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THIOL MODULATION OF THE CHLOROPLAST PROTONMOTIVE ATPase AND ITS EFFECT ON PHOTOPHOSPHORYLATION

JOHN D. MILLS * and PETER MITCHELL

Glynn Research Institute, Glynn House, Bodmin, Cornwall, PL30 4AU (U.K.)

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Thiol modulation of the chloroplast protonmotive ATPase (CF_0-CF_1) by preillumination of broken chloroplasts in the presence of dithiothreitol (or preillumination of intact chloroplasts in the absence of added thiols) had the following effects on photophosphorylation. (1) When assayed at pH 8 and saturating light, the initial rate of photophosphorylation was increased by 10–40%. There was an accompanying increase in the rate of coupled electron transport with no significant change in the overall $P/2e$ ratio. (2) On lowering the pH of the assay medium to pH 7, the stimulatory effect of thiol modulation on photophosphorylation and coupled electron flow was enhanced. At pH 7, there was also a small increase in $P/2e$ ratio. (3) Addition of a non-saturating amount of uncoupler to the assay medium enhanced the stimulatory effect of thiol modulation on photophosphorylation. In the presence of 1 mM NH_4Cl, there was only a small increase in coupled electron flow and a correspondingly larger increase in $P/2e$ ratio. (4) Lowering the light intensity, or inhibiting electron transport, diminished the stimulatory effect of thiol modulation on photophosphorylation, coupled electron transport and $P/2e$ ratio. (5) Under all the above conditions, the ΔpH maintained across the thylakoid membrane was lower after thiol modulation, even when photophosphorylation markedly increased in rate. (6) Thiol modulation of CF_0-CF_1 increased the observed Michaelis constant for ADP (K_m(ADP)) and the apparent maximum rate (V_{app}) of photophosphorylation by the same factor, so that ratio V_{app}/K_m was not altered. V_{app}/K_m was also unaffected by changing the medium pH, but was significantly decreased upon addition of uncouplers to the medium. These results indicate that the observed rate of ATP synthesis catalysed by thiol demodulated chloroplasts is limited kinetically by the fraction (α) of enzyme molecules that are active during photophosphorylation. A model based on a dual pH optimum requirement for activation of CF_0-CF_1 is presented to explain the dependence of α on ΔpH. Thiol modulation of CF_0-CF_1 is proposed to stimulate photophosphorylation by causing the enzyme to become active over a lower range of ΔpH, thereby reducing the kinetic limitation on ATP synthesis imposed by the activation process.

* Permanent address: Department of Biological Sciences, University of Keele, Keele, Staffordshire ST5 5BG, U.K., to which correspondence should be addressed.

Abbreviations: Chl, chlorophyll; CF_0-CF_1, reversible protonmotive ATPase; $P/2e$, number of moles of ATP synthesized per 2e transported from H_2O to methyl viologen; pH_N and pH_P, pH values of the external medium and intrathylakoid space, respectively; $\Delta\bar{\mu}H^+$, ΔpH, difference in the electrochemical potential of protons, or in the pH, between the intrathylakoid and aqueous medium; ΔG_p, free energy change of ATP synthesis or hydrolysis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Introduction

The mechanism by which the reversible protonmotive ATPase of chloroplasts (CF_0-CF_1) catalyses synthesis and hydrolysis of ATP is still the subject of much study. Recent work has revealed that the enzymic activity of CF_0-CF_1 is highly regulated. Studies using envelope-free chloroplasts (thylakoids) have shown that CF_0-CF_1 must undergo an activation prior to the observation of any catalytic activity [1–7]. This activation normally occurs when an electrochemical potential difference of protons ($\Delta\bar{\mu}H^+$) develops across the thylakoids, and the activation process is associated with conformational changes in the structure of CF_0-CF_1 [8] and with the release of tightly bound adenine nucleotides [3–6,9–12]. Mitchell has suggested [13] that the activation process simply reflected a dual pH optimum requirement of CF_0-CF_1, whereby the pH optimum of the stromal, or *N*, side of CF_1 was around pH_N 8 and the optimum of the CF_0, or *P*, side of CF_1 (at the bottom of a proton well through CF_0) was around pH_P 5. The differential pH conditions would thus normally exist only when a $\Delta\bar{\mu}H^+$ was present across CF_0-CF_1, and deactivation of CF_1 would occur when the pH at either the *P*- or *N*-poles deviated from the optimum.

In addition to, and superimposed upon, this pH activation of CF_0-CF_1, enzymic activity is also reversibly modulated by the oxido-reduction state of certain thiol/disulphide groups within the enzyme structure [14]. In the presence of reduced dithiols such as dithiothreitol [2,4,11] or thioredoxin [15], the pH-activated state of CF_0-CF_1 is modified. The resulting thiol-modulated form of CF_0-CF_1 also undergoes pH activation or deactivation [2,4,7]. The thiol modulation process can be reversed by adding mild oxidants such as ferricyanide or oxidised thioredoxin [7]. We have termed this latter process 'thiol demodulation' of CF_0-CF_1.

The thiol-modulated form of CF_0-CF_1 (when pH activated) is observed to catalyse ATP synthesis or hydrolysis, depending on the conditions, but the demodulated form cannot be observed to catalyse net ATP hydrolysis (in our hands) under any condition. The failure to observe net ATP hydrolysis (or $P_i \rightleftarrows$ ATP exchange) using thiol-demodulated chloroplasts has yet to find a completely satisfactory explanation. Graber et al. [3] observed that the initial rate of ATP synthesis at low ΔG_p was kinetically determined by the fraction, α, of CF_0-CF_1 complexes that were pH activated. As discussed by Schlodder et al. [6], this implies that CF_0-CF_1 becomes active under these conditions at a value of $\Delta\bar{\mu}H^+$ that is higher than that required thermodynamically for net ATP synthesis, thus precluding the possibility of observing net ATP hydrolysis. This explanation is valid when ΔG_p is small, but requires elaboration to explain the apparent lack of net ATP hydrolysis when ΔG_p is poised at a high level.

