rnal of   Zeitschrift für Naturforschung C

Biosciences

nded 1946 in the Institutes
e Max-Planck-Gesellschaft

Volume 42 Number 1/2
January/February 1987

lag der Zeitschrift für Naturforschung   Tübingen

ZEITSCHRIFT FÜR NATURFORSCHUNG

Section C

A Journal of Biosciences

ISSN 0341-0382

Managing Editor:

H. Hausen, Tübingen

Council:

E. Bünning, Tübingen
A. Butenandt, München
M. Eigen, Göttingen

Editors:

A. Hager, Tübingen
K. Hahlbrock, Köln
W. Hasselbach, Heidelberg
P. Karlson, Marburg
F. Kaudewitz, München
J. Klein, Tübingen
J. St. Schell, Köln
E. Wecker, Würzburg

Associate Editors:

N. Amrhein, Bochum
B. A. Askonas, London
W. Barz, Münster
P. Böger, Konstanz
G. Bornkamm, Freiburg
D. Bückmann, Ulm
K. G. Götz, Tübingen
G. Gottschalk, Göttingen
P. Gruss, Heidelberg
G. Isenberg, Köln
R. Jaenicke, Regensburg
V. ter Meulen, Würzburg
G. F. Meyer, Tübingen
M. Rajewsky, Essen
H. Schimassek, Heidelberg
D. Schulte-Frohlinde, Mühlheim/R.
G. Schulz, Freiburg
F. F. Seelig, Tübingen
J. Seelig, Basel
H. Simon, München
W. Steglich, Bonn
H. Stieve, Aachen
J. Suko, Wien
A. Trebst, Bochum
G. Weissenböck, Köln
G. Wick, Innsbruck
V. Zimmermann, Würzburg

Information for Contributors

Contributions to Z. Naturforsch., Section C (biological sciences), may be (i) original papers, (ii) research notes not exceeding two pages in print and (iii) reports on current research of special interest. Two copies of each contribution should be sent to Dr. Helga Hausen, P.O. Box 2645, D-7400 Tübingen. (Section B (chemical sciences): Dr. Heide Voelter, P.O. Box 2645, D-7400 Tübingen; Section A (physical sciences): Mrs. Tamara Littmann, Lenzhalde 21, D-7082 Oberkochen).

Contributors are encouraged to suggest possible reviewers of their papers. The manuscript should be type written using double spacing throughout.

The title should be concise but informative. The names of the authors, with first names and initials, and of the institution where the work was carried out follow the title. A running title with not more than 60 characters should be indicated if the title is longer than this.

In a footnote on the first page, please give an address for reprint requests.

The title, a selfconsistant abstract, suitable for direct use by the abstracting journals (no references to the main text), and five keywords, all in English, must precede the main text of each contribution.

The main text should meet the highest standards as to novelty of the material, organization and conciseness. A qualified colleague and, if the text is in a foreign language, a person who thoroughly knows it, should have been given the opportunity to check the paper before its submission for publication.

Papers, reporting mainly spectroscopic, X-ray or other data of no general relevancy should not be submitted.

References and footnotes should be numbered (e.g. [1]) and listed at the end of the paper (e.g. [1] A. Meyer, Z. Naturforsch. **30c,** 633 (1976)).

Tables with the appropriate captions and a list of the figure legends should follow at the end of the paper.

Allowance should be made for the reduction in printing of the drawings (line thickness, lettering!). Original drawings larger than 21 × 30 cm should be replaced by copies of reduced size, when the manuscript is submitted and only be sent in when the manuscript is accepted. On all illustrations, the figure number and the author's name must be written in pencil.

The authors will receive page proofs.

Changes in the text after acceptance of the paper and drawings not fit for direct reproduction cause delay and create extra costs which may be charged to the author.

50 reprints are free of charge.

Information for Subscribers

The subscription prices per year are for normal subscribers:

Section A	Section B	Section C
DM 510.–	DM 610.–	DM 425.–

for authors and their institutions if they order directly from the publishers:

Section A	Section B	Section C
DM 408.–	DM 488.–	DM 340.–

plus postage and handling

Section A	Section B	Section C
DM 35.–	DM 35.–	DM 25.–

Single copies and back-numbers are available.

Subscriptions will remain standing for the following year unless cancellations are made by postage October 1st.

Informations for Advertisers

The price for a small advertisement (breadth 43 mm, height 57 mm) in all three sections of the journal is DM 60.–.

For larger advertisments please order the price list from the publishers.

VERLAG DER ZEITSCHRIFT FÜR NATURFORSCHUNG, TÜBINGEN
P.O. Box 2645, D-7400 Tübingen (Postscheck-Konto Stuttgart 8039-700).

Bayerische
Staat

ZEITSCHRIFT FÜR NATURFORSCHUNG

SECTION C

A EUROPEAN JOURNAL OF

BIOSCIENCES

Council

E. BÜNNING, Tübingen
A. BUTENANDT, München
M. EIGEN, Göttingen

Editorial Board

A. HAGER, Tübingen
K. HAHLBROCK, Köln
W. HASSELBACH, Heidelberg
P. KARLSON, Marburg
F. KAUDEWITZ, