Sec-dependent Thylakoid Protein Translocation

ΔpH REQUIREMENT IS DICTATED BY PASSENGER PROTEIN AND ATP CONCENTRATION*

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A Sec-type system is responsible for the translocation of a subset of proteins across the thylakoid membrane in higher plant chloroplasts. Previous studies have suggested that the thylakoidal ΔpH plays a minor role in this translocation mechanism, but we show here that it can be essential for the translocation process, depending on the identity of the passenger protein and the concentration of ATP. Studies using chimeric proteins show that, whereas the presequence dictates the translocation pathway, the ΔpH requirement is dictated exclusively by the passenger protein; some passenger proteins are virtually ΔpH-independent whereas others are absolutely dependent. ΔpH requirement is not related to charge characteristics of the passenger proteins, ruling out an electrophoretic effect. Analysis of the 33-kDa photosystem II protein reveals an inverse relationship between ΔpH requirement and ATP concentration; import into isolated thylakoids is inhibited 14-fold by nigericin at moderate ATP concentrations, and totally inhibited into isolated thylakoids is inhibited 14-fold by nigericin

In plants and green algae, a number of photosynthetic proteins are synthesized in the cytosol and targeted across all three chloroplast membranes into the thylakoid lumen. This complex import pathway can be broadly divided into two phases, the first of which involves the transport of a cytosolically synthesized precursor protein into the stroma, after which the stromal form is transported across the thylakoid membrane into the lumenal space (Hageman et al., 1986; James et al., 1989; Ko and Cashmore, 1989; Hageman et al., 1990). The two translocation events are directed by distinct signals in the presequences of luminal proteins. The first “envelope transit” signals resemble the presequences of imported stromal proteins in both structural and functional terms, whereas the second “thylakoid transfer” signals have differing properties which are more reminiscent of the signal peptides which direct transport across the bacterial plasma membrane (von Heijne et al., 1989; Bassham et al., 1991).

The available evidence suggests that most proteins are transported across the envelope membranes by a common mechanism, but recent studies have pointed to the operation of at least two completely different mechanisms for protein transport across the thylakoid membrane. The development of assays for the import of proteins into isolated thylakoids has shown that luminal proteins fall into two clear groups in terms of import requirements. A subset, including plastocyanin (PC) and the extrinsic 33-kDa photosystem II protein (33K), require the presence of a stromal protein factor and nucleoside triphosphates (NTPs) for their transport across the thylakoid membrane (Hulford et al., 1994; Robinson et al., 1994), whereas neither of these elements is required for transport of the extrinsic 23- and 16-kDa photosystem II proteins (23K and 16K), photosystem II subunit T, and photosystem I subunit N; these proteins appear to be dependent only on the transthylakoidal ΔpH (Mould et al., 1991; Kloögen et al., 1992; Cline et al., 1992; Nielsen et al., 1994; Henry et al., 1994). In contrast, both 33K and PC can be transported across the thylakoid membrane of intact chloroplasts in the complete absence of a ΔpH (Theg et al., 1989; Cline et al., 1992; Nielsen et al., 1994).

Other studies have shown that these differing requirements reflect the operation of separate thylakoidal protein translocation systems. Robinson et al. (1994) used chimeric proteins to show that the presequences of 23K and 16K are able to direct translocation of mature PC solely by the 23K/16K-type pathway, indicating that the presequences of 23K and PC contain different types of targeting signal which almost certainly specify translocation by distinct translocases. A similar conclusion was reached by Cline et al. (1993), who showed that 33K and PC compete for transport across the thylakoid membrane, and 23K competes with 16K, but the two groups do not compete with each other. These findings are remarkable in view of the similarities between thylakoid transfer signals and typical signal sequences, all of which contain hydrophobic core regions and apparently identical terminal cleavage sites for a signal-type peptidase (von Heijne et al., 1989; Halpin et al., 1989). However, recent studies have shown that transfer signals for the ΔpH-driven system contain a twin arginine motif which is critical for translocation across the thylakoid membrane

