The chloroplast Tat pathway transports substrates in the dark*

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Photosynthetic electron transport pumps protons into the thylakoid lumen, creating an electrochemical potential called the proton motive force (PMF). The energy of the thylakoid PMF is utilized by such machinery as the chloroplast F0F1-ATPase as well as the chloroplast Tat (cpTat) pathway (a protein transporter) to do work. The bulk phase thylakoid PMF decays rapidly after the termination of actinic illumination, and it has been well established via potentiometric measurements that there is no detectable electrical or chemical potential in the thylakoid after a brief time in the dark. Yet, we report herein that cpTat transport can occur for long periods in the dark. We show that the thylakoid PMF is actually present long after actinic illumination of the thylakoids ceases and that this energy is present in physiologically useful quantities. Consistent with previous studies, the dark-persisting thylakoid potential is not detectable by established indicators. We propose that cpTat transport in the dark is dependent on an electrochemical potential in the thylakoid held out of equilibrium with those in the bulk aqueous phase.

The cpTat3 transporter located on the thylakoid membrane transports fully folded proteins from the stroma of chloroplasts to the lumen of thylakoids. The cpTat translocon is seemingly composed of three subunits: TatC, Hcf106, and Tha4 (1–3). The subunits TatC and Hcf106 are each present at approximately one copy/cpTat translocon (4, 5), and both subunits are involved in substrate binding (6, 7). Tha4 has been postulated to form a custom-sized channel for transporting the folded cpTat substrates across the thylakoid membrane (8, 9); however, structural or mechanistic evidence to support this model is not conclusive, and other models have been proposed to explain the mechanism of transport (10).

The energy to accomplish transport on the cpTat pathway is derived exclusively from the trans-thylakoid proton motive force (PMF) (11), which arises during photosynthetic electron transport. Nucleotide-binding motifs are not found on any of the cpTat subunits, and transport is unaffected by NTP addition or elimination (12). Consistent with the dependence of the cpTat pathway on the thylakoid PMF, transport is sensitive to both the electroneutral H+ /K+ antiporter nigericin and the electrogenic K+ ionophore valinomycin (11–15). Although the process of transporting a folded cpTat substrate does not generally render the thylakoid membrane permeable to ions (16), the proton/protein stoichiometry of a single substrate transport event has been estimated to be quite high; ~80,000 protons are drained from the gradient for every Tat substrate that enters (17).

PMF-dependent processes have a minimum thermodynamic threshold below which the process cannot occur. For instance, phosphorylation of ADP on the chloroplast F0F1-ATPase has a threshold of ~2 ΔpH units (18–20) when employing thylakoid conditions similar to those we commonly use in our laboratory (cf. Ref. 21). Considering that the maximum ΔpH that can be maintained in a thylakoid is ~3.5 units (22, 23), a large proportion of the thylakoid’s energy is not available to the chloroplast F0F1-ATPase. Additionally, a disulfide bond is formed within the chloroplast F0F1-ATPase when the thylakoid PMF drops below a certain magnitude, locking it in a nonfunctional form (24, 25). These two features make phosphorylation by the chloroplast F0F1-ATPase a suboptimal tool for investigating the thylakoid PMF at a wide range of potentials. The cpTat pathway appears to have substrate-dependent thermodynamic thresholds for transport, which can be lower than those of phosphorylation by the chloroplast F0F1-ATPase. Two subunits of the oxygen-evolving complex of photosystem II, OE17 and OE23, are cpTat substrates and have thermodynamic thresholds of transport measured to be ~1 and 2 ΔpH units, respectively (17). Furthermore, the cpTat pathway is not known to undergo an inactivation reaction at low electrochemical potentials. For these reasons, the cpTat pathway may be a superior tool for probing the work that can be done by low values of the thylakoid PMF.

