Energy Requirement for the Import of Protein into Plastids from Developing Endosperm of *Ricinus communis* L.¹

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ABSTRACT

Leucoplasts isolated from developing endosperm of *Ricinus communis* L. will import the precursor of the small subunit of ribulose bisphosphate carboxylase from pea shoots and process it to its mature molecular weight (SA Boyle, SM Hemmingsen, DT Dennis [1986] Plant Physiol 81: 817–822). This process requires energy in the form of ATP. GTP, CTP, and UTP are inactive. ADP will also satisfy the energy requirement, probably through the action of adenylate kinase in the envelope. Fatty acid biosynthesis which occurs within these leucoplasts also requires ATP for maximal activity. Phosphoenolpyruvate will stimulate fatty acid biosynthesis approximately three times as effectively as ATP through the generation of ATP within the organelle by the action of the plastid pyruvate kinase. However, phosphoenolpyruvate under similar conditions will not stimulate the uptake of the small subunit of ribulose bisphosphate carboxylase into leucoplasts. These results indicate that ATP is required outside the leucoplast for protein uptake and that internally generated ATP is not effective in this process.

Leucoplasts from the developing endosperm of the castor plant (*Ricinus communis* L.) are differentiated plastids in which fatty acid biosynthesis occurs (5). They are nonphoto-synthetic but contain ribulose, 1,5-bisphosphate carboxylase which is identical with the enzyme from chloroplasts (1). Leucoplasts and chloroplasts are probably differentiated from the same proplastid progenitor since they have identical genomes (A. Thompson, unpublished data).

Many proteins localized within the chloroplast are synthesized as precursor proteins on free cytosolic ribosomes and imported posttranslationally into the organelle (reviewed in refs. 14 and 15). The import of the small subunit of ribulose bisphosphate carboxylase has been studied extensively. In peas, it is synthesized as a precursor protein (prSS²) of *M*ₙ 21,000 (3, 12). On uptake into the chloroplast, the N-terminal transit peptide is removed by a stromal protease to yield the mature protein of *M*ₙ 14,000. The uptake requires energy in the form of ATP (4, 10) but the site at which it acts is in dispute. It has been reported to be required within the chloroplast (21, 26), but it has also been reported to be required externally (9, 23). Etioplasts will also import protein by a mechanism that appears to be identical with that found in chloroplasts (24). Uptake of proteins into plastids requires only ATP whereas uptake into mitochondria requires both ATP and a membrane potential (6, 14).

Leucoplasts from castor endosperm will import prSS synthesized from hybrid selected mRNA from developing pea seedlings (2). The imported prSS is processed to the mature size of *M*ₙ 14,000 which is identical with that in pea chloroplasts and is located within the organelle as determined by resistance to protease digestion (2). Uptake into both pea chloroplasts and castor leucoplasts requires an energy source. In chloroplasts, both ATP and light can satisfy this requirement, but only ATP can be used by the leucoplasts as might be expected since they have no photosynthetic components or pigments. Preliminary data indicate that prSS initially binds to the leucoplast envelope and is then transported into the organelle and processed. The membrane-bound precursor remains susceptible to protease treatment.

The pathway of fatty acid synthesis that occurs within these leucoplasts requires ATP (18). Hence, fatty acid synthesis can be used to monitor ATP levels within the organelle. The high levels of ATP required for fatty acid synthesis are probably derived from an active glycolytic pathway that exists within these leucoplasts (17, 25). Thus, not only can high levels of internal ATP be generated by supplying a transportable glycolytic intermediate, but also the predicted increase in internal ATP concentration can be verified by assaying fatty acid synthesis in organello.

We have exploited the high fatty acid biosynthetic capability and high glycolytic activity of leucoplasts to address the controversy over the site of the ATP requirement for protein uptake into plastids (9, 21, 23, 26). ATP generated within the organelle through the action of the organellar glycolytic pathway (17, 25) can be used for fatty acid biosynthesis but will not stimulate prSS uptake. This indicates that ATP is required outside plastids for protein uptake.

MATERIALS AND METHODS

Materials

Castor oil plants (*Ricinus communis* L. cv Baker 296) were glasshouse grown under natural light supplemented with 16 h fluorescent light.

²¹⁵³Methionine (1360 Ci/mmol) and [U-¹⁴C]acetate acid (sodium salt) (54 mCi/mmol) were from ICN. All other materials were obtained as described previously (2).