* This work was supported by a Biotechnology and Biological Sciences Research Council Studentship (to A. M.) and a grant from the British-German Academic Research Collaboration Programme, funded by the British Council and the German Academic Exchange Scheme. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PC, plastocyanin; 33K, 23K, 16K, 33-, 23-, and 16-kDa proteins of the oxygen-evolving complex of photosystem II; 133K, intermediate size form of 33K; NTP, nucleoside triphosphate; 33/16, PC/16, chimeric proteins comprising the presequences of spinach 33K or PC, followed by the spinach 16K mature protein, AMP-PCP, adenylyl (β,γ-methylene)diphosphate; AMP-PNP, 5'-adenylyl imidophosphate.
The origins of the $\Delta pH$-driven translocation mechanism are presently unclear, but there are indications that the ATP-dependent, azide-sensitive mechanism may have been inherited from a Sec-related mechanism in the cyanobacterial-type progenitor of the chloroplast. 33K and PC are present in cyanobacteria, where they are synthesized with presequences resembling signal peptides and transfer signals and (probably) transported across the thylakoid membrane by a Sec-dependent mechanism (Kuwabara et al., 1987; Briggs et al., 1990). Furthermore, the transport of these proteins across the chloroplast thylakoid membrane is blocked by azide (Knott and Robinson, 1994; Henry et al., 1994), a known inhibitor of bacterial SecA proteins (Oliver et al., 1990), and a SecA homolog has been shown to participate in the translocation process (Nakai et al., 1994; Yuan et al., 1994). Recent studies by Mant et al. (1994) and Karnauchov et al. (1994) on the import of PSI-F are consistent with this model: like 33K and PC, PSI-F is present in cyanobacteria (Chitnis et al., 1991) and is targeted in chloroplasts by the 33K/PC-type pathway. None of the proteins targeted by the other, ATP-independent pathway have been found in cyanobacteria, and it has therefore been suggested that their appearance in chloroplasts may have been accompanied by the emergence of a novel mechanism for their translocation across the thylakoid membrane.

Because PC, 33K, and PSI-F can all be transported across the thylakoid membrane of intact chloroplasts in the absence of a $\Delta pH$, this pathway has tended to be regarded as essentially "$\Delta pH$-independent." In this study we have used two approaches to examine the energetics of the Sec-type mechanism more closely. First, we show through the use of chimeric proteins that some proteins are completely dependent on a $\Delta pH$ for transport across the thylakoid membrane and that the $\Delta pH$ dependence is strictly linked to the identity of the passenger protein rather than the presequence. Second, we show that a protein which appears to be $\Delta pH$-independent in intact chloroplasts becomes wholly reliant on the $\Delta pH$ when the ATP concentration is lowered to micromolar levels.

**EXPERIMENTAL PROCEDURES**

Chloroplast Import Assays—Precursors of thylakoidal proteins were synthesized in vitro by transcription of cDNA clones followed by translation in a wheat-germ cell-free system (Ames et al., 1989; Clausmeyer et al., 1993). Import assays were carried out as detailed in Clausmeyer et al. (1993). When present, nigericin was added from a 100 x stock in ethanol; equivalent concentrations of ethanol were added to control incubations.

Thylakoid Import Assays—Import assays were as described by Hulford et al. (1994) with the following modifications applied when desalting of the translation mix was involved. Chloroplasts were lysed at 1.0 mg ml$^{-1}$ chlorophyll, and 900 $\mu l$ of stromal extract was mixed with 100 $\mu l$ of 33K translation mix. This mixture was loaded on a NAP 10 desalting column and was eluted with 1.5 ml of 10 mM HEPES-KOH, pH 7.5, 0.1 M NaCl, (HM buffer), also used to pre-equilibrate the column. Generally 300–400 $\mu l$ of eluate was discarded before collecting the next 600 $\mu l$ and discarding the final 500 $\mu l$. This middle fraction was used to resuspend twice-washed thylakoids, to which we added HM buffer, various concentrations of NTP, or non-hydrolyzable analogs of ATP. Controls consisted of twice-washed thylakoids resuspended in HM buffer or untreated stromal extract, which was diluted to approximate the concentration of stromal extract eluted from the NAP 10 column. In the latter two incubations, ATP was present in the assay as part of the translation mix (60 or 120 $\mu l$).

In ATP-depletion experiments, agarose-linked hexokinase (2U) was used to treat 50 $\mu l$ of stromal extract, in the presence of 10 mM glucose for 10 min on ice. The mixture was agitated to ensure the agarose beads remained in suspension. After the incubation, the beads were pelleted in a microfuge, and the stromal supernatant was removed to be used in a thylakoid import assay. The presence of 10 mM glucose had no adverse effects on control incubations.