If, however, the lack of observed ATP hydrolysis can be caused by the pH-activation requirements of CF_0-CF_1, then one of the effects of thiol modulation might be to lower the effective $\Delta\bar{\mu}H^+$ at which CF_0-CF_1 becomes active. This idea originated with the observation that thiol modulation of CF_0-CF_1 lowered the apparent 'threshold' ΔpH required to observe ATP synthesis after an acid/base jump [16]. Those results indicated that in demodulated chloroplasts, acid/base induced phosphorylation was limited (at low ΔpH) by α, the fraction of activated CF_0-CF_1 complexes, and that thiol modulation of CF_0-CF_1 increased α under those conditions.

Our previous results suggest that thiol modulation of CF_0-CF_1 should also stimulate photophosphorylation, when the rate is limited by a low $\Delta\bar{\mu}H^+$. To date, however, no reports of such a thiol-dependent stimulation of photophosphorylation have appeared. Harris and Crofts [4] showed that thiol modulation of CF_0-CF_1 decreased the lag period of flash-dependent photophosphorylation, but their steady-state rates were unaffected.

In this report, we outline several conditions under which the predicted stimulation of steady-state photophosphorylation may be observed. The results are interpreted using the hypotheses outlined above and provide a basis for understanding the in vivo regulation of CF_0-CF_1 by thiols.

Materials and Methods

Washed, broken chloroplasts were prepared from intact chloroplasts from *Pisum sativum* (var. Meteor) as described [16]. Thiol modulation was

carried out by suspending chloroplasts (at a concentration of approx. 250 μg chlorophyll/ml) in a medium comprising 0.4 M sorbitol/15 mM Tricine/5 mM $MgCl_2$/100 μM methyl viologen/10 mM dithiothreitol/2.5 μM diadenosine pentaphosphate/catalase (750 units/ml) (pH 8.0) and illuminating at 40–70 W/m^2 (blue light) for 6 min. Demodulated chloroplasts were suspended in the same medium but kept in the dark. During the course of this investigation we discovered that care must be taken to prevent an irreversible photo-inhibition of chloroplast activity during the thiol-modulation procedure. The light intensity must be above 30 W/m^2 to achieve a reasonable rate of thiol modulation, but above 100 W/m^2 photo-inhibition occurs. A temperature around 20°C is optimal. Lower temperatures cause slow thiol modulation and higher temperatures cause photo-inhibition. We also observed that high concentrations of dithiothreitol cause light-dependent irreversible inhibition of the chloroplasts. Although we did not pursue the cause of the photo-inhibition, a parallel decrease in electron transport and photophosphorylation activity (with less change in *P*/2e ratio) suggested that the inhibition was due to an inhibition of electron transport (probably PS II units).

Assays were carried out by diluting 100 μl of pretreated chloroplasts with 0.9 ml of a medium comprising 50 mM buffer/1 mM KH_2PO_4/0.5 mM ADP/100 μM methyl viologen/5 mM $MgCl_2$/4 μM diadenosine pentaphosphate. Buffers used were Mes (pH 6.5), Hepes (pH 7–7.5) and glycylglycine (pH 8–9.5). Illumination was usually 500 W/m^2 (Corning 3-97 glass filter) and the reaction was stopped after 30 s by adding 0.5 ml 10% trichloracetic acid. ATP was measured by the luciferin/luciferase technique as described [16] or by a ^{32}P method. In the latter case, the assay medium included 0.5 μCi $^{32}P_i$ (Amersham International, Amersham, Bucks., U.K.), 4 units hexokinase and 15 mM glucose. Unreacted $^{32}P_i$ was removed by precipitation with ammonium molybdate/triethylamine [5]. After removing the precipitate by centrifugation, 0.5 mM unlabelled P_i was added, after which a second precipitation spontaneously occurred. After recentrifugation, aliquots were counted via Cerenkov radiation in an LKB Rackbeta scintillation counter. This double-precipitation procedure removed better than 99.9% of the $^{32}P_i$ under the described conditions (sorbitol less than 50 mM) giving controls barely above background levels (50–80 cpm in Cornwall).

P/2e ratios were measured as above except that catalase was reduced to 30 U/ml in the preillumination stage and the assay medium (2.0 ml) contained 0.5 mM azide (to inhibit catalase carried over) and 5 mM ascorbate. Assays were carried out by an O_2 electrode (Rank Bros, Cambridge, U.K.) and O_2 consumption was estimated after 2 min illumination. An O_2 uptake/2e ratio of 1.5 was assumed [17]. Aliquots were taken from the same assay mixtures and ATP formed was measured by the lucerifin/luciferase technique. *P*/2e was estimated directly by dividing the ATP synthesis rate by the electron transport rate measured.

9-Aminoacridine fluorescence was measured as described [7]. In this case, the volume of assay medium was increased to 2 ml and contained 24 μM 9-aminoacridine. Fluorescence quenching was measured after 2 min illumination (Corning 2-72 filter) at 185 W/m^2 [7]. The value of ΔpH was calculated according to Ref. 18 and corrected for binding by multiplying by 0.875 [19].

Results

Effect of thiol modulation of CF_0-CF_1 on photophosphorylation

We predicted that, under conditions where α (the fraction of active CF_0-CF_1 complexes) is small, thiol modulation of CF_0-CF_1 would stimulate photophosphorylation by increasing α. We have therefore studied photophosphorylation under several conditions where $\Delta\bar{\mu}H^+$ was made suboptimal and α was correspondingly small. Fig. 1a shows the initial rate of photophosphorylation observed over a range of pH in the suspending medium (pH_N). Chloroplasts were either thiol modulated or demodulated as described in Materials and Methods, but otherwise treated identically so that a proper comparison of the effects of thiol modulation could be made.