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² Abbreviations: prSS, precursor of the small subunit of ribulose 1,5-bisphosphate carboxylase; SS, small amount of ribulose 1,5-bisphosphate carboxylase.
Uptake of Pea prSS into Isolated Castor Bean Endosperm Leucoplasts

mRNA coding for the prSS was isolated by hybrid selection and translated in vitro (2). ATP and other low mol wt components were removed from the translation mixture by gel filtration (2). Products of in vitro protein synthesis (10^6 cpm per incubation) were incubated with leucoplasts (2.0 mg protein) which were then treated with 62.5 μg/mL proteinase K as described (2). This treatment removed radioactive proteins that bound to the envelope of the leucoplast but were not imported (data not shown).

The radioactive products of uptake were separated on 15% polyacrylamide gels (11). Initially, these were analyzed both by fluorography and by excision of the gel band coincident with the mature SS. This band was extracted and the radioactivity in it measured by liquid scintillation counting (2). Both methods gave very similar results with low background and no radioactivity in prSS (2). The results described in this report are representative of data from several independent protein uptake experiments.

Fatty Acid Synthesis

Isolated plastids (4 mg protein) were resuspended in 100 μl 50 mM Hepes-KOH (pH 8.0) buffer containing 1 mM MgCl₂, 0.4 M sorbitol, 1 mM MnCl₂, 20 mM KHCO₃, 0.2 mM NADH, 0.1 mM NADPH, 1 mM NADPH, 1 mM CoA, 10 mM NaHCO₃, 5 mM NaOAc, and 1 μCi ¹⁴C-acetate and incubated for 20 min at room temperature (25°C). Reactions were terminated with 10 volumes of ice-cold chlorormethanol (2:1 v/v) and the mixture was centrifuged 5 min. The pellet was reextracted once with 500 μL CHCl₃:CH₃OH (2:1), and the organic phases were combined and extracted three times with 1 mL 0.5 KCl. The washed organic phase was dried and dissolved in 300 μL CHCl₃, then the radioactivity was measured in a liquid scintillation counter (Searle 300) using toluene/Triton X-100 liquid scintillation cocktail. Counts per minute were converted to disintegrations per minute using the internal standard channels ratio method. These experiments were repeated four times. The representative results of one experiment are presented here.

RESULTS AND DISCUSSION

Energy Requirement for prSS Uptake into Leucoplasts

Of the four nucleotide triphosphates tested (ATP, GTP, CTP, and UTP), only externally added ATP stimulates protein uptake (Fig. 1). This is consistent with the results of Flugge and Hinz (9) and Schindler et al. (23). This result with plastids is in contrast to that found in mitochondria in which GTP is also effective for protein uptake (6). AMP does not stimulate prSS uptake but ADP is as effective as ATP. This implies either that ADP itself is used as a substrate for prSS uptake, or, more likely, that it is converted to ATP by adenylate kinase which has been reported to be present in the intermembrane space of plastids from other species (19).

Figure 1. Nucleotide requirement for uptake of in vitro synthesized pea prSS by isolated castor bean endosperm leucoplasts. Pea prSS mRNA was translated in vitro in the presence of [³⁵S]methionine and incubated with isolated castor bean endosperm leucoplasts. Incubation was for 20 min in the absence (-----) or presence of 2 μM of the indicated nucleotides. Products were electrophoresed on 15% SDSPolyacrylamide gels and the [³⁵S]methionine activity in mature SS was determined by excising the appropriate band from the gel and by liquid scintillation counting. BG is the background radioactivity in a gel slice of equal size that did not contain radioactive protein.

Energy Requirement for Fatty Acid Biosynthesis in Leucoplasts

Fatty acid biosynthesis occurs in leucoplasts (18). In this pathway, ATP is required as a substrate for both acetyl-CoA carboxylase and acetyl CoA synthase, enzymes found in the stroma of the leucoplast (8). When acetate is the precursor, 16 molecules of ATP are required to synthesize one molecule of oleate, the final product of fatty acid biosynthesis in this organelle. Isolated leucoplasts will synthesize fatty acids from acetate in vitro when supplied with ATP (18; and Fig. 2). These plastids will also synthesize fatty acids using glycolytic intermediates as carbon sources, indicating a glycolytic pathway is functioning within the organelle (18) as had been predicted from enzyme localization studies (25).