**RESULTS**

Translocation across the Thylakoid Membrane by the Azide-sensitive Pathway Can Be $\Delta pH$-dependent or -independent, According to Passerger Protein—The protonmotive force plays an important role in bacterial Sec-dependent translocation, but its precise role in the thylakoidal mechanism is less clear. The effects of dissipating the thylakoidal $\Delta pH$ vary according to the precursor protein under study and vary even among the same precursor from different plant species. Fig. 1 shows the effects of nigericin, an ionophore which specifically dissipates the $\Delta pH$, on the translocation of the precursor proteins used in this study. The data show that nigericin has no apparent effect on the translocation of spinach PC into the lumen but significantly inhibits the translocation of spinach 33K across the thylakoid membrane, with the result that the stromal intermediate form becomes the predominant imported form in the chloroplasts (lane S). The results with PC are as expected since previous studies have shown that dissipation of the $\Delta pH$ has no apparent effect on the targeting of pea or spinach PC into the lumen of intact chloroplasts (Theg et al., 1989; Robinson et al., 1994). Translocation of spinach 33K, however, is inhibited to a somewhat surprising degree, since transport of the pea protein is affected to a much lesser extent by nigericin (Cline et al., 1992). The wheat 33K precursor is imported rather more efficiently in the presence of nigericin because very little stromal intermediate form is apparent (lower panel).

In order to examine the influences of the presequence and mature protein on the energetics of transport by the Sec-type pathway, a variety of chimeric proteins were tested in assays for the import of proteins by intact chloroplasts. We analyzed the import characteristics of two constructs (33/16 and PC/16) in which the presequences of spinach 33K or PC are followed by

[Image 334x472 to 524x742]
mature size spinach 16K, a protein which is usually transported by the ΔpH-dependent pathway (Klösken et al., 1992). In the case of 33/16, the precursor form is imported into chloroplasts and converted to two forms in the control incubations: a stromal intermediate form, which presumably results from processing by SPP, and mature-size 16K which is located in the thylakoid fraction (lane T). The mature size 16K is resistant to proteolysis of the thylakoids (lane T+) demonstrating that it has been correctly targeted into the lumen. In the presence of nigericin, only the stromal intermediate form is found, indicating that translocation across the thylakoid membrane has been completely blocked.

Similar results are obtained with the PC/16 fusion protein (lower panel). In the control panel, the chimera is imported into chloroplasts and mature size 16K is again found in the thylakoid lumen in a protease-protected form. In addition, two prominent polypeptides are found in the stromal fraction, one of which (denoted iPC/16) probably results from the action of SPP. The second main stromal form (denoted i2) is only slightly larger than mature size 16K, and we believe that this polypeptide results from proteolysis or aberrant cleavage by SPP. In the presence of nigericin, translocation across the thylakoid membrane is again completely inhibited, and only the stromal polypeptides are detectable.

The likely conclusion from the results shown in Fig. 2 is that the thylakoid ΔpH is essential for the translocation of 16K by the Sec-dependent mechanism, but this conclusion is valid only if the 33K and PC presequences do indeed transport 16K by this pathway. An alternative interpretation is that in these chimeras the 16K mature protein is dictating translocation by the ATP-independent pathway. Additional tests were therefore carried out to determine the pathway followed by these chimeras; our previous studies have shown that the presequences of 23K and 16K are able to direct PC onto the ATP-independent pathway, but reciprocal types of construct (in which the presequences originate from Sec-dependent substrates) were not analyzed. Previous studies have shown that the 33K, PC-type mechanism is azide-sensitive (Knott and Robinson, 1994), and Fig. 3 shows that azide blocks translocation of 33/16 across the thylakoid membrane, suggesting that the 33K presequence is directing translocation of 16K by the Sec-dependent mechanism. We also carried out control tests to verify that azide had no effect on the ΔpH-dependent pathway, and, rather than using pre-23K or pre-16K in these tests, we analyzed the transport of another fusion protein consisting of the presequence of 23K linked to mature size 16K. This was deemed useful in order to test whether fusion proteins containing 16K are fortuitously sensitive to azide irrespective of import pathway. The lower panel of Fig. 2 shows that this fusion protein (23/16) is efficiently targeted into the thylakoid lumen in both the absence and presence of azide, indicating that translocation is taking place by the ΔpH-dependent pathway as expected. Finally, we have found that PC/16 does not compete with over-expressed 23K for translocation across the thylakoid membrane, demonstrating that translocation is not taking place by the ΔpH-dependent pathway (not shown). In each of these chimeras, therefore, the presequence dictates the import pathway followed.