Fig. 1a shows that, in thiol-demodulated chloroplasts, the observed optimum for photophosphorylation (in the presence of hexokinase and glucose) was at pH_N 8.5. Thiol modulation had the effect of

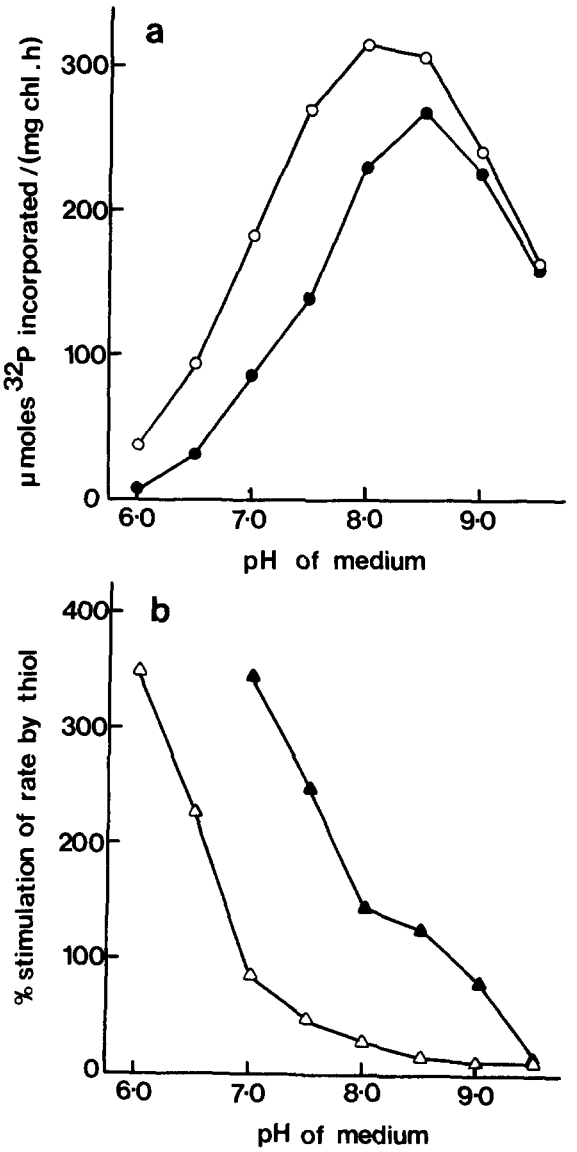

Fig. 1. (a). Effect of thiol modulation of CF_0-CF_1 on the observed initial rate of photophosphorylation at varying pH_N. Photophosphorylation was measured by the ^{32}P technique (see Materials and Methods for other details). Each data point represents the mean of two experiments; ○, thiol modulated chloroplasts; ●, demodulated chloroplasts. (b) Percentage stimulation of rate induced by thiol modulation in the absence (△) or presence (▲) of $1 \cdot 10^{-7}$ M nigericin. In the presence of nigericin, the assay media also contained 50 mM KCl.

shifting the optimum to pH_N 8 and broadening the profile on the acid side of the pH_N optimum. As shown in Fig. 1b, the effect of thiol modulation was therefore to stimulate the observed rate of photophosphorylation and this stimulation became relatively greater as pH_N was lowered. At pH_N 8 and saturating light, thiol modulation of CF_0-CF_1 stimulated photophosphorylation by 10–40%, the magnitude of the stimulation varying between different chloroplast preparations and depending somewhat on the age of the chloroplasts. Below pH_N 7, the relative stimulation was greater than 100%. It should be noted that lowering of the medium pH is thought to decrease the magnitude of $\Delta\bar{\mu}H^+$ under saturating light [20] (see also Table III).

Fig. 2 shows the effect of adding uncoupler, or lowering light intensity, on the initial rate of photophosphorylation in thiol modulated and demodulated chloroplasts. Addition of a progressively higher concentration of NH_4Cl to chloroplasts at pH_N 8 (saturating light) inhibited phosphorylation as expected (Fig. 2a). However, as the degree of uncoupling increased, the relative stimulation by thiol modulation was enhanced (Fig. 2c). Nigericin and ethanol exhibited similar effects. The enhanced stimulatory effect of thiol modulation under partially uncoupled conditions was observed over the whole range of pH_N studied (see Fig. 1b).

In contrast to the effects of the uncouplers, lowering the light intensity, which also decreased the rate of photophosphorylation (Fig. 2b), caused the stimulatory effect of thiol modulation to diminish (Fig. 2(c)). Partial inhibition of non-cyclic electron transport by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) had a similar effect to lowering the light intensity (results not shown).

In summary, thiol modulation of CF_0-CF_1 has a small stimulatory effect on photophosphorylation at pH_N 8 and saturating light. This stimulation is enhanced by lowering pH_N or by partial uncoupling, and is abolished by raising pH_N or by inhibiting electron transport.