To our surprise, we occasionally observed transport of cpTat substrates in samples held in the dark, i.e. in our “no-PMF” controls. Upon investigation, we were able to identify the conditions that allow this phenomenon to occur reproducibly. We demonstrate here that the cpTat pathway genuinely transports substrates in the dark and is dependent on a thylakoid PMF, which persists in the thylakoid at energetically useful levels for long periods of time after illumination has ceased. We propose that the energy for cpTat transport in the dark comes from a...
tightly held pool of protons that is not in equilibrium with the bulk aqueous proton pool.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The iOE17 and iOE23 clones used in this study were described previously (17, 26). The i2×OE17 chimera was prepared by including a SacII restriction site at the end of the coding region of an iOE17 clone. On a separate parallel IOE17 clone, the lumen-targeting signal peptide was replaced with a SacII restriction site. Subsequent digestion and ligation of the two modified iOE17 clones resulted in a construct containing a single signal peptide followed by tandem mature regions.

Preparation of Radiolabeled cpTat Substrates—Clones contained on pET23a plasmids (Novagen) were subject to in vitro transcription with T7 polymerase (Promega). The iOE17 and i2×OE17 clones were each translated in the presence of [35S]methionine, and iOE23 was translated in the presence of 10% load lanes of radioactive Tat sub-...
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FIGURE 1. Transport of a cpTat substrate in the dark is sensitive to nigericin and valinomycin. Thylakoids were pre-illuminated for 3 min and then kept on ice in darkness for 10 min before the first transport reaction was initiated; time = -10 corresponds to the moment the pre-illumination light was extinguished. iOE17 transport was initiated at 10-min intervals, and the reactions were each of 5-min duration. A, control reactions without the addition of nigericin and valinomycin. mOE17, mature OE17. B, reactions in which 1 µM both nigericin and valinomycin (nig/val) were added just before initiation of the transport reactions. The first lanes in A and B were loaded with 10% of the iOE17 translation product added to the transport reactions. C, normalized means ± S.E. from three time course experiments for untreated control samples (circles) and samples treated with 1 µM both nigericin and valinomycin just before initiation of the transport reaction (diamonds). An exponential regression line is drawn through the control points. The transport of iOE17 was calculated by comparing the densities of the mature bands to those of the 10% load lanes.

10 min at a chlorophyll concentration of 40 µg/ml in the dark and additionally in the light as a control treatment. The reactions were subsequently treated with thermolysin to digest substrate that had not been transported or were treated with mock solutions. Protease protection of the mature bands of OE17 and 2×OE17 indicates that they were inaccessible to thermolysin and thus located in the thylakoid lumen (Fig. 2, A and B).

Compared with iOE17 and 12×OE17, iOE23 transported considerably less well in the dark at a chlorophyll concentration of 40 µg/ml. By reducing the volume of transport buffer in the reaction and thus concentrating the thylakoids to a chlorophyll concentration of 330 µg/ml, iOE23 exhibited significant transport in the dark (Fig. 2C); the mature protein formed was similarly resistant to digestion by thermolysin.

The cpTat substrate OE17 appeared as a doublet in its mature size in many of our experiments. One obvious explanation is that there is an unknown OE17-processing or degradation event that occurs in the thylakoid lumen, but this has not been explored. Because both bands of the doublet are protease-protected, both bands reflect genuine protein transport.

The cpTat Pathway Is Responsible for Transport of cpTat Substrates in the Dark—To verify that the cpTat pathway is responsible for the dark transport we observed and thus rule out substrate passage on another transport pathway, the kinetics of cpTat transport in the dark was compared with the kinetics of protease-protected, both bands reflect genuine protein transport. The cpTat Pathway Is Responsible for Transport of cpTat Substrates in the Dark—To verify that the cpTat pathway is responsible for the dark transport we observed and thus rule out substrate passage on another transport pathway, the kinetics of cpTat transport in the dark was compared with the kinetics of protease-protected, both bands reflect genuine protein transport.

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mum transport at ~6 min. Transport in the light produced roughly double the amount of mature protein compared with transport in the dark (Fig. 3A). After averaging in additional data sets and recasting the plots to normalize for the extent of substrate transported, it is obvious that both dark and light transport occur with similar kinetics ($k = 0.36$ and $0.26 \text{ s}^{-1}$, respectively) (Fig. 3B). This is consistent with the idea that both transport processes utilize the same pathway.

