Thiol modulation of CF_0–CF_1 stimulates acid/base-dependent phosphorylation of ADP by broken pea chloroplasts

John D. Mills and Peter Mitchell

Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, England

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1. INTRODUCTION

There is now considerable evidence that the reversible protonmotive ATPase of chloroplasts (CF_0–CF_1) 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_0–CF_1 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 thylaloid [3–10]. This activation is accompanied by the release of 1 mol bound ADP/mol CF_1, by which means the activation process has been extensively studied [5–10]. Deactivation of CF_0–CF_1 occurs upon the collapse of $\Delta\bar{\mu}H^+$ and is associated with the rebinding of ADP [8–10]. Activation of CF_0–CF_1 may simply reflect a dual pH optimum requirement of CF_1, whereby the stromal, or *N* pole, of CF_1 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_1, and its rate would be influenced by the presence of bound nucleotides. We call this type of regulation of CF_0–CF_1 'pH (de)activation'.

In addition to pH activation, CF_0–CF_1 activity is also modulated by reduced dithiols [3, 13–17]. In the absence of reduced dithiols, CF_0–CF_1 is in a demodulated state and is observed to catalyse net synthesis of ATP under the appropriate conditions, but little ATPase or $ATP \rightleftharpoons P_i$ exchange activity is observed. In contrast, the presence of reduced dithiols modulates the pH-activated CF_0–CF_1 [3] and ATP hydrolysis [3,13] and $P_i \rightleftharpoons ATP$ exchange [14] are readily observed.

Work from this laboratory has shown that thiol modulation of CF_0–CF_1 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_0–CF_1 and both these processes are readily reversed in the dark [17], providing that the chloroplasts remain intact. In an attempt to understand why CF_0–CF_1 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_0–CF_1 increases the yield of ATP synthesis by chloroplasts subjected to an acid/base transition. The stimulation is mainly observed under conditions of limiting ΔpH, suggesting that, in demodulated chloroplasts , the ΔpH required for

Abbreviations: CF_0–CF_1, 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_N, pH_P bulk pH of the extra- and intrathylakoid aqueous phases; $\Delta\bar{\mu}H^+$, ΔpH, 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_0–CF_1

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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 μl stock suspension with 0.8 ml of a medium containing 15 mM tricine, 5 mM $MgCl_2$, 12 mM dithiothreitol, 100 μM methyl viologen, 1600 U catalase (type C40, Sigma Chemical Co.) and 2.5 μM diadenosine pentaphosphate (to inhibit adenylate kinase). The chloroplasts were illuminated for 6 min at 80 W/m^2 (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 μl treated chloroplasts were mixed with 100 μl 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 μM ADP, 0.5 mM KH_2PO_4, 2.5 μM 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% trichloracetic acid, usually 10 s after addition of the basic medium. ATP synthesis was assayed by either luciferase or ^{32}P methods. For the luciferase technique, the solution was neutralized with 0.5 ml 0.6 M KOH, then 100 μl was withdrawn and directly assayed for ATP with the aid of LKB monitoring reagent and luminometer. In the ^{32}P assay, the P_i content of the base stage was reduced to 120 μM, and 0.5 μCi $^{32}P_i$ (Amersham International) was included per assay. Unreacted $^{32}P_i$ 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 $^{32}P_i$ 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 ΔpH was limiting. In contrast, for large ΔpH values

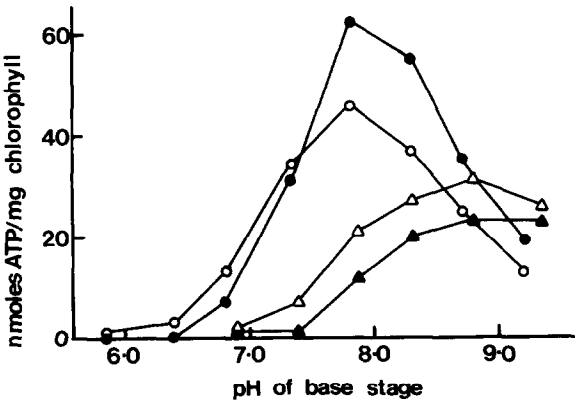

Fig.1. Comparison of the yield of ATP observed after an acid/base transition of thiol-modulated (○,△) and demodulated (●,▲) chloroplasts. The pH of the acid stage was either 4.1 (○,●) or 5.3 (△,▲). ATP was assayed by luciferase technique.

(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 ΔpH 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 ΔpH 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 ΔpH required for acid/base-induced synthesis of ATP.

The latter result is shown in more detail in fig.2, where the observed threshold ΔpH 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 ΔpH for ATP synthesis depended on the pH of the acid stage, and

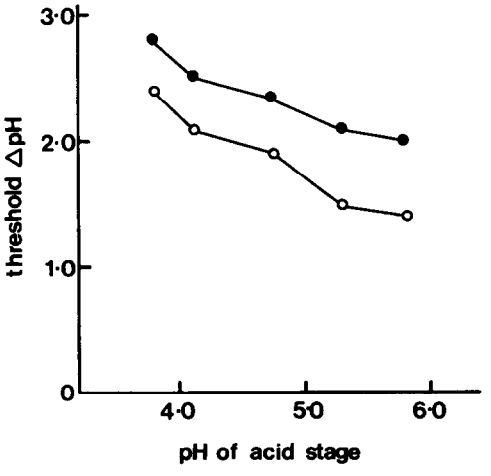

Fig.2. Comparison of the threshold ΔpH (at which ATP synthesis is first observed) of thiol-modulated (○) and demodulated (●) chloroplasts. Other details as in fig.1.