As shown in Table I, a similar pattern of effects was observed when thiol modulation was carried out by illuminating intact chloroplasts in the absence of dithiothreitol and methyl viologen [7,15]. Overall rates were higher using freshly lysed chloroplasts compared with twice-washed, broken chloroplasts and quantitatively, the effect of thiol modulation on photophosphorylation was smaller. The latter observation is presumably caused by

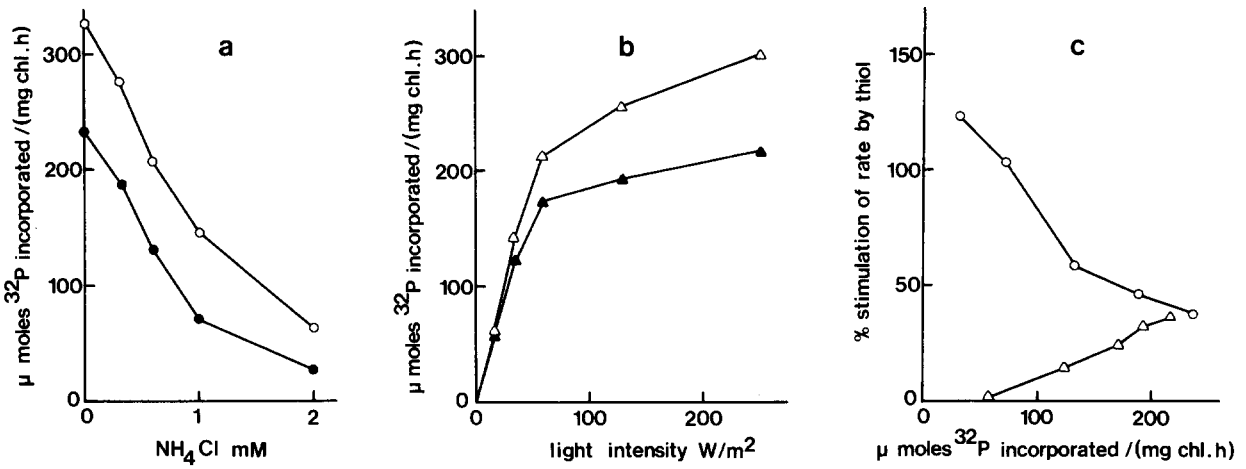

Fig. 2. Effect of thiol modulation of CF_0-CF_1 on the initial rate of photophosphorylation observed at pH_N 8; (a) varying NH_4Cl concentration in the assay medium and (b) varying light intensity (using neutral-density filters). Photophosphorylation was measured by the ^{32}P method; ○ and △, thiol-modulated chloroplasts; ● and ▲, demodulated chloroplasts; (c) percentage stimulation of rate induced by thiol modulation as a function of the observed rate in demodulated chloroplasts. Data calculated from a (○) or b (△).

incomplete thiol modulation of CF_0-CF_1, since only CF_0-CF_1 in those chloroplasts that were initially intact (60–70%) undergo thiol modulation in these conditions. Nevertheless, the results show that the effects of thiol modulation are the same whether CF_0-CF_1 is modulated by dithiothreitol in broken chloroplasts, or by the physiological thioredoxin system in intact organelles.

TABLE I

THIOL MODULATION OF CF_0-CF_1 VIA THE ENDOGENOUS THIOREDOXIN IN INTACT CHLOROPLASTS

Thiol modulation was carried out by illuminating intact chloroplasts for 2 min in 0.4 M sorbitol/15 mM Tricine (pH 8.0) containing 1500 U catalase/ml. Demodulated chloroplasts were not illuminated. Lysis of the organelles occurred on transfer into the assay medium, and the initial rate of photophosphorylation was measured by the luciferin/luciferase technique. Low light intensity was 30 W/m^2 obtained with neutral-density filters.

Assay conditions	Observed rate of photophosphorylation (μmol ATP/mg Chl per h)		
	Thiol modulated	Demodulated	Percentage stimulation
pH_N 8	486	439	11
pH_N 7	273	190	44
pH_N 8 +1 mM NH_4Cl	305	239	28
pH_N 8, low light	188	192	−2

Effect of thiol modulation on the rate of electron transport and P/2e

We further studied the effects of thiol modulation on photophosphorylation by measuring the rates of coupled electron flow and overall $P/2e$ ratio, and the results are shown in Table II. At pH_N 8 and saturating light, the increased rate of photophosphorylation upon thiol modulation of CF_0-CF_1 was accompanied by an increase in the rate of coupled electron flow. There was an increase in observed $P/2e$ ratio also, but this increase is not statistically significant. At pH_N 7, where the increase in photophosphorylation was relatively larger, the stimulation was again accompanied by a large increase in the rate of coupled electron flow, but also by a small but significant increase in the $P/2e$ ratio. We estimate that approx. 90% of the thiol-induced increase in photophosphorylation at pH_N 7 was contributed by increased electron transport, and 10% by an increased $P/2e$ ratio. At pH_N 8 and in the presence of 1 mM NH_4Cl, the large thiol-induced increase in photophosphorylation was accompanied by a small increase in the rate of electron flow, which was already near to the maximum uncoupled rate. However, there was a correspondingly larger increase in the $P/2e$ ratio under these conditions. In contrast to the results obtained at pH_N 7, most (75%) of the thiol-induced increase in

TABLE II

EFFECT OF THIOL MODULATION OF CF_0-CF_1 ON THE INITIAL RATE OF PHOTOPHOSPHORYLATION, ELECTRON TRANSPORT AND OVERALL $P/2e$ RATIO

Photophosphorylation was measured by the luciferin/luciferase method. Each value is the mean of 16 experiments. Error estimates represent the standard deviation between experiments. Unstarred values indicate that thiol modulation of CF_0-CF_1 had no significant effect. Rates of photophosphorylation are in μmol ATP/mg Chl per h and of electron transport are in μmol O_2 consumed/mg Chl per h. Low light intensity was 17.5 W/m^2.

Assay conditions	Thiol modulated			Demodulated		
	Photophos	Electron transport	$P/2e$	Photophos	Electron transport	$P/2e$
pH_N 8	286 ± 42 [b]	274 ± 55 [a]	1.58 ± 0.13	236 ± 38 [b]	236 ± 40 [a]	151 ± 0.16
pH_N 7	160 ± 36 [b]	155 ± 28 [b]	1.55 ± 0.16 [b]	88 ± 20 [b]	93 ± 16 [b]	1.41 ± 0.13 [b]
pH_N 8 + 1 mM NH_4Cl	168 ± 31 [b]	317 ± 51	0.80 ± 0.10 [a]	111 ± 20 [b]	290 ± 39	0.58 ± 0.09 [a]
pH_N 8, low light	66 ± 11	77 ± 17	1.32 ± 0.25	63 ± 13	78 ± 14	1.24 ± 0.25

[a] The means are significantly different at the 5% level of confidence (Student's t-test).
[b] The means are significantly different at the 1% level of confidence (Student's t-test).

photophosphorylation in the presence of uncoupler was contributed by the increase in $P/2e$ ratio. At low light intensity and pH_N 8, thiol modulation had no significant effect on photophosphorylation, coupled electron flow or $P/2e$ ratio.