To further verify that the cpTat pathway is responsible for transporting these substrates in the dark, we disabled this pathway and looked for an effect on dark transport. It has previously been shown that cpTat transport is inhibited by treating thylakoids with antibodies to Tha4, one of the subunits of the cpTat translocon (34). Incubation of iOE17 with thylakoids pretreated with anti-Tha4 antibody revealed that its transport in the dark was similarly inhibited by this treatment (Fig. 3C). Used as a control, treatment of thylakoids with an irrelevant antibody (fourth lane) did not inhibit dark transport. Inclusion with the antibody of an equimolar amount of Tha4 protein fragment antigen resulted in a modest recovery of dark transport. This demonstrates that dark transport is specifically dependent on Tha4 and that dark transport thus occurs on the cpTat pathway.

**Dark Transport Is the Result of neither Hydrolysis of Exogenous ATP nor Mixing Artifacts**—We investigated the effect of extrathylakoidal ATP on the creation of the long-lasting PMF in thylakoids. The chloroplast F$_0$F$_1$-ATPase can hydrolyze ATP to pump protons across the thylakoid membrane (35), resulting in the formation of a PMF in the absence of actinic illumination. This can result in the transport of cpTat substrates (Fig. 4) (12). The in vitro translated cpTat substrates we used to investigate dark transport introduced a certain amount of ATP (36, 37), as well as an ATP regeneration system. We sought to determine whether the ATP added with our cpTat substrate to the transport reactions (estimated at a final concentration of 25–50 μM) supplied the energy to accomplish dark transport. Compared with the control treatment in Fig. 4A (lane 2), dark transport was unaffected by 3 units of apyrase and 12 μM tentoxin, compounds that nonproductively hydrolyze extrathy-
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lkoidal ATP and that bind to and inactivate the chloroplast £0F1-ATPase, respectively. Although externally added ATP is able to boost cpTat transport in the dark, this effect was eliminated by apyrase and tentoxin additions (compare lanes 6 and 9–11). Because apyrase and tentoxin had no effect on the existing PMF, their inclusion did not affect the amount of dark transport, which remained at control levels. Curiously, the addition of dithiothreitol to reduce the inhibitory regulatory disulfide in the CF1-ATPase had no apparent effect on increasing ATP hydrolysis and subsequent proton pumping by the £0F1-ATPase in the dark, which would have been manifested by increased protein transport (24, 25, 38).

One additional concern we had regarding the origins of the PMF in dark-adapted thylakoids was that the electrochemical potential that drives cpTat transport in the dark could possibly be the result of a buffer transition. The classic pH-jump experiments of Jagendorf and Uribe (39) showed that a potential can arise in thylakoids by rapidly changing the pH of the buffer in which the thylakoids are suspended. A similar experimental design can create a work-producing ionic diffusion potential (40). Although the thylakoids used in this investigation were equilibrated in transport buffer before use and were diluted into a reaction primarily composed of transport buffer, we wanted to be sure that ionic or pH changes did not occur upon dilution of the thylakoids into the reaction medium. To this end, the iOE17 substrate was added directly to the stock dark-adapted thylakoid suspension (at 1 mg/ml chlorophyll) and dark transport was subsequently assessed (Fig. 4B). Efficient cpTat transport under these conditions demonstrated that the energy for dark transport did not arise from effects relating to thylakoid buffer transitions.

The PMF Utilized for Dark Transport Arises during Electron Transport—We next investigated whether proton pumping by the photosynthetic electron transport chain is the source of the PMF for dark cpTat transport. To this end, we performed an experiment in which the capacity of thylakoids for dark transport was monitored over time. After 60 min, when dark transport was significantly diminished, the thylakoids were again exposed to actinic light for 3 min and then placed in darkness on ice for another 12 min before a final cpTat transport reaction was initiated (Fig. 5). The untreated series exhibited an increase in thylakoid potential over time. After 60 min, when dark transport was significantly diminished, the thylakoids were again exposed to actinic light for 3 min and then placed in darkness on ice for another 12 min before a final cpTat transport reaction was initiated (Fig. 5). The untreated series exhibited an increase in thylakoid potential over time.

