloroplasts were treated as above but no preillumination was given. Acid/base-induced phosphorylation was measured as follows: 100  $\mu$ l treated chloroplasts were mixed with 100  $\mu$ l 30 mM succinate, 5 mM  $MgCl_2$  for 30 s in a well-stirred tube (acid stage), then 0.8 ml 100 mM buffer, 5 mM  $MgCl_2$ , 100  $\mu$ M ADP, 0.5 mM  $KH_2PO_4$ , 2.5  $\mu$ M diadenosine pentaphosphate was added. Buffers used were MES (pH 6.0–7.0); Hepes (7.0–8.0) and glycylglycine (8.0–9.6). These buffers were chosen because of their lack of inhibitory side-effects at the high concentrations used.

The reaction was terminated by addition of 0.5 ml 10% trichloroacetic acid, usually 10 s after addition of the basic medium. ATP synthesis was assayed by either luciferase or <sup>32</sup>P methods. For the luciferase technique, the solution was neutralized with 0.5 ml 0.6 M KOH, then 100  $\mu$ l was withdrawn and directly assayed for ATP with the aid of LKB monitoring reagent and luminometer. In the <sup>32</sup>P assay, the  $P_i$  content of the base stage was reduced to 120  $\mu$ M, and 0.5  $\mu$ Ci <sup>32</sup>P<sub>i</sub> (Amersham International) was included per assay. Unreacted <sup>32</sup>P<sub>i</sub> was removed by a modification (to be described elsewhere) of the ammonium molybdate/triethylamine precipitation procedure [6]. Samples were counted via Cerenkov radiation in an LKB Rackbeta scintillation counter. This procedure ensured that

> 99.9% of <sup>32</sup>P<sub>i</sub> was removed and resulted in controls that were barely above background levels (50–80 cpm compared to typically 1000 cpm for maximal acid/base-dependent phosphorylation).

## 3. RESULTS

Acid/base-induced phosphorylation was discovered by Jagendorf and coworkers [19] and has been extensively studied using broken, demodulated chloroplasts [7,19,20]. To our knowledge, only one study has compared the yield of ATP synthesized by demodulated and thiol-modulated chloroplasts and this showed an apparent decrease in yield of ATP after thiol modulation of the chloroplasts [15]. The authors recognized that the apparent decrease in yield may have resulted from hydrolysis of the newly formed ATP by  $CF_0$ - $CF_1$  ATPase which was activated by the acid/base procedure [15], but they did not study the problem further.

Fig.1 shows the results of an experiment where the yield of ATP observed after an acid/base transition is compared for demodulated and thiol-modulated chloroplasts and for two values of pH of the acid stage ( $pH_P$ ). The pH of the base stage ( $pH_N$ ) was varied from 6.0–9.3. At  $pH_P = 4.1$ , thiol-modulated chloroplasts were observed to synthesize more ATP than demodulated chloroplasts when  $\Delta pH$  was limiting. In contrast, for large  $\Delta pH$  values

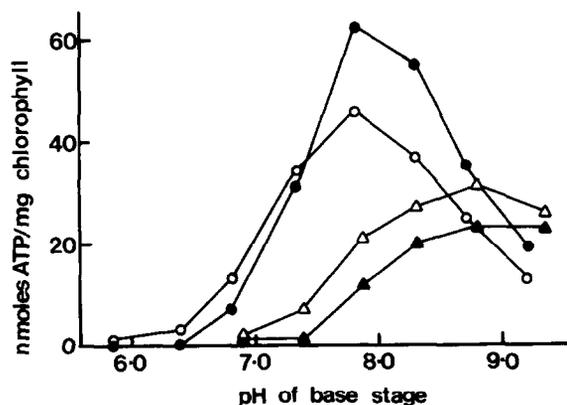


Fig.1. Comparison of the yield of ATP observed after an acid/base transition of thiol-modulated (○,△) and demodulated (●,▲) chloroplasts. The pH of the acid stage was either 4.1 (○,●) or 5.3 (△,▲). ATP was assayed by luciferase technique.