It is important to note that the effects of thiol modulation on electron transport only occurred under phosphorylating conditions. Fig. 3 shows that in the absence of ATP synthesis, thiol modulation of the chloroplasts had comparatively little effect on coupled or uncoupled electron transport.

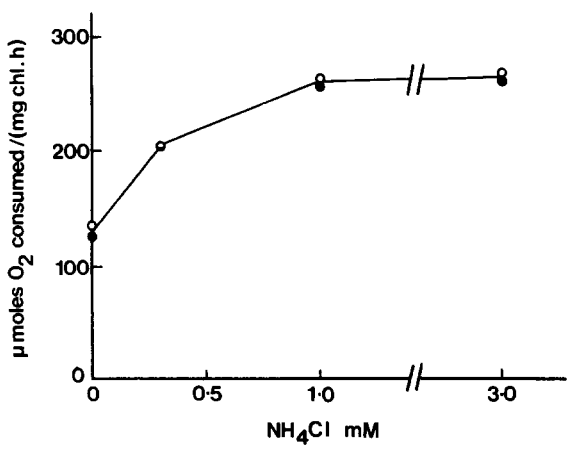

Fig. 3. Effect of thiol modulation of CF_0-CF_1 on the rate of electron transport observed at pH_N 8 under non-phosphorylating conditions (ADP omitted from the assay media); ○, thiol-modulated; ●, demodulated chloroplasts.

The results suggest that the effects of thiols are due to modulation of CF_0-CF_1, and that there is no direct interaction of dithiothreitol with components of the electron transport chain. A small increase in coupled electron flow induced by thiols under non-phosphorylating conditions (Fig. 3) may indicate a slight uncoupling effect of the thiol modulation procedure.

Effect of thiol modulation on steady-state ΔpH during photophosphorylation

The results presented so far support the prediction that lowering $\Delta\bar{\mu}H^+$ (by partial uncoupling or decreasing medium pH) causes the thiol-induced stimulation to be more pronounced. The exception to this prediction has been the effect of lowering light intensity (or inhibiting electron transport) which abolished the thiol effects. This initially surprising result may be explained if, under the latter conditions, photophosphorylation were primarily limited by the slow rate of electron flow, and if ΔpH were lower after thiol modulation of the chloroplasts (see Discussion). Table III shows that this is in fact the case. In the absence of ADP, ΔpH (calculated from steady-state quenching of 9-aminoacridine [18]) was observed to be slightly smaller after thiol modulation of the chloroplasts. Upon adding ADP to the medium, the ΔpH fell as expected, since photophosphorylation increases the rate of efflux of protons from the thylakoids [21].

TABLE III

EFFECT OF THIOL MODULATION OF CF_0-CF_1 ON STEADY-STATE 9-AMINOACRIDINE FLUORESCENCE QUENCHING AND ΔpH

In these experiments the final medium (2 ml) comprised 120 μM ADP, 3 U hexokinase per ml, 12 mM glucose and chloroplasts at 20 μg chlorophyll per ml (phosphorylating conditions). ADP was omitted under non-phosphorylating conditions. Each value is the mean of two experiments. Low light intensity was 16.5 W/m^2.

Assay conditions	Percentage fluorescence quenched		ΔpH (calculated)	
	Thiol	Demodulated	Thiol	Demodulated
Non-phosphorylating				
pH_N 8	66.5	68	3.50	3.52
pH_N 7	41.5	42	3.11	3.11
Phosphorylating				
pH_N 8	47	56	3.19	3.33
pH_N 7	34.5	40	2.99	3.08
pH_N 8 + 1 mM NH_4Cl	30	33	2.91	2.97
pH_N 8, low light	29	38	2.90	3.05

However, the steady-state ΔpH established in thiol-modulated chloroplasts under phosphorylating conditions was significantly lower than that observed with demodulated chloroplasts under low-intensity illumination. Table III also lists the ΔpH calculated from observed 9-aminoacridine fluorescence quenching under other conditions. During photophosphorylation, ΔpH was always observed to be lower after thiol modulation of the chloroplasts. It is interesting to note that the effect of thiol modulation on ΔpH was at its greatest when the observed stimulation of photophosphorylation was small or absent (pH_N 8 high or low light intensity). It is quite clear that the rate of photophosphorylation is not controlled thermodynamically by the magnitude of ΔpH, since, for example, at pH_N 7, thiol modulation of CF_0-CF_1 results in a higher rate of photophosphorylation but a smaller ΔpH. Experiments in the presence of valinomycin (which collapses any membrane potential) indicate that these effects are not due to changes in the membrane potential ($\Delta\psi$), which in any case, is probably small under these conditions.

Effect of thiol modulation of CF_0-CF_1 on apparent K_m and V_{app}

To complete this study, we observed the effect of thiol modulation of CF_0-CF_1 on the apparent K_m(ADP) and maximum rate (V_{app}) estimated from double-reciprocal plots of initial phosphorylation rate against ADP concentration. The results are summarised in Table IV. It can be seen that

TABLE IV

EFFECT OF THIOL MODULATION OF CF_0-CF_1 ON THE APPARENT K_m FOR ADP AND V_{app} FOR PHOTOPHOSPHORYLATION

ATP synthesis was measured by the ^{32}P method and ADP was varied from 3 to 40 μM. Linear-regression analysis of double-reciprocal plots (1/(rate) against 1/[ADP]) gave straight-line fits to the data with very high correlation coefficients (> 0.99), and K_m and V_{app} were estimated from the intercept at the appropriate axis. Error estimates represent the standard deviation between experiments. K_m is expressed in μM and V_{app} in μmol ATP/mg Chl per h.

