*ADP KINASE AND ATPASE IN CHLOROPLASTS**

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Abstract.—Treatment of chloroplasts with trypsin activates a light-requiring ATPase whose properties are strikingly similar to those of the light-requiring ADP kinase of chloroplasts. The observations here presented suggest that there exists, in chloroplasts, a reducible enzyme which, in its reduced state, catalyzes the reversible reaction:

$$P_i^{-2} + ADP^{-3} + H^+ \rightleftharpoons ATP^{-4} + H_2O.$$

By reduction and protonation of the catalytic site of this enzyme, light-driven electron flow in the chloroplast drives the reaction to the right. Hydrolysis of ATP proceeds only when the enzyme is reduced and when the proton concentration within the chloroplast is kept at low levels, viz., in the absence of light, in the presence of uncoupling agents which decrease the concentration of internal H^+, or in the presence of electron acceptors which by oxidizing the internal electron acceptors also decrease the proton potential. Activation of the enzyme requires light; it remains active only in the presence of ATP. Hydrolysis of all the ATP results in inactivation of the ATPase. The membrane-bound protein CF_2 limits the reversibility of the reaction by excluding ATP and H_2O from the enzyme site. It also facilitates the ability of the chloroplasts to accumulate and to maintain high internal concentrations of such ions as ADP, P_i, PMS^+, and imidazole.

Previous studies[1] have shown that maintenance, within chloroplasts, of both a large redox potential and a high internal concentration of protons is required for photophosphorylation. This report offers evidence that suggests that catalysis of the reversible reaction,

$$H^+ + ADP^{-3} + P_i^{-2} \rightleftharpoons ATP^{-4} + H_2O$$

is the fundamental event in photophosphorylation and that the catalytic competence of this single responsible enzyme is affected by its own state of oxidation. This enzyme must be in the reduced state to be active, both as an ADP kinase or as an ATPase. The direction of this reaction is controlled by (1) H^+ pressure and (2) a protein factor, CF_2,[2, 3] which specifically inhibits the reaction to the left. The function of electron flow is to reduce and to protonate the catalytic site and thereby to drive the reaction to the right. Since the back reaction is completely inhibited by CF_2, ATPase activity can be observed only in the absence of CF_2. CF_2 of intact chloroplasts can be easily and specifically inactivated by brief exposure of the chloroplasts to trypsin.[2, 3] Maximal rates of phosphorylation can proceed in the absence of CF_2; however, much higher concentrations of ADP, and P_i, as well as of electron acceptor (PMS^+) and hypertonic conditions are required in the absence of CF_2. Thus CF_2 restricts not only ATP, but also H_2O,

ADP, and P_i from the catalytic site. Uncoupling agents, all of which lower the internal concentration of H^+,[1] shift the equilibrium of the reaction to the left. Oxidants, such as ferricyanide or benzoquinone, inactivate the eznyme, that is, no activity is observed in either direction.

It is concluded from the data in this report and from the studies of Racker[4] that phosphorylation is the result of the reduction and protonation of enzyme sites in chloroplasts which can serve as reversible ADP kinase/ATPases.

Methods.—Spinach chloroplasts were prepared as before[5] except that 0.2 mg of soybean trypsin inhibitor was added to the buffered sucrose in which the spinach was homogenized. Trypsin-treated chloroplasts were prepared by exposing previously illuminated chloroplasts, containing 0.45 mg of chlorophyll, under phosphorylating conditions to 2×-crystallized trypsin (30 μg) for 1 min. Maximal activation of the ATPase occurs within 1 to 2 min in the dark or within 20 sec in light. Soybean trypsin inhibitor (60 μg) was then added and the chloroplasts were isolated by centrifuging at 10,000 × g for 5 min.

Protonation and reduction of neutral red were measured as before.[6] Rates of oxidation and reduction of cytochrome b_{559}, cytochrome f_{552}, P_{700}, and methylene blue were measured spectrophotometrically, as before.[1] Rate and extent of H^+ uptake were assayed titrimetrically, as before.[5] Rates of photophosphorylation were assayed titrimetrically and enzymatically.[1] Rates of electron flow were assayed titrimetrically, using ferricyanide as acceptor.[5] Rates of hydrolysis of ATP were measured titrimetrically and the extent of hydrolysis of ATP was measured titrimetrically at periodic intervals by addition of hexokinase and glucose.[5]

Spectrophotometric measurements were performed at 15° in 3 ml of 0.4 M sucrose, containing 0.005 M $MgCl_2$, 0.01 M NaCl, 0.0008 M sodium phosphate, and either ADP or ATP or both.