FIGURE 5. Energy utilized for dark transport arises from proton pumping by the photosynthetic electron transport chain. Dark transport reactions were performed at the indicated intervals until 60 min, at which time thylakoids were re-exposed to actinic illumination for 3 min and then dark-adapted for an additional 12 min before a final transport reaction was conducted. Where indicated, thylakoids were treated prior to the zero time point (but in the dark) with 80 µM methyl viologen or 20 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). 10% TP denotes a lane loaded with translation product at 10% that placed into the transport reaction; dp indicates a degradation product of mature OE17 (mOE17).

The restoring effect of light re-exposure on cpTat transport in the dark was prevented by the addition of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethyurea prior to the second light treatment, further demonstrating that electron transport is the ultimate source of the PMF utilized during dark protein transport.

Traditional Indices of the Thylakoid PMF Do Not Detect a Lingering Energetic Potential in the Dark—We sought to determine whether the thylakoid energetic potential we detected indirectly by cpTat protein transport in the dark could also be observed by established potentiometric measurements. The traces shown in Fig. 6 are of light and dark treatments that mimicked our thylakoid handling immediately before transport: 3 min of actinic light followed by 10 min in the dark. We used four thylakoid potential indicators: phenol red, a membrane-impermeable pH-indicating dye, reports the pH in the solution outside of the thylakoid vesicles (Fig. 6A) (29); neutral red, a membrane-permeable dye, monitors the interior thylakoid pH when the external pH is strongly buffered (30) (Fig. 6B), although measurements made in constant light are not quantitative (31); 9-aminoacridine, a permeable fluorescent amine, indicates the ΔpH across the thylakoid membrane (Fig. 6C) (23); and the potential-dependent electrochromic shift of carotenoid absorbance reports the transmembrane electric potential (Fig. 6D) (32, 41). With the exception of the electrochromic shift, each indicator showed the development of a thylakoid potential at the onset of actinic illumination and a rapid recovery to the basal potential within a few minutes after actinic illumination ended, which remained steady during the 10 min in the dark. Not surprisingly, the carotenoid electrochromic shift was less well behaved, exhibiting a slow downward drift in the post-illumination dark period. Nonetheless, the immediate drop in signal at the cessation of illumination that crossed the base line within 1 min of darkness indicates that the electric potential was similarly not maintained to an appreciable extent in the dark. Thus, like many before us, we are unable to demonstrate the existence of the dark PMF using the well-established techniques that report on thylakoid energetics.
**DISCUSSION**

The aim of this investigation was to determine whether the cpTat pathway is able to accomplish genuine protein transport in the dark and to probe the energetics of the thylakoid that underlie this phenomenon. Our experiments focused on verifying that dark cpTat transport is authentic, ruling out energy artifacts, and investigating the nature of the long-lived PMF underlying this phenomenon and the results we report here remains to be determined.

Our first experiments were aimed at establishing the conditions that lead to the reproducible formation of a mature-sized product in protein transport reactions conducted in the dark. To that end, we settled on a procedure whereby we exposed thylakoids to light for 3 min and then placed them in complete darkness for at least 10 min (a length of time sufficient for the dissipation of all measurable proton gradients) prior to starting the reaction. We then verified that the mature proteins had indeed been transported to a protease-resistant compartment (Fig. 2) and that this transport had occurred on the cpTat pathway (Fig. 3). Most convincing were the experiments demonstrating that this transport phenomenon could be observed with a number of cpTat substrate proteins and that it was sensitive to antibodies directed against Tha4, a subunit of the cpTat translocon.

Curiously, although we showed a PMF dependence of cpTat transport in the dark by demonstrating sensitivity to nigericin and valinomycin, the addition of these ionophores at 1 μM each did not always result in complete inhibition of transport under the conditions of our experiments (Fig. 1). Further examination revealed that it took longer than we expected for valinomycin and nigericin to eliminate all protein transport in the dark, typically taking ~20 min of thylakoid exposure for complete inhibition of the reaction, although this time was variable (data not shown).