Assay conditions	Number of experiments	Thiol-modulated			Demodulated		
		K_m	V_{app}	V_{app}/K_m	K_m	V_{app}	V_{app}/K_m
pH_N 8	5	15 ±4	339±70	22.6	11±4	261±67	23.7
pH_N 7	2	7.5±2	190±16	25.3	4±1	90±25	22.5
pH_N 8 + 1 mM NH_4Cl	4	18 ±5	139±50	7.7	10±2	82±21	8.2

thiol modulation of CF_0-CF_1 increased both V_{app} and K_m under the conditions listed. The results rule out the possibility of explaining the stimulatory effects of thiol modulation in terms of changes in the binding constant of ADP. The effects of thiols are due to an increase in apparent maximal rate.

The data of Table IV contain an interesting feature noted by others [22]. These workers found that the ratio V_{app}/K_m was constant when photophosphorylation was varied by changing light intensity or inhibiting electron transport. We note that V_{app}/K_m is also unaffected by thiol modulation of CF_0-CF_1 (since both V_{app} and K_m change proportionately). However, partial uncoupling decreases V_{app}/K_m significantly and the new value is again unaffected by thiol modulation. The results are related to those of Vinkler [23], who observed that in demodulated chloroplasts inhibition of electron transport decreased K_m, whilst uncouplers increased K_m. Although we do not observe a consistent increase in K_m in the presence of uncoupler, the trend is readily apparent in our data, and becomes clear when V_{app}/K_m is considered.

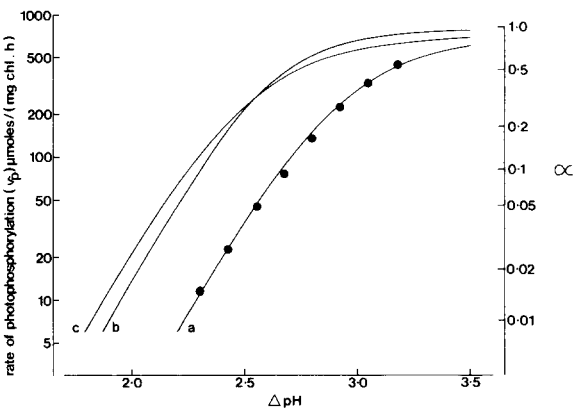

Fig. 4. Theoretical dependence of the rate of ATP synthesis (v_p) (or of α, the fraction of active CF_0-CF_1 molecules) on ΔpH. The value of α was calculated from Eqn. 5 and v_p then obtained from Eqn. 1 (see Appendix for details). Curve a represents thiol demodulated CF_0-CF_1 and has been fitted to data from [26] depicted by the points assuming that the 9-aminoacridine fluorescence technique used [26] overestimates ΔpH by 0.25 units. Values of the constants were $K_P^0 = 1 \cdot 10^{-8}$ M; $K_N^0 = 1 \cdot 10^{-11}$ M; $K_d = 0.1$. Curves b and c represent thiol-modulated CF_0-CF_1 and were generated by b reducing K_d to 0.012 and c decreasing K_P^0 to $6.025 \cdot 10^{-9}$ M and increasing K_N^0 to $1.585 \cdot 10^{-11}$ M.

Discussion

Early studies reported a decrease [24] or no change [4] in the steady-state rate of photophosphorylation upon thiol modulation of CF_0-CF_1. As outlined in Materials and Methods, care must be taken during the thiol modulation procedure (even when using dithiothreitol) to prevent inhibition of chloroplast activity from obscuring subsequent observations. We show here that, after careful thiol modulation of CF_0-CF_1, the rate of photophosphorylation is stimulated, especially at suboptimal pH_N or partial uncoupling in the assay media. Even at pH_N 8 and saturating light, a small increase in the rate of photophosphorylation can be consistently observed.

It has been suggested that, when $\Delta\bar{\mu}H^+$ is low, the observed catalytic activity of CF_0-CF_1 is determined kinetically by the fraction, α, of CF_0-CF_1 complexes that are in the active state [6]. At saturating ADP and P_i, the initial rate of ATP synthesis (v_p) is therefore given by:

$$v_p = \alpha V_p \qquad (1)$$

Where V_p is the true maximum rate governed by the catalytic turnover time of CF_0-CF_1. The observed initial rate, v_p, and the apparent maximum initial rate, V_{app}, (obtained from double-reciprocal plots at non-saturating substrate levels) will depend on the value of α at saturating light intensity. The observation that thiol modulation of CF_0-CF_1 increased v_p and V_{app} under otherwise constant conditions suggests that α had been increased by the thiol-modulation procedure. This effect can be understood if thiol modulation of CF_0-CF_1 causes the enzyme to become active at lower values of $\Delta\bar{\mu}H^+$.

We have derived a model based on a dual pH optimum requirement for activation of CF_0-CF_1 that describes the dependence of α on ΔpH (see Appendix for details). The model assumes that any small membrane potential component of $\Delta\bar{\mu}H^+$ is converted into ΔpH by a 'proton well' through CF_0. Curve a of Fig. 4 illustrates the dependence of α on ΔpH predicted by the model for thiol-demodulated CF_0-CF_1. The solid points represent data taken from Pick et al. [26] to which curve a

has been fitted. Thiol modulation of CF_0-CF_1 is proposed to enable the enzyme to become active over a lower range of ΔpH, and this is represented in Fig. 4 by curves b or c (see Appendix for a mathematical explanation of this effect). Assuming that the rate of ATP synthesis is kinetically limited by α even after thiol modulation of CF_0-CF_1 (which may only be true at very low values of ΔG_p), the model predicts the following effects of thiol modulating the enzyme: (i) the threshold ΔpH at which ATP synthesis is first observed should be lowered; (ii) the ΔpH maintained at equivalent rates of ATP synthesis should be lowered; (iii) the rate of ATP synthesis observed at equivalent ΔpH should be increased, but the relative stimulation of rate should be smaller at higher values of ΔpH (as α approaches the value of 1).