(high  $pH_N$ ), the yield was apparently diminished, as reported in [15]. When the pH of the acid stage was raised to 5.3, a stimulation of yield throughout the  $pH_N$  range was observed after thiol modulation of the chloroplasts (fig. 1). Two other points may be noted.

- (i) When  $\Delta pH$  was large (i.e., low  $pH_P$ ), the optimal yield of ATP was observed when the base stage was around pH 8.0, and the yield declined above pH 8. When  $\Delta pH$  was limiting (high  $pH_P$ ), the optimal yield was shifted to  $pH_N = 9$ , but the overall yields at pH 9 for both values of  $pH_P$  were similar.
- (ii) The threshold  $pH_N$  (at which net ATP synthesis is first observed) was, as expected, shifted to more alkaline values as the pH of the acid stage was raised. However, for both the values of  $pH_P$  given in fig.1, thiol modulation of the chloroplasts resulted in a lower threshold  $pH_N$  relative to demodulated chloroplasts. In other words, thiol modulation appears to lower the threshold  $\Delta pH$  required for acid/base-induced synthesis of ATP.

The latter result is shown in more detail in fig.2, where the observed threshold  $\Delta pH$  for ATP synthesis is plotted against the pH of the acid stage for several experiments of the kind depicted in fig.1. It is of interest to note that the threshold  $\Delta pH$  for ATP synthesis depended on the pH of the acid stage, and

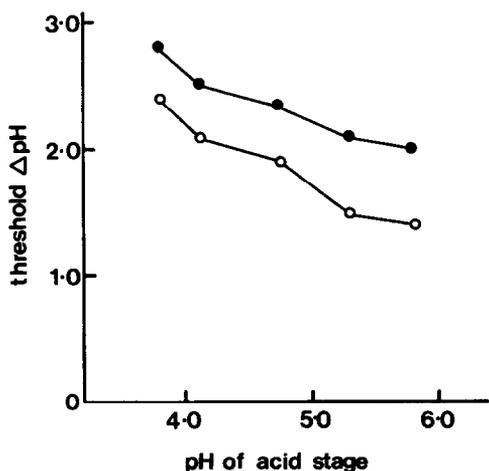


Fig.2. Comparison of the threshold  $\Delta pH$  (at which ATP synthesis is first observed) of thiol-modulated (○) and demodulated (●) chloroplasts. Other details as in fig.1.

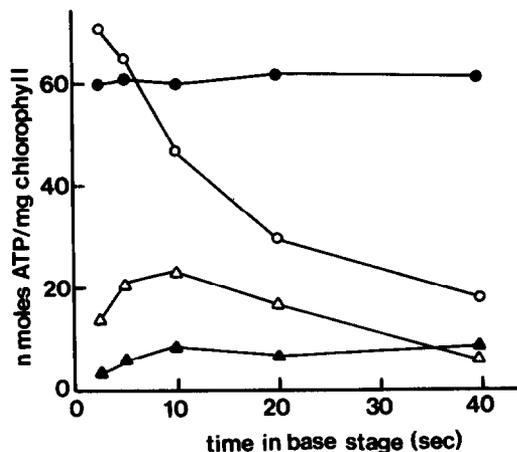


Fig.3. Effect of time in the base on the observed yield of ATP following an acid/base transition of 4.3/8.0 (○,●) or 5.3/8.0 (△,▲). Chloroplasts were thiol-modulated (○,△) or demodulated (●,▲). ATP was assayed by the luciferase technique.

apparently decreased as  $pH_P$  increased. In all cases however, the observed value was lower when chloroplasts were thiol-modulated.