CF_2 was prepared from freshly isolated chloroplasts, using the method of Livne *et al.*[2]

Results and Discussion.—Activation of ATPase by trypsin: Brief exposure of freshly prepared, previously illuminated chloroplasts (made in the presence of trypsin inhibitor) to trypsin in the dark at pH 8.0 results in the marked activation of ATPase (Fig. 1). Inactivation of the added trypsin by trypsin inhibitor yields a stable preparation of chloroplasts in which phosphorylation rate is al-

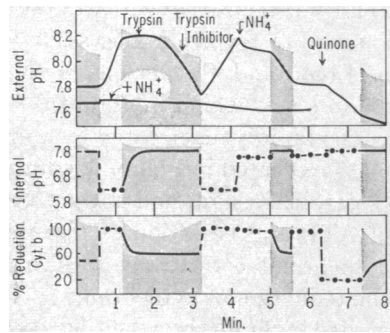

FIG. 1.—Activation of ATPase by trypsin. Chloroplasts (0.46 mg chlorophyll) were incubated at 15° in 20 ml of 0.4 M sucrose as in *Methods*. In addition, 2 μmoles of ADP and ATP and 3 × 10^{-6} M PMS^+ were present. Saturating white light was added where indicated, light areas indicate illumination. Trypsin (30 μg), 60 μg of soybean trypsin inhibitor, 3 × 10^{-3} M, NH_4Cl (pH 8.2), and 2.0 μmole benzoquinone were added where indicated. Aliquots (3 ml) of the above solution, containing 3 × 10^{-6} M neutral red, were used to measure changes with illumination in internal pH (see *Methods*). Other aliquots, not containing neutral red, were used to measure changes in reduction of cytochrome b_{559} cytochrome f, and P_{700} (see *Methods*). Changes in cytochrome b only were shown, but changes similar to those of cytochrome b were observed for cytochrome f and P_{700}. Changes in per cent reduction of cytochrome b_{559}, using ferricyanide and ascorbate, were calculated as before.[3] Since changes of internal pH or of per cent reduction of cytochrome could not be accurately assessed in the light, the points on the graph represent absorbance changes that were observed during the first second after termination of illumination, as before.[3] Changes in external pH were monitored titrimetrically (see *Methods*). In the presence of 3 × 10^{-3} M NH_4Cl, as indicated in the top figure, activation of ATPase by trypsin was not observed.

most maximal despite the fact that ATPase activity in the dark is very high. Light-induced electron flow (measured with ferricyanide), internal acidification (measured with neutral red), and reduction of cytochrome b_{559} were all unaffected by trypsin treatment.

Addition of uncoupling agents, such as NH_4^+, salicylanilides, TTFB,† desaspedin, nigerian plus KCl, or an inhibitor of electron transport, that is, CMU, before addition of trypsin, blocks the apparent activation of this ATPase by trypsin (Fig. 1). However, addition of the above uncoupling agents after activation of the ATPase and in the presence of ATP was without effect on the ATPase activity, assayed in the dark. In the light, in the presence of uncoupling agents, neither ATPase nor ADP kinase activity is observed. However, addition of benzoquinone in the presence of NH_4^+, which completely eliminates internal acidification and also partially oxidizes the internal electron carrier, inhibits ATPase in the dark. Under this condition, ATPase activity is observed in light only. Thus, it appears that ATPase functions only under conditions in which the internal H^+ activity is very low. Uncoupling agents, which diminish the internal redox potential in the light as indicated by the state of reduction of neutral red, methylene blue, or PMS attainable, also partially inhibited internal acidification[1] (Fig. 1); hence, apparently, they poise the enzyme in a state in which it is partially protonated and reduced, so that during illumination, no net synthesis or hydrolysis of ATP occurs. If the internal H^+ concentration is further decreased by addition of benzoquinone, which elevates the internal redox potential (Fig. 1), the enzyme then functions, in the light, as an ATPase.