All these features have been observed in our recent study of acid/base-dependent phosphorylation [16] and the data presented in this paper are broadly in agreement with the above predictions. However, in order to understand fully the effects of thiol modulation of CF_0-CF_1 on photophosphorylation, and especially on coupled electron transport and $P/2e$ ratios, it is necessary to consider also the effects of ΔpH and pH_P on the rate of electron transport and passive H^+ efflux from the thylakoid. The assumption that the rate of catalysis by CF_0-CF_1 is determined kinetically by α implies that the ΔpH maintained across the thylakoid during steady-state photophosphorylation is greater than that thermodynamically required to support the observed rate of ATP synthesis. Such a 'supra optimal' ΔpH would generate a low pH_P that would be expected to inhibit the rate of electron transport (which seems to be controlled mainly by pH_P [20,27]), and this in turn would further limit the rate of photophosphorylation. The actual ΔpH maintained during photophosphorylation will therefore be a compromise between a high level, required for pH activation of CF_0-CF_1, and a low level, which allows the rate of electron transport to be maximal. Consider then the effect of thiol modulation of CF_0-CF_1 on such a system. Thiol modulation of CF_0-CF_1 would initially allow a given rate of photophosphorylation to proceed at a lower ΔpH, thereby reducing the constraint on electron transport. Consequently, both the rates of electron transport and photophosphorylation would rise, with little change in $P/2e$ ratio. This is observed at pH_N 8 and saturating light. At pH_N 7, the effects of thiol modulation should be even more pronounced since pH_P is correspondingly lower and the initial inhibition of electron transport more severe. We observed that lowering pH_N enhanced the stimulatory effect of thiol modulation on photophosphorylation and coupled electron flow.

The observations made under low-intensity illumination can also be explained in this way. At low light, the rate of electron flow is limited primarily by the quantum efficiency of PS I and II and less by the effects of pH_P. Under these conditions, therefore, thiol modulation of CF_0-CF_1 would cause a decrease in ΔpH, but electron flow would not increase correspondingly, and thus we observed the same rate of photophosphorylation but under a diminished ΔpH.

In the presence of uncoupler, the effects of thiol modulation are changed, with electron flow rates being largely unaffected, whereas $P/2e$ ratios increase after thiol modulation. Under these conditions, the observed $P/2e$ ratio depends on the relative flux of protons passing through CF_0-CF_1 (phosphorylating H^+ efflux) compared with that carried by the uncoupler (non-phosphorylating efflux). If thiols cause an increase in α, then there is a higher probability at a given $\Delta\bar{\mu}H^+$ that proton efflux will occur via the phosphorylating pathway relative to the non-phosphorylating efflux. Thus, we would expect that thiol modulation would (a) increase the rate of photophosphorylation, (b) increase $P/2e$ with less effect on the rate of coupled electron flow, and (c) only slightly decrease the magnitude of ΔpH maintained during photophosphorylation, as observed.

The qualitative explanation given above can be described mathematically, using a model based on the assumption that both the rates of photophosphorylation and electron transport are controlled kinetically. The simulated results agree rather well with observations reported in this paper and details will be published elsewhere [28].

Finally, these results are important when considering the in vivo regulation of CF_0-CF_1. We have shown that CF_0-CF_1 interacts with the thioredoxin system of chloroplasts and that CF_0-CF_1 is therefore probably in the thiol-modulated

form in the light, but in the demodulated form in the dark [7,15]. The ability of the enzyme to assume these two forms may be related to a need to prevent net hydrolysis of ATP during darkness. The demodulated enzyme is efficiently pH-deactivated when conditions favour net hydrolysis and this would prevent ATP hydrolysis in vivo in the hours of darkness. However, this form of the enzyme is at a kinetic disadvantage in the light, and thiol modulation makes the enzyme kinetically more competent in ATP synthesis, especially under conditions when $\Delta\bar{\mu}H^+$ may be limited. We would emphasize, therefore, that studies designed to understand the mechanism of ATP synthesis should be concentrated more on the thiol-modulated state of CF_0-CF_1, which is likely to be the state in which the enzyme functions during in vivo photophosphorylation.

Appendix. Dependence of α on ΔpH, pH_P and pH_N

We assume that the rate of photophosphorylation (v_p) is determined kinetically by α, the fraction of CF_0-CF_1 complexes that are active [6]:

$$v_p = \alpha V_p \tag{A-1}$$

where V_p is the maximum rate of ATP synthesis observed when $\alpha = 1$, and corresponds to the turnover number of an active CF_0-CF_1. Experimental studies in which v_p has been measured as a function of pH_P and pH_N suggest that v_p depends mainly on the difference between pH_P and pH_N, i.e., on ΔpH, and (within limits) less [16,26] or not at all [5,6] on the absolute values of pH_P or pH_N. (In the above and all further discussion it is assumed that any membrane potential ($\Delta\psi$) component of $\Delta\bar{\mu}H^+$ is converted by a 'proton well' in CF_0 into an equivalent ΔpH.) Assuming that the dependence of v_p on ΔpH reflects the dependence of α on ΔpH [6], this behaviour may be explained by a model based on a dual pH optimum requirement of CF_0-CF_1 [13].

The model requires the following assumptions.