The data of fig.1 and 2 indicate that thiol-modulation of  $CF_0$ - $CF_1$  increases the efficiency of phosphorylation when  $\Delta pH$  is limiting. We considered whether this might also be true when  $\Delta pH$  is not limiting (i.e., large  $\Delta pH$  jumps), but that hydrolysis of the newly formed ATP by  $CF_0$ - $CF_1$  was obscuring the observations. Fig.3 shows an experiment where the reaction time in the base stage was varied from 2.5–40 s. It is clear that extensive hydrolysis of ATP occurred subsequent to acid/base mixing when chloroplasts were thiol-modulated, especially at the lower value of  $pH_P$ . At short reaction times, thiol-modulation of  $CF_0$ - $CF_1$  stimulated the yield of ATP synthesized at both  $pH_P = 4.3$  and 5.3 (fixed  $pH_N \sim 8.0$ ) but the relative stimulation is much smaller in the former case (i.e., at the larger value of  $\Delta pH$ ). However, the rapid rate of hydrolysis of ATP precludes a firm conclusion from this type of experiment. Therefore, we have re-examined this question using glucose and hexokinase to trap the newly formed ATP before subsequent hydrolysis can take place. The results are shown in fig.4 and they qualitatively confirm the results already presented. It can be seen that thiol-modulation of the chloroplasts stimulated the yield of ATP

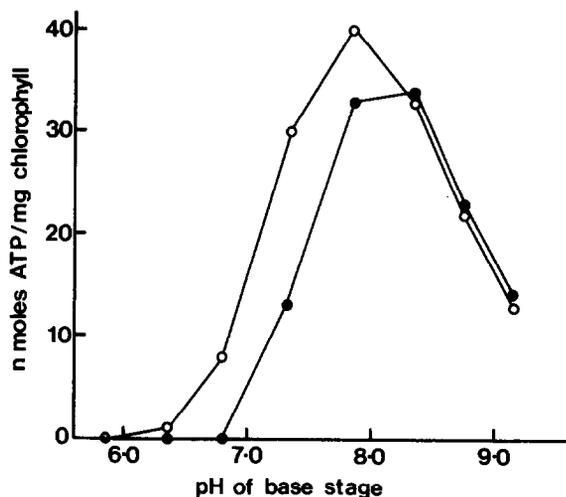


Fig.4. Comparison of the yield of ATP observed after an acid/base transition of thiol-modulated (○) and demodulated (●) chloroplasts. The pH of the acid stage was 4.15. The base stage additionally contained 4 units of hexokinase (type F-300, Sigma Chemical Co.) and 12 mM glucose. ATP was estimated by the  $^{32}\text{P}$  method.

synthesis at relatively small acid/base transitions (low  $\Delta\text{pH}$ ), but the stimulation diminished to zero as  $\Delta\text{pH}$  was increased. Furthermore, thiol-modulation lowered the observed threshold  $\Delta\text{pH}$  for net synthesis of ATP. Quantitatively, the yields of ATP observed by this method (which involves assay of glucose 6-phosphate by incorporation of  $^{32}\text{P}$ ) are lower than those assayed by the luciferase method (cf. fig.1). This can mostly be ascribed to the non-saturating concentration of  $\text{P}_i$  used in the media in the former case. ADP at  $\sim 5 \mu\text{M}$  and  $\text{P}_i$  at  $\sim 30 \mu\text{M}$  gave half-maximal yields; thiol-modulation of the chloroplasts slightly increased these values (not shown).

#### 4. DISCUSSION

It has long been known that thiol-modulation of  $\text{CF}_0\text{--CF}_1$  stimulates the observed rate of ATP hydrolysis [13] and  $\text{ATP} \rightleftharpoons \text{P}_i$  exchange [14] reactions, and we now show that this treatment also stimulates the yield of ATP observed after an acid/base transition. The stimulation of acid/base-induced phosphorylation is most marked at limiting  $\Delta\text{pH}$ , and the effect tends to diminish as the size of the pH

transition is increased (fig.4). These results cannot be explained in terms of changes in the binding constant of ADP or  $\text{P}_i$ . Therefore, in order to explain this behavior, it is necessary to determine what limits the yield of ATP synthesis at low  $\Delta\text{pH}$ .