Activation of the ATPase has been achieved only with trypsin; chymotrypsin, papain, leucine aminopeptidase, and pepsin were all ineffective. Nagarse did activate the ATPase briefly, but photophosphorylation, H^+ uptake, and electron transport were rapidly impaired also. Inactivation of trypsin activity by boiling, treatment with DFP or with trypsin inhibitor completely eradicated the ability of trypsin to activate the ATPase. Thus, it appears that the proteolytic activity of trypsin is necessary for its action on chloroplasts. Previous studies of Livne and Racker[2] have shown that CF_2, a protein factor obtained from chloroplasts which inhibits the thiol-requiring, isolated ATPase of chloroplasts, is also rapidly inactivated by trypsin.

As indicated in Figure 2, addition of benzoquinone to trypsin-activated chloroplasts, in the light and in the absence of uncoupling agents, also caused hydrolysis of ATP and cessation of phosphorylation associated with marked oxidation of cytochrome *b*. However, once the benzoquinone was reduced, as indicated by the reduction of cytochrome *b*, ATPase activity stopped and ATP synthesis proceeded. Similarly, when benzoquinone was added in the dark, phosphorylation did not commence until reduction of the benzoquinone and cytochrome *b* was accomplished.

Under hypotonic conditions, activation of ATPase by trypsin resulted in preparations which were unable to phosphorylate (Fig. 3). However, ATPase was active in the dark. Addition of uncoupling agents under hypotonic conditions inhibited the ATPase in the dark, and under these conditions, ATPase activity was observed only in light. Addition of benzoquinone again inactivated this

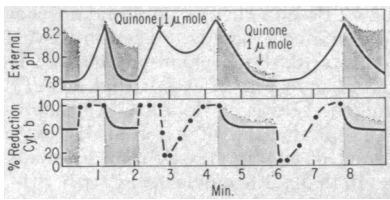

FIG. 2.—Effect of benzoquinone on trypsin-treated chloroplasts. Trypsin-treated chloroplast (prepared as in *Methods*) were illuminated as indicated in the presence of 3.0 μmoles ADP and 3×10^{-6} *M* PMS^+ as in Fig. 1. No ATP was added. Benzoquinone (1 μmole) was added when indicated. Rate of ATP synthesis and ATP hydrolysis and changes in per cent reduction of cytochrome b_{559} were monitored as in Fig. 1.

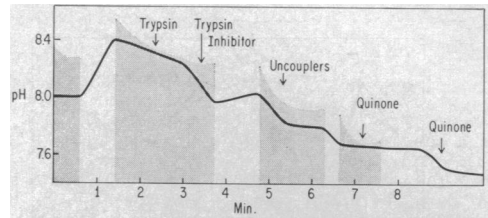

FIG. 3.—Effect of hypotonicity on trypsin-treated chloroplasts. Chloroplasts (0.53 mg chlorophyll) were illuminated as in Fig. 1, except that no sucrose was present. 30 μg trypsin and 60 μg trypsin inhibitor were added when indicated. Uncoupling agents (3×10^{-3} *M* NH_4Cl, 5×10^{-3} *M* salicylanilide, or 5×10^{-8} *M* nigericin plus 10^{-2} *M* KCl) were added where indicated. Since essentially identical traces were observed with all uncoupling agents, only one line is shown in the figure. Benzoquinone (1.0 μmole) was added when indicated. Changes in external pH were recorded. No changes in external pH occurred in the absence of added ATP, either in the presence or absence of uncoupling agents or benzoquinone.

light-requiring ATPase. Thus, under hypotonic conditions and with agents which partially diminish the internal redox potential, light is obligatorily required for ATPase activity and agents which minimize the internal redox potential completely inhibit the light-requiring ATPase. Thus, it appears that CF_2 may be required not only to restrict ATP from this catalytic site, but also to restrict H_2O and OH^- as well. Since the rates of phosphorylation in these trypsin-treated chloroplasts are nearly maximal with saturating light, it appears that CF_2 is not an obligatory "coupling agent." Rather, it serves to inhibit the hydrolysis of ATP in the absence of illumination. Although maximal rates of phosphorylation can be observed in trypsin-treated chloroplasts, higher concentrations of ADP, P_i, and PMS^+ are required. The concentration of ADP, P_i, and PMS^+ required for 50 per cent activity in intact chloroplasts under conditions illustrated in Figure 1 are 2.5×10^{-5} *M*, 1.0×10^{-4} *M*, and 1.5×10^{-6} *M*, respectively. In trypsin-treated chloroplasts the concentrations required are 6.5×10^{-5} *M* ADP, 7×10^{-4} *M* P_i, and 5×10^{-6} *M* PMS.