(i) For activation to proceed, the enzyme requires that three groups on CF_1, protonatable only from the intrathylakoid space, or P side, be protonated, and three groups protonatable only from the stromal, or N, side be deprotonated.

(ii) Each P-side group is functionally linked with one N-side group such that the operational pK of either group is influenced by the protonation state of the corresponding group on the opposite pole of CF_1. Thus each N-side group may assume one of two operational pK values, or pK_N^0 or pK_N^1, depending on whether the corresponding P-side group is deprotonated or protonated, respectively. Similarly, the pK of a P-side group may be pK_P^0 or pK_P^1, depending on whether the corresponding N-side group of the pair is deprotonated or protonated.

(iii) Each pair of P- and N-side groups functions independently of other pairs.

(iv) Activation of CF_0-CF_1 occurs only when the enzyme attains the correct protonation state and is described by a simple equilibrium:

$$E_{\text{inactive}} \overset{K_d}{\rightleftharpoons} E_{\text{active}} \tag{A-2}$$

(v) ΔG_p is poised at a minimum and Mg^{2+}, ADP and P_i are saturating, so that the active CF_0-CF_1 molecules exist only as the enzyme-substrate complex and the tight nucleotide-binding sites contain ADP [22]. Under the above assumptions, α is given by Eqn. 3:

$$\begin{aligned}\frac{1}{\alpha} = 1 + K_d \Bigg[& \left(1 + \frac{H_N^+}{K_N^1}\right)^3 \\ & + 3\left(\frac{K_P^0}{H_P^+}\right)\left(1 + \frac{H_N^+}{K_N^1}\right)^2\left(1 + \frac{H_N^+}{K_N^0}\right) \\ & + 3\left(\frac{K_P^0}{H_P^+}\right)^2\left(1 + \frac{H_N}{K_N^1}\right)\left(1 + \frac{H_N^+}{K_N^0}\right)^2 \\ & \times \left(\frac{K_P^0}{H_P^+}\right)^3\left(1 + \frac{H_N^+}{K_N^0}\right)^3\Bigg]\end{aligned} \tag{A-3}$$

Two cases exist where Eqn. 3 reduces to a simpler form.

(1) If assumption (ii) does not apply, i.e., all protonation/deprotonation events are independent, then $K_P^0 = K_P^1$ and $K_N^0 = K_N^1$, and Eqn. 3 becomes:

$$\frac{1}{\alpha} = 1 + K_d\left(1 + \frac{H_N^+}{K_N}\right)^3\left(1 + \frac{K_P}{H_P^+}\right)^3 \qquad \text{(A-4)}$$

This case corresponds to the original concept of a dual pH optimum for enzyme activity [13], and α varies independently with H_P^+ and H_N^+. However, an apparent dependence on ΔpH will be generated if $K_P > K_N$ because most CF_0-CF_1 will be active only when $H_P^+ > K_P$ and $H_N^+ < K_N$, and these conditions will only be satisfied by a ΔpH across the membrane.

(2) If values for the various constants in Eqn. 3 are chosen so that only terms containing H_N^+/H_P^+ are significant, then Eqn. 3 reduces to:

$$\frac{1}{\alpha} = 1 + K_d\left(1 + \frac{K_P^0}{H_P^+}\frac{H_N^+}{K_N^0}\right)^3 \qquad \text{(A-5)}$$

The conditions for Eqn. 5 to be a valid approximation of Eqn. 3 are:

$$\left(\frac{H_N^+}{K_N^1}\right) \ll 1, \quad \left(\frac{K_P^0}{H_P^+}\right) \ll 1 \quad \text{and} \quad \left(\frac{H_N^+}{K_N^0}\right) \gg 1 \qquad \text{(A-6)}$$

In this case, protonation of the *P*-side group is fully concerted with deprotonation of the *N*-side group and, in consequence, α is purely a function of ΔpH. Curve c of Fig. 4 shows a plot of log α against ΔpH given by Eqn. 5. The range of ΔpH over which α becomes significant is determined both by K_d and K_P^0/K_N^0. Pick et al. [26] measured simultaneously the rate of photophosphorylation and ΔpH in thiol-demodulated chloroplasts. The points depicted in Fig. 4 represent the mean of the lines drawn by those authors through the experimental points of Figs. 2a and 4 in [26]. Curve a has been fitted to those data using Eqn. 1, assuming V_p equals 833 μmol ATP/mg Chl per h and with the additional assumption that the 9-aminoacridine fluorescence technique used overestimated ΔpH by 0.25 units. When α is small, curve a approaches linearity with a slope of 3. McCarty and colleagues [20,24] have repeatedly shown that experimental plots of log v_p against ΔpH are linear, with a slope of 3.

Curve a of Fig. 4 represents the dependence of α on ΔpH for thiol-demodulated chloroplasts. The effect of thiol modulation can be simulated by decreasing K_d (curve b) or K_P^0/K_N^0 (curve c), either of which shifts the dependence of α on ΔpH to a lower range. Decreasing K_d may also affect the kinetics of deactivation and might account for the increased stability of the activated state after thiol modulation of CF_0-CF_1 [2].

Schlodder et al. [6] have also presented a model describing the dependence of α on ΔpH. In their model, all protonation/deprotonation events as well as activation of CF_0-CF_1 occur in one completely concerted reaction. This is likely to be an unnecessary and unrealistic simplification and provides no clues to the mechanistic details of the process. The advantage of the model presented here is that it is based on defined protonation/deprotonation reactions incorporating only the minimum cooperativity required to generate a dependence of α on ΔpH. The model can be easily explained in terms of the known structure of CF_0-CF_1 if, for example, each $\alpha\beta$ pair of subunits within CF_1 carries one *P*- and one *N*-side group whose p*K* values are mutually influenced by conformational changes within the $\alpha\beta$ subunit pair.

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