The overall conclusion from Figs. 2 and 3 is that the ΔpH is critical for the translocation of the 16K passenger protein by the Sec-type mechanism. This is the first indication that translocation by this pathway can be absolutely ΔpH-dependent, and there is no doubt that it is the passenger protein which dictates the translocation energetics in this case: pre-PC and PC/16 contain the same presequence but exhibit diametrically opposite ΔpH dependences. This phenomenon is further exemplified by studies with chimeras in which the presequences and mature proteins of spinach pre-PC and pre-33K are exchanged (33/PC and PC/33). The top panel of Fig. 4 shows that 33/PC is efficiently imported into the thylakoid lumen (there is no evidence for a stromal intermediate form), and that nigericin has no apparent effect on the translocation process, in that a stromal intermediate does not appear. Translocation of PC/33 across the thylakoid membrane, on the other hand, is completely inhibited by nigericin. When these results are compared with those obtained with the authentic precursor proteins (Fig. 1), it is again apparent that the passenger protein dictates the ΔpH dependence of translocation. There is a slight difference in the effects of nigericin on the translocation of pre-33K and PC/33 across the thylakoid membrane, in that the latter is...
totally inhibited whereas the former is almost completely inhibited. However, this is a minor difference which may simply reflect the fact that PC/33 is a poor substrate for the translocation machinery; this construct is translocated with the lowest efficiency among those used in this study. As with the 33/16 and PC/16 fusion proteins, azide sensitivity and competition tests were used to confirm that the PC/33 and 33/PC are translocated into the thylakoid membrane by the Sec-type machinery; this construct is translocated with the lowest efficiency among those used in this study. As with the 33/16 and PC/16 fusion proteins, azide sensitivity and competition tests were used to confirm that the PC/33 and 33/PC are translocated across the thylakoid membrane by the Sec-type mechanism (data not shown).

The ΔpH Is Essential for the Translocation of Wheat 33K into Thylakoids at Low ATP Concentrations: Hydrolysis of Nucleoside Triphosphates Is Also Essential—On the basis of the data shown in Figs. 1–4, it is clear that different mature proteins rely on the ΔpH to markedly different extents for translocation by the azide-sensitive mechanism. However, is the translocation of some proteins genuinely unaffected by the absence of a ΔpH? In the case of pre-PC, for example, the absence of a stromal intermediate in intact chloroplasts does not prove that translocation is unaffected by nigericin; meaningful kinetic studies can not be performed because translocation across the thylakoid membrane is effectively instantaneous within an intact chloroplast. To address this point, we used an assay for the import of proteins by isolated thylakoids because changes in translocation efficiency would be immediately apparent. Wheat 33K was used as a substrate because this protein (like spinach PC) is not affected by the presence of nigericin in intact chloroplast import assays (Nielsen et al., 1994), and a truncated, artificial intermediate form of wheat pre-33K (i33K) is the most efficiently imported substrate in this type of assay (Hulford et al., 1994).

Fig. 5 shows the results of assays for the import of i33K by pea thylakoids. As shown by Hulford et al. (1994), stromal extract is required for efficient import of i33K, although washed thylakoids are capable of a low level of import. The presence of nigericin reduces import efficiency to ~7% of the control level, showing that the ΔpH does indeed stimulate the translocation process to a significant extent under these conditions. The ATP concentration in this assay is 120 μM, and additional MgATP does not improve import efficiency in the absence of nigericin; the presence of 1 mM MgATP does, however, rescue import efficiency to some extent in the presence of nigericin (up to 15% of the control value).