As indicated in Figure 4, prior illumination of trypsin-treated chloroplasts is required to activate the ATPase. ATP, added to trypsin-activated chloroplasts, is hydrolyzed only in the postillumination period; when most of the ATP has been hydrolyzed, ATPase activity declines and reactivation requires another round of illumination. Such reactivation can occur under conditions which block ATP synthesis (presence of arsenate) or which block H^+ uptake (uncoupling agents). However, in the presence of uncoupling agents, which limit the degree of reduction obtainable by illumination, only partial reactivation of the ATPase is observed (Fig. 4).

In the presence of sufficient CMU to inhibit completely reduction of ferricyanide, partial activation of ATPase by light was observed. Light at 680 and

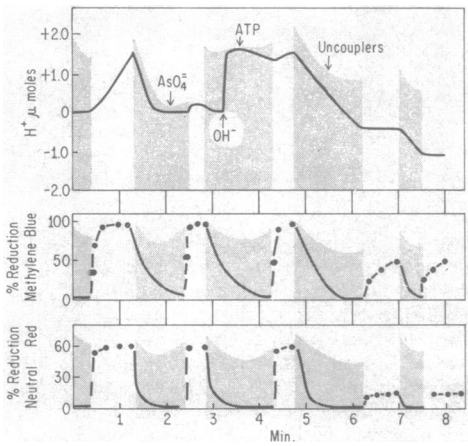

FIG. 4.—Requirement of light for activation of ATPase in the presence and absence of uncoupling agents. Trypsin-treated chloroplasts (0.51 mg chlorophyll) were illuminated as in Fig. 2, except that no ATP was added. Initial pH, 7.91. Consumption and production of external H^+ were monitored as in Fig. 1. Sodium arsenate, 2.5×10^{-3} *M*, 1.8 μmoles ATP (pH 8.1), and uncoupling agents (same as in Fig. 3) were added when indicated. Per cent reduction of methylene blue (8×10^{-6} *M*) was assayed in 3-ml aliquots of the above chloroplasts, as before.[3] Per cent reduction of neutral red (2×10^{-6} *M*) was assayed at pH 5.5 on 3-ml aliquots of the above chloroplasts (pH adjusted to 5.5 with dilute HCl) as before.[5] Since all three uncoupling agents had essentially identical effects on these chloroplasts, only one trace is indicated in the figure.

715 mμ were equally effective. Thus, reduction of the electron carrier associated with system I is associated with the partial activation of ATPase. Addition of oxidants, such as benzoquinone or ferricyanide, in the presence of CMU and light again causes inactivation of the ATPase. Reactivation by light occurs if sufficient ascorbate or dithiothreitol is added to reduce the added benzoquinone or ferricyanide (Fig. 5). As seen in Figure 5, neither ascorbate nor dithiothreitol are capable of activating the ATPase of the trypsin-treated chloroplasts in the dark, although dithiothreitol has been shown to activate the isolated ATPase (CF_1) of chloroplasts.[2, 3]

Evidence that the ATPase and ADP kinase are the same enzyme: (1) Organometals, for example, tributyl tin, tributyl germanium, and tetraphenyl lead, completely inhibit the trypsin-activated ATPase (data not shown). The concentrations of these agents required to inhibit ATPase by 50 per cent are also the concentrations required to inhibit photophosphorylation by 50 per cent.[7] Neither electron flow nor extent of H^+ uptake are inhibited at these concentrations of organo-metals.[1] Preincubation of trypsin-treated chloroplasts with carbodiimide for varying periods of time[1] also inactivates both the ADP kinase and the trypsin-activated ATPase to the same extent (data not shown).