When phosphoenolpyruvate is added to the leucoplasts in place of ATP, fatty acid synthesis from acetate is stimulated to a rate 3.7-fold that found with ATP (Fig. 2). This indicates that ATP generated by the plastid isozyme of pyruvate kinase (13) is more effective in fatty acid biosynthesis than externally added ATP and indicates that internally generated ATP could reach a higher concentration than is accomplished when ATP is supplied external to the organelle. The phosphoenolpyruvate probably enters the leucoplast via the phosphate translocator which appears to be present in this organelle (18). Fatty acid biosynthesis can also be activated by 2-phosphoglycerate, but it is not as effective as phosphoenolpyruvate. Other glycolytic intermediates resulted in no significant fatty acid synthesis above background. When these experiments were repeated with plastids in which the osmoticum, sorbitol, was not included, no incorporation of acetate into fatty acids was detected, indicating that intact plastids were required for this effect. Phosphoenolpyruvate and 2-phosphoglycerate have
been reported to be effective in supplying energy for fatty acid biosynthesis in chloroplasts in a very similar fashion to the results reported here (16).

Site of Energy Requirement for Protein Uptake

In contrast to fatty acid biosynthesis, neither phosphoenolpyruvate nor 2-phosphoglycerate will stimulate prSS uptake into leucoplasts, but ATP will act as an energy source (Fig. 3). Since the conditions in this experiment were identical with those in which fatty acid synthesis was stimulated by phosphoenolpyruvate (except for the absence of those substrates and cofactors necessary for fatty acid biosynthesis), it can be assumed that internal ATP was generated but was not available for protein uptake. Schindler et al. (23) also reported that phosphoenolpyruvate could not be used to support prSS uptake into chloroplasts. However, the effect of phosphoenolpyruvate on the internal concentration of ATP in these chloroplasts was not measured.

These data are not consistent with a sole requirement for internal ATP for protein uptake because, in such a case, internally generated ATP should be available for both protein import and fatty acid synthesis. It is possible that there are separate pools of internal ATP that might not be accessible to both the uptake mechanism and to fatty acid synthesis. The possibility of microcompartmentation is difficult to determine experimentally, but it is unlikely that ATP generated through the pyruvate kinase reaction inside the leucoplast is in a pool that is less accessible to the protein import mechanism than is ATP that has been directly imported from the cytosol.

Apparently, ATP generated internally cannot be exported from the leucoplasts and used for protein uptake. This contrasts with chloroplasts where ATP generated internally can stimulate protein uptake (10, 23). This difference may result from a low rate of ATP export from the leucoplast resulting in a low external concentration. It is noteworthy that, though internal ATP derived through photophosphorylation can supply energy for protein uptake in chloroplasts, the level of uptake can be substantially increased by external ATP (9, 21).

The conclusion that ATP is required external to the plastid would suggest that protein uptake in plastids is similar to that in mitochondria (6). In mitochondria, ATP appears to be required for a conformational change in the protein to be imported to a configuration that favours transport across the membrane (7). It has been proposed that a phosphorylation-dephosphorylation cycle may also be involved in chloroplast protein uptake (9).

Binding of prSS to Leucoplasts in Vitro

Previous reports have shown that binding of precursors to plastids is rapid and independent of ATP (4, 22). To determine if prSS uptake into leucoplasts showed the same characteristics, isolated leucoplasts were incubated with prSS in the absence of ATP, then centrifugation and resuspended in media with and without ATP. Substantial amounts of prSS associated with the membranes after incubation for 5 min with prSS and subsequent incubation without ATP (data not shown). At least some of this prSS was susceptible to protease treatment and hence on the outside of the plastid. This bound prSS was capable of being imported into the leucoplast and becoming protected from protease treatment when ATP was supplied.

The specificity of the binding process could not be established. However, a protein that is synthesized in the rabbit reticulocyte lysate in the absence of added mRNA did not...
become associated with the plastids. This supports the contention that prSS is specifically bound to the envelope. These preliminary results suggest that binding of prSS to the plastid is not dependent on ATP, in accord with other studies on protein uptake (4, 22), although the requirement for very low concentrations of ATP has also been reported (20).

LITERATURE CITED
25. Simcox PD, Reid EE, Camwin DT, Dennis DT (1977) Enzymes of the glycolytic and pentose phosphate pathways in plastids from the developing endosperm of Ricinus communis L. Plant Physiol 59: 1128–1132