These experiments made it clear that a thylakoid PMF drove the dark protein transport reactions we observed. Thus, we turned our attention to the origin of the long-lived PMF.

The experiments of Figs. 4 and 5 indicate that this potential did not arise as an artifact of our experimental design, but remained as a residual driving force generated by photosynthetic electron transport during prior incidental light exposure. This conclusion is confounded by our finding that this potential is not detected by any known indicators of the thylakoid PMF. In contrast to 9-aminoacridine, which does not respond to ΔpH values below ~1.8 units, phenol red, neutral red, and the carotenoid electrochromic shift were each capable of detecting small potential changes close to the original base line under our experimental conditions. Thus, a lingering potential equivalent to a ΔpH of 1 unit or more between the internal and external bulk aqueous phases would have been detected by these indicators had it been present. In addition, in agreement with the entire body of work on thylakoid energetics, every potential-indicating measurement we performed showed a collapse of the transmembrane proton chemical potential in the dark with a half-time in the range of 10–20 s (faster for the electrochromic shift). Given that seven half-lives (1–2 min) would lead to a >99% dissipation of such gradients, it is clear that the protons giving rise to the PMF controlling our experiments do not equilibrate with those probed by the bulk phase indicators we used. Interestingly, it has recently been reported that a newly developed energy-dependent in vitro Tat transport assay in bacterial inverted membrane vesicles also continues beyond the time at which energy gradients can be detected (51). The relationship between that phenomenon and the results we report here remains to be determined.

The principal cpTat substrate used in our experiments (iOE17) has a thermodynamic threshold for transport of ~5.7 J/mol H⁺ (1 ΔpH unit) (17) and was transported well in the dark under a variety of reaction conditions. In contrast, iOE23 has been shown to have a thermodynamic transport threshold of ~11.4 J/mol H⁺ (2 ΔpH units), thereby requiring a higher PMF before transport can be initiated. With our standard dark transport reaction conditions, iOE23 was transported poorly in the dark. Nevertheless, we did observe robust dark iOE23 transport at higher thylakoid concentrations. The chimeric substrate i2×OE17 has a transport threshold measured in the light similar to that of iOE17 (data not shown), leading to the expecta-
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tion, realized in our experiments, that i2×OE17 would be transported in the dark with similar efficiency as iOE17.

The poor transport in the dark of the cpTat substrate iOE23 and the robust transport of iOE17 might indicate the energy content of thylakoids in the dark. On the basis of the respective thermodynamic thresholds of transport for iOE17 and iOE23, we can estimate that the meta-stable thylakoid PMF is between a ΔpH of 1 and 2 units in the dark under our experimental conditions. ATP formation by the chloroplast F0F1-ATPase also occurs in the dark; however, it lasts only for a short time after actinic illumination (42). Because ATP synthesis and iOE23 transport have similar thermodynamic thresholds (18–20), we can infer that earlier studies of chloroplast F0F1-ATPase activity may not have detected the long-lived low energy thylakoid PMF in the dark, as it appears to exist below these thresholds.

The existence in thylakoids of a slowly equilibrating pool of protons held out of equilibrium with those in the bulk aqueous phases has been demonstrated before (cf. Ref. 43 and references therein). These so-called localized protons have been measured directly with a pH electrode as they are released from dark-adapted thylakoids in response to uncoupler addition (44), and they have been implicated in processes as varied as ATP synthesis (45, 46) and establishing the environment of the active site of the water-splitting enzyme (47, 48). Our data are consistent with the hypothesis that the transport of cpTat substrates in the dark is an additional manifestation of the presence of these localized protons. The physical location of this pool of slowly equilibrating protons remains enigmatic.

Recently, the energetic requirements of transport on the cpTat pathway have been drawn into question by two groups (49, 50), each of which have suggested that the PMF dependence of transport observed in vitro may not apply in vivo. We believe that the issues brought up in those publications have been resolved by our previous study demonstrating that both the ΔpH and ΔΨ components of the PMF contribute to cpTat energetics (11). The work presented herein contributes further to that discussion by underscoring the uncertainties and complexities of thylakoid energetics in general and its application to protein transport on the cpTat pathway in particular.

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