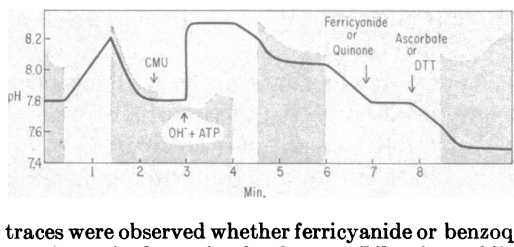

FIG. 5.—Effect of CMU on trypsin-treated chloroplasts. Trypsin-treated chloroplasts were illuminated as in Fig. 2. No ATP was added. CMU (5×10^{-5} *M*), either 1 μmole ferricyanide, or 1 μmole benzoquinone, and either 1.5 μmoles sodium ascorbate or 2 μmoles dithiothreitol (pH 7.8) were added when indicated. Since identical traces were observed whether ferricyanide or benzoquinone were added, only the trace for benzoquinone is shown in the figure. Likewise, addition of ascorbate or dithiothreitol resulted in identical traces. At 3 min, 2.0 μmoles of NaOH and 3 μmoles of ATP (pH 8.0) were added. Changes in external pH were monitored. At periodic intervals, content of ATP was also measured on aliquots of the reaction mixture as indicated in *Methods*.

(2) The maximal rates of ATPase and ADP kinase activities, in the presence of excess nucleotides, are the same under similar conditions (Figs. 1, 2, and 6). The K_m^{ATP} for ATPase and the K_m^{ADP} for ADP kinase in trypsin-treated chloroplasts are quite close, approximately, $9 \times 10^{-5} M$.

(3) The rates of photooxidation of the two enzymatic activities, with either ascorbate or rose bengal in the presence of PMS^+, also closely parallel each other, as shown in Table 1. The marked sensitivity of these enzymic activities to photooxidation by rose bengal may indicate that the imidazole ring of histidine is involved in these catalytic activities.[1] Under the condition of Table 1, neither rate of electron flow nor rate and extent of H^+ uptake are affected by three minutes of illumination in the presence of rose bengal. Nor does photoinactivation of the ADP kinase/ATPase occur under anaerobic conditions.

TABLE 1. *Photooxidation of ADP kinase-ATPase activities.*

Illumination time	ADP Kinase (% activity)			ATPase (% activity)		
	Control	+ Ascorbate	+ Rose bengal	Control	+ Ascorbate	+ Rose bengal
5 sec	100	100	100	100	100	100
15 sec	100	100	67	100	100	70
1 min	100	100	18	100	100	15
5 min	100	81	0	100	79	0
10 min	87	44	0	8	42	0
20 min	59	12	0	62	9	0

Trypsin-activated chloroplasts (0.45 mg chlorophyll) were illuminated at 15° for varying periods of time as in Fig. 1, except that no ATP was added and only 1 μmole of ADP was present. Concentration of PMS^+ was $3 \times 10^{-6} M$. Initial pH 7.9. Either sodium ascorbate, $2 \times 10^{-4} M$, or rose bengal, 2 μg/ml, were added at zero time, as indicated in the table. After varying periods of illumination, rate of ATP synthesis with saturating white light and rate of ATP hydrolysis were measured titrimetrically in the postillumination period at pH 7.9, as in Fig. 1, in the presence of 2 μmoles of ADP and 2 μmoles of ATP.

(4) Both enzymic activities are evident only after reduction of either system II or system I electron carriers and both are much more active when both major systems are reduced by light (Fig. 5).

(5) Both enzymatic activities exhibit an obligatory requirement for Mg^{++}. Replacement of Mg^{++} with Ca^{++}, under the conditions given for Figure 1, resulted in complete inhibition of both ADPase kinase and ATPase in trypsin-activated chloroplasts. Ca^{++} ions are not inhibitory *per se*, however, since full activity of both the ATPase and the ADP kinase were restored by addition of $MgCl_2$ ($10^{-3} M$) in the presence of $3 \times 10^{-3} M$ $CaCl_2$. Vambutas and Racker[9] have previously observed a Ca^{++}-activated ATPase in chloroplasts but prolonged pretreatment with trypsin was required.

(6) As predicted from the stoichiometry of the reaction,

$$H^+ + ADP^{-3} + P_i^{-2} \rightleftharpoons ATP^{-4} + H_2O.$$

ATPase activity is observed only under conditions in which the internal H^+ concentration about the reduced catalytic site is maintained at low levels either by uncoupling agents or by hypotonic conditions (Figs. 1, 3, and 4). At concentrations of internal H^+ greater than the external concentration of H^+, net ATPase activity is nil, whether or not ATP synthesis can occur, for example in the presence of arsenate or absence of P_i (Fig. 4), or in the presence of uncoupling agents.

As previously shown,[3] the above reaction can be driven to the right only at high concentration of internal H^+. At the low internal concentrations of H^+ which obtain in the presence of uncoupling agents and light, the equilibrium of the reaction is maintained close to unity, since neither net synthesis nor hydrolysis of ATP is observed (Figs. 1 and 4).