Thylakoid import assays were also used to address another important question: is the importance of the ΔpH related to the prevailing ATP concentration? The two sources of energy overlap to an extent in bacteria, in that the $K_m$ for ATP is much lower in the presence of a proton motive force (Shiozuka et al., 1990), and the requirement for a ΔpH can be overcome by adding an excess of SecA (Yamada et al., 1989b). A detailed understanding of the thylakoidal Sec-type system similarly requires an understanding of the precise contributions of ATP and ΔpH. Again, the intact chloroplast assay is unsuitable because fairly high ATP concentrations (above 100 μM) are required to first translocate proteins across the envelope membranes (Theg et al., 1989). For these studies we developed a low ATP variant of the thylakoid import assay, in which the mixed stromal extract and 133K translation mixture were passed through a NAP 10 desalting column in order to remove the vast majority of free ATP. These columns are designed to remove small molecules from DNA-protein samples, but we found that the actual concentration of ATP in the eluate was 1–2 μM (possibly because some ATP is weakly bound to macromolecules in the concentrated stromal extract). Fig. 6 shows that the import efficiency remains high after desalting of the stromal extract-translation mixture (lanes S and D), indicating that 2 μM ATP is sufficient to drive translocation. Significantly, nigericin completely inhibits import under these conditions (lanes N) showing that the ΔpH is essential at low ATP concentrations.

The efficient import evident in lane D raises the possibility that the required ATP is actually bound to the stromal translocation factor during passage through the NAP 10 column, and we have attempted to determine whether this is the case. The desalted stromal extract-translation mix was preincubated with a mixture of hexokinase and glucose in order to hydrolyze free ATP, and lane H shows that this treatment totally blocks import. We therefore believe that micromolar levels of free ATP are sufficient to drive the translocation process, although we cannot exclude the possibility that prebound ATP does in fact contribute to the translocation process in lane D (the presence of hexokinase/glucose may shift the equilibrium between bound and free ATP).

Fig. 7 shows a more detailed assessment of the role of NTPs in the translocation reaction, using the same type of assay. Two points emerge from the data. First, the inhibition by nigericin can be relieved to a substantial extent by the inclusion of 1 mM MgATP in the assay mixture. The average import efficiency in the presence of nigericin, 1 mM MgATP is 31% of the control value, and we conclude that the ΔpH dependence is linked to ATP concentration. Additional ATP does not improve import efficiency in the absence of nigericin, suggesting that the available 2 μM ATP is sufficient to drive translocation at high rates in the presence of a ΔpH. Second, we find that 100 μM 5′-adenyl-1,5′-imido-1,5′-diphosphate (AMP-PNP, a non-hydrolyzable ATP analog) completely inhibits 33K translocation in the presence of a ΔpH, and it therefore appears extremely likely that hydrolysis of ATP is an essential step in the translocation of 33K. A second analog, adenylyl-(β,γ-methylene)-diphosphate (AMP-PCP) is less effective at inhibiting import at this concentration, but is likewise capable of inhibiting import completely at 1 mM concentrations (not shown). It should be emphasized that the figures shown in Fig. 7 were averaged from several experiments, for the following reason. We find that the effects of nigericin and AMP-PNP under these conditions are fully reproducible: import is invariably completely blocked. However, the extent to which added ATP is able to restore import in the presence of nigericin varies between experiments. The
cause of this variability is presently unknown.

Is the translocation of PC likewise dependent on the \( \Delta pK \) at low ATP concentrations? Fig. 8 shows the effects of nigericin in "standard" thylakoid import assays (ATP concentration, 120 \( \mu M \)) and after desalting of the translation mix-stromal extract as described above. In the standard import assay, PC translocation is affected to a somewhat lesser extent by nigericin (import efficiency is reduced by a factor of 6), and transport continues at an appreciable rate in the low ATP assay (again, a 6-fold reduction of import efficiency) whereas import of 33K was totally inhibited under these conditions. These data are fully consistent with the data obtained with the chimeric proteins and reinforce the conclusion that PC is least reliant on a \( \Delta pK \) among the proteins studied to date, although the \( \Delta pK \) stimulates translocation even in this case.

**DISCUSSION**

In this study we have sought to examine the precise contributions of the \( \Delta pK \) and NTPs to the azide-sensitive protein translocation mechanism, in order to build a more detailed picture of the energetics of this translocation process. The role of the \( \Delta pK \), in particular, has been a source of confusion in recent studies, and no details have emerged concerning the type of NTP required (or whether NTP hydrolysis is a prerequisite for translocation). In the present study we have used chimeric proteins and more controlled in vitro translocation assays to examine these requirements. The overall message
from these studies is clear: the presequence dictates the translocation pathway inside the chloroplast, whereas the passenger protein dictates the ΔpH requirement for transport across the thylakoid membrane when targeted by the Sec-dependent mechanism.