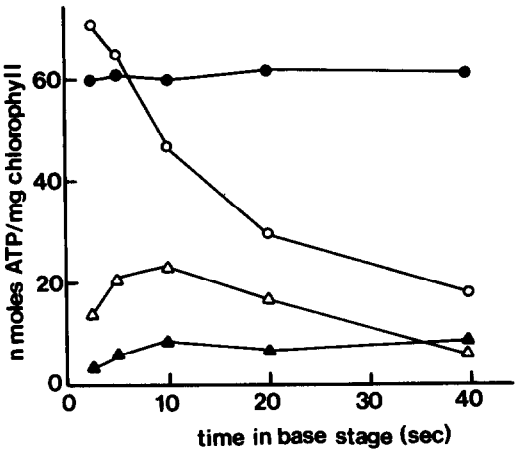

Fig.3. Effect of time in the base on the observed yield of ATP following an acid/base transition of 4.3/8.0 (○,●) or 5.3/8.0 (△,▲). Chloroplasts were thiol-modulated (○,△) or demodulated (●,▲). ATP was assayed by the luciferase technique.

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 ΔpH is limiting. We considered whether this might also be true when ΔpH is not limiting (i.e., large ΔpH 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 ΔpH). 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

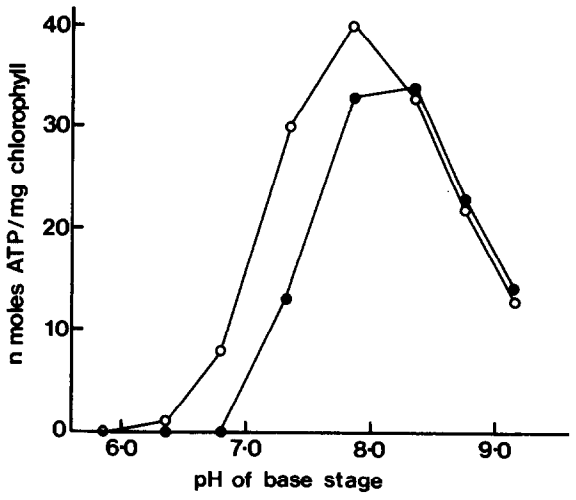

Fig.4. Comparison of the yield of ATP observed after an acid/base transition of thiol-modulated (○) and demodulated (●) chloroplasts. The pH of the acid stage was 4.15. The base stage additionally contained 4 units of hexokinase (type F-300, Sigma Chemical Co.) and 12 mM glucose. ATP was estimated by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

synthesis at relatively small acid/base transitions (low ΔpH), but the stimulation diminished to zero as ΔpH was increased. Furthermore, thiol-modulation lowered the observed threshold ΔpH 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}P) are lower than those assayed by the luciferase method (cf. fig.1). This can mostly be ascribed to the non-saturating concentration of P_i used in the media in the former case. ADP at ~5 μM and P_i at ~30 μM 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 CF_0–CF_1 stimulates the observed rate of ATP hydrolysis [13] and ATP ⇌ 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 ΔpH, 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 P_i. Therefore, in order to explain this behavior, it is necessary to determine what limits the yield of ATP synthesis at low ΔpH.

It has been suggested that phosphorylation in demodulated chloroplasts at low $\Delta\bar{\mu}H^+$ is limited by the fraction (α) of CF_0–CF_1 complexes that are active [5–7]. On this assumption, CF_0–CF_1 activity is kinetically controlled, and $\Delta\bar{\mu}H^+$ may not be in equilibrium with ΔG_P, the free energy of ATP hydrolysis. Therefore, thiol-modulation of CF_0–CF_1 may stimulate phosphorylation by causing α 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 α is initially small, i.e., at low $\Delta\bar{\mu}H^+$ (ΔpH) as observed here. One would also predict that photophosphorylation should also be stimulated when ΔpH 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 α 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 $\Delta\bar{\mu}H^+$ required to activate CF_0–CF_1 ($\Delta\bar{\mu}H^+_{act}$). Such a reduction in $\Delta\bar{\mu}H^+_{act}$ could occur by a change in the pH optimum at either the *P* or *N* poles of CF_1. At low ΔG_P, this may cause a $\Delta\bar{\mu}H^+_{act} > \Delta G_P$ whilst after thiol-modulation, a $\Delta\bar{\mu}H^+_{act} < \Delta G_P$. This situation would then explain the appearance of net ATP hydrolysis only after thiol modulation of CF_0–CF_1. However, it should be noted that the effect of thiols would tend to disappear as $\Delta\bar{\mu}H^+$ increases. Therefore, this mechanism cannot explain the lack of ATP hydrolysis or ATP ⇌ P_i exchange at high $\Delta\bar{\mu}H^+$ where α is close to 1.

The data of fig.1 and 2 show that the threshold ΔpH at which ATP synthesis is first observed is lower after chloroplasts have been thiol-modulated. The H^+/ATP stoichiometry may be calculated from this threshold value and the values obtained are 3.4 (demodulated chloroplasts, ΔpH = 2.0) and 4.6 (thiol-modulated chloroplasts, ΔpH = 1.5). The latter value is higher than the values of 2 [21] or 3 [7,23] obtained by other methods. The threshold ΔpH 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 ΔpH may be in error for two reasons.

(i) The threshold ΔpH 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 ΔpH) 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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