(7) At high external concentrations of H^+ (pH 6.4 or lower), essentially no ATPase activity is observed with trypsin-activated chloroplasts in the light or dark (data not shown); while the ADP kinase remains active. Thus, as predicted, ATPase activity is observed only at low concentrations of H^+. H^+ concentration within chloroplast can be increased either by electron flow or by addition of external H^+.[3]

(8) Exposure of trypsin-treated chloroplasts to preparations of CF_2 restored their ability to phosphorylate at relatively low concentrations of ADP and PMS and also caused an inhibition of the light-activated ATPase as seen in Table 2. Thus the effect of CF_2 is to decrease the apparent K_m^{ADP}, while markedly increasing the apparent K_m^{ATP} at the same site.

TABLE 2. *Inhibition of ATPase by CF_2.*

Additions	Concentrations (μM) ADP	PMS	ADP kinase (μmoles/min)	ATPase (μmoles/min)
None	40	2.5	3.1	0.14
Trypsin	40	2.5	1.1	1.6
Trypsin	40	9.0	1.7	2.6
Trypsin	200	9.0	2.4	2.7
Trypsin + CF_2	40	2.5	2.1	0.7
Trypsin + CF_2	40	9.0	2.8	0.6
Trypsin + CF_2	200	9.0	3.0	0.6

Experimental conditions were as in Fig. 1. Rates of ATP synthesis and hydrolysis were measured with and without trypsin treatment, as in Fig. 1, at various concentrations of ADP and PMS^+. Reconstitution of the trypsin-treated chloroplasts with CF_2 was accomplished by adding the chloroplasts (0.4 mg chlorophyll in 15 ml of the assay mixture) to 2 ml of CF_2[2] at 5°, pH 7.6. After 5 min, the chloroplasts were isolated by centrifugation (see *Methods*) and resuspended in fresh assay medium.

These results, together with the reconstitution experiments of Racker *et al.* on chloroplasts and mitochondria,[2-4] strongly suggest that the ATPase and ADP kinase of chloroplasts are manifestations of the same enzyme, that this enzyme must somehow be reduced to be active, that the hydrolysis of ATP maintains the enzyme in its active form even in the absence of electron flow by supplying internal H^+ to the site,[1] and that the function of electron flow in these particles is to reduce and to protonate this enzymic site. Since ATP synthesis consumes H^+, continuous electron flow is required to maintain a continuous supply of H^+ to this catalytic site. Under conditions in which the enzyme is reduced but unprotonated and in the absence of CF_2, hydrolysis of ATP results.

The effects of trypsin are probably the consequence of proteolysis of CF_2[1] that normally serves to restrict ATP and H_2O from access to this enzymic site by itself binding to the working protein.[2] Thus the presence of CF_2 shifts the apparent equilibrium of the above reaction toward the right.

Whether or not a free-radical imidazole, as proposed by Wang,[8] is involved in the protonation and dehydration of P_i remains unknown. Preliminary studies concerning the nature of the group on the trypsin-treated ATPase site within

chloroplasts, which is reduced by light, indicate that this group is not inactivated by such sulfhydryl inhibitors as iodoacetate, *n*-ethyl maleimide, or PCMS. In view of the photoinactivation of this site by rose bengal (this report), imidazole groups of the ATPase probably function in ATP synthesis and hydrolysis and may also undergo oxido-reduction during electron flow. The marked sensitivity of this proposed site to organo-metal halides, which in nonaqueous environments are known to undergo exchange reactions with imidazole, is also consistent with the suggestion that imidazole groups or some other ionic groups are involved in the ATPase–ADP kinase site.

These observations are not consistent with either the membrane theory of Mitchell and Moyle[10] or the various theories of oxidative phosphorylation which include a series of high potential intermediates. Rather, it appears that the protonation and reduction of an ATPase site, which is protected from external ATP and H_2O by membrane proteins,[2, 4] supplies sufficient energy to drive the H^+-consuming reaction to the right.

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† Abbreviations used: TTFB, tetrachloro trifluoromethyl benzimidazole; CMU, 3-(*p*-chloro-phenyl)-1,1-dimethylurea; PMS, phenasinemethosulfate; DFP, diisopropyl fluorophosphate; PCMS, *p*-chloromercuriphenyl sulfonic acid.

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