A significant point to emerge from this study is that the ΔpH probably stimulates, to a large extent, the transport of most or all of the substrates for the Sec-type mechanism. In previous studies using intact chloroplasts, nigericin was found to have no effect at all on the transport of pre-PC or wheat pre-33K into the lumen (Theg et al., 1989; Robinson et al., 1994; Nielsen et al., 1994), suggesting that the translocation of some proteins is truly ΔpH-independent. The use of thylakoid import assays has shown that this is clearly not the case; translocation of both spinach pre-PC and wheat pre-33K is severely affected when the ΔpH is collapsed. We now suggest that the translocation of these proteins is similarly affected in intact chloroplasts, but that the efficiency of intraorganellar sorting is such that stromal intermediates still fail to accumulate in many cases. This idea is supported by observations on the translocation efficiency of pre-33Ks in intact chloroplasts. Under standard assay conditions, nigericin almost totally blocks translocation of spinach 33K in intact chloroplasts, has a less marked effect on pea 33K (about half of the imported protein is present as stromal intermediate; Cline et al., 1992), and has little or no apparent effect on wheat 33K. We suggest that the transport of all three species of 33K is probably inhibited to a similar extent but that the proteins are transported across the thylakoid membrane with varying efficiencies. Support for this possibility comes from the observation that, even in the absence of nigericin, a prominent stromal form of spinach 33K can be detected (suggesting relatively slow translocation across the thylakoid membrane) whereas no stromal form of wheat 33K is usually apparent. Thus, it may well be the case that translocation of the wheat protein is much more efficient, and under these circumstances a ~10-fold inhibition of transport by nigericin (which is the extent to which translocation is inhibited in standard thylakoid import assays) may not be detectable in intact chloroplasts because translocation is still too efficient for the stromal form to accumulate.

The second point is that the Sec-dependent passenger proteins vary greatly in terms of their requirement for the ΔpH. The least responsive is PC, which can be transported across the thylakoid membrane in the complete absence of a ΔpH, either in intact chloroplasts or in isolated thylakoids at low ATP concentrations as shown in this study. We have shown that the PC mature protein is likewise least reliant on a ΔpH (in chloroplasts) when fused to the spinach 33K presequence because no stromal intermediate can be detected during import of 33/PC in the presence of nigericin. 33K, on the other hand, demonstrates an intermediate level of sensitivity. In intact chloroplasts nigericin does induce the appearance of a stromal intermediate but only with precursor proteins from some plant species. The effects of nigericin in standard thylakoid import assays are more pronounced with 33K than with pre-PC and, critically, translocation is totally blocked in low ATP assays whereas that of pre-PC is not. The third natural substrate of the azide-sensitive mechanism is PSI-F (Mant et al., 1994; Karnauchov et al., 1994). We have not been able to demonstrate import of this protein into isolated thylakoids, but uncouplers slightly inhibit the transport of this protein across the thylakoid membrane in intact chloroplasts, suggesting that this protein also exhibits an intermediate level of ΔpH requirement. Other proteins, however, are totally dependent on the ΔpH for translocation to occur, even in intact chloroplasts. The example illustrated in this study is 16K, which is completely reliant on a ΔpH for translocation across the thylakoid membrane when directed onto the Sec-dependent pathway. The data therefore indicate that passenger proteins are not simply passively translocated across the thylakoid membrane, but instead influence to a major extent the physiological conditions required during the translocation process. The actual translocation route, on the other hand, is specified entirely by the presequence.

Why do some proteins need a ΔpH more than others? An electrophoretic effect appears unlikely since the electrical potential component (Δψ) of the thylakoidal proton motive force is small under steady state conditions, and dissipation of the Δψ does not block translocation of 33K or PC (Mould and Robinson, 1991; Cline et al., 1992). One obvious possibility is that proton flux is coupled to protein translocation, in which case it might be expected that protons re-entering the thylakoids from the stroma might help to protonate acidic residues before translocation. Unfortunately, there is no obvious correlation between ΔpH requirement and the charge characteristics of the various passenger proteins studied to date. PC, which is least dependent on a ΔpH, has a net negative charge (~9 in spinach), but so has spinach 33K (~5) which is almost completely dependent on a ΔpH. Equally striking is the observation that barley PSI-F and spinach 16K have very similar overall charges (~3 in each case) but notably different ΔpH dependences: PSI-F can be efficiently translocated in the absence of a ΔpH (Mant et al., 1994; Karnauchov et al., 1994) whereas 16K is absolutely dependent on a ΔpH. Finally, there is no correlation between ΔpH requirement and polypeptide size, since PC, PSI-F, and 16K are not dissimilar in terms of chain length. One possibility is that the ΔpH has to be harnessed to drive the translocation of proteins which are more highly folded, and previous work on bacterial systems would favor this idea (see below).

Another intriguing aspect of the ΔpH requirement (at least in the case of 33K) is that it depends to some extent on the concentration of ATP in the reaction mixture. At very low ATP concentrations the ΔpH becomes essential for the translocation of wheat 33K, and we therefore conclude that there is some overlap between the roles of the two sources of energy, as in bacterial systems. It is tempting to speculate that, in the absence of sufficient energy from ATP hydrolysis, the ΔpH provides an input of free energy which enables the translocase to overcome the activation energy for the translocation reaction. Nevertheless, there is little doubt that the predominant driving force in this mechanism is provided by ATP hydrolysis because removal of ATP using hexokinase or apyrase completely blocks translocation even at high ΔpH values, and ATP analogs are potent inhibitors of translocation. The ΔpH is a prerequisite only for the translocation of a few proteins at physiological ATP concentrations.

How do the energetics of this translocation reaction compare with those of prokaryotic Sec-dependent translocation mechanisms, from which the chloroplast mechanism is supposed to have evolved? It appears that there are numerous similarities, suggesting that the basic mechanism has been highly conserved. As with the chloroplast system, both an energized membrane and ATP are involved in driving protein translocation in bacteria (reviewed in Driessen, 1992), although both the ΔpH and Δψ contribute to the proton motive force, Δψ (Bakker and Randall, 1984). Furthermore, the importance of the ΔpH also varies between different proteins. In a study of three precursors, Ernst et al. (1994) found that the rates of import of three proteins (precursors of LamB, OmpA, and Skp) fell to between 10-40% when the ΔpH was collapsed, and Yamada et al. (1989a) reported that transport of pro-OmpA was reduced to 25% of the control value. It was not established in these studies whether...
the presequences or the mature proteins dictate Δp dependence, but other experiments using constructs strongly suggest that the Δp is required to drive the translocation of folded domains in the mature proteins, since the introduction of a disulfide bond into a passenger protein increases the Δp dependence of translocation (Tani et al., 1990; Tani and Mizushima, 1991). These similarities notwithstanding, it may be that the two mechanisms rely on the Δp to different extents in vivo. An energized membrane is essential for bacterial protein export in vivo but merely stimulates translocation in vitro. The reasons for this discrepancy are not entirely clear, but possible explanations suggested by Driessen (1994) are that excess SecA in the in vitro assays may suppress the Δp dependence of translocation, that the in vitro assays are able to generate only low levels of Δp, and that the collapse of the Δp in vivo has the effect of lowering the internal pH, thereby inactivating the translocase. Alternatively, it may be that those proteins which are absolutely dependent on a Δp rapidly jam the translocation machinery and block translocation of all other substrates. The situation may be somewhat different in chloroplasts. The ΔpH is essential for efficient translocation into isolated thylakoids but is less important (at least for PC and some species of 33K) within intact chloroplasts in which the conditions are usually regarded as being near to in vivo for thylakoid protein translocation. Nigericin has no detectable effect on the translocation of PC or wheat 33K within intact chloroplasts. The thylakoidal system has evolved to become less reliant on a ΔpH. This may be that the thylakoidal system is more efficient than the bacterial system or that the thylakoidal system has evolved to become less reliant on a Δp, perhaps because the thylakoidal ΔpH fluctuates enormously during the lifetime of the chloroplast. It will be of interest to study thylakoid protein translocation in intact plants or algae to ascertain the true importance of the ΔpH in vivo.

Acknowledgment—We are extremely grateful to Arnold Driessen for helpful advice throughout this work.

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