It has been suggested that phosphorylation in demodulated chloroplasts at low  $\Delta\bar{\mu}\text{H}^+$  is limited by the fraction ( $\alpha$ ) of  $\text{CF}_0\text{--CF}_1$  complexes that are active [5–7]. On this assumption,  $\text{CF}_0\text{--CF}_1$  activity is kinetically controlled, and  $\Delta\bar{\mu}\text{H}^+$  may not be in equilibrium with  $\Delta G_p$ , the free energy of ATP hydrolysis. Therefore, thiol-modulation of  $\text{CF}_0\text{--CF}_1$  may stimulate phosphorylation by causing  $\alpha$  to increase, and studies of the steady levels of bound nucleotides suggest that this is indeed the case [9]. Furthermore, the effect of thiol modulation would be most marked when  $\alpha$  is initially small, i.e., at low  $\Delta\bar{\mu}\text{H}^+$  ( $\Delta\text{pH}$ ) as observed here. One would also predict that photophosphorylation should also be stimulated when  $\Delta\text{pH}$  is limiting. We have studied and verified this prediction, and details will be published elsewhere.

The mechanism by which thiol-modulation causes an increase in  $\alpha$  under any particular set of conditions is not clear at this stage. A likely possibility is that a change in enzyme structure results in a lowering of the  $\Delta\bar{\mu}\text{H}^+$  required to activate  $\text{CF}_0\text{--CF}_1$  ( $\Delta\bar{\mu}\text{H}_{\text{act}}^+$ ). Such a reduction in  $\Delta\bar{\mu}\text{H}_{\text{act}}^+$  could occur by a change in the pH optimum at either the *P* or *N* poles of  $\text{CF}_1$ . At low  $\Delta G_p$ , this may cause a  $\Delta\bar{\mu}\text{H}_{\text{act}}^+ > \Delta G_p$  whilst after thiol-modulation, a  $\Delta\bar{\mu}\text{H}_{\text{act}}^+ < \Delta G_p$ . This situation would then explain the appearance of net ATP hydrolysis only after thiol modulation of  $\text{CF}_0\text{--CF}_1$ . However, it should be noted that the effect of thiols would tend to disappear as  $\Delta\bar{\mu}\text{H}^+$  increases. Therefore, this mechanism cannot explain the lack of ATP hydrolysis or  $\text{ATP} \rightleftharpoons \text{P}_i$  exchange at high  $\Delta\bar{\mu}\text{H}^+$  where  $\alpha$  is close to 1.

The data of fig.1 and 2 show that the threshold  $\Delta\text{pH}$  at which ATP synthesis is first observed is lower after chloroplasts have been thiol-modulated. The  $\text{H}^+/\text{ATP}$  stoichiometry may be calculated from this threshold value and the values obtained are 3.4 (demodulated chloroplasts,  $\Delta\text{pH} = 2.0$ ) and 4.6 (thiol-modulated chloroplasts,  $\Delta\text{pH} = 1.5$ ). The latter value is higher than the values of 2 [21] or 3 [7,23] obtained by other methods. The threshold  $\Delta\text{pH}$  obtained with thiol-modulated chloroplasts should be a better estimate of the thermodynamic

equilibrium point than that obtained with demodulated chloroplasts where  $CF_0$ – $CF_1$  is certainly under kinetic control. However, the  $H^+$ /ATP stoichiometry calculated from the observed threshold  $\Delta pH$  may be in error for two reasons.

- (i) The threshold  $\Delta pH$  was observed to vary with the pH of the acid stage (which may in fact be caused by a suboptimal pH in the base stage).
- (ii) The acid/base transition will certainly generate ionic diffusion potentials (in addition to  $\Delta pH$ ) which, although probably small, should be taken account of in calculating  $H^+$ /ATP.

Finally, these results are important in understanding the *in vivo* regulation of  $CF_0$ – $CF_1$ . The ability of  $CF_0$ – $CF_1$  to interact with the thioredoxin system suggests that the *in vivo* enzyme is thiol-modulated in the light but demodulated in the dark [17]. Therefore, in the dark  $\Delta\bar{\mu}H_{act}^+ > \Delta G_P$  and  $CF_0$ – $CF_1$  is able to deactivate rapidly and completely whereas in the light,  $\Delta\bar{\mu}H_{act}^+ < \Delta G_P$  and ATP synthesis may proceed at maximal efficiency. In this way, the chloroplast may avoid unnecessary hydrolysis of ATP during darkness without affecting the efficiency of ATP synthesis during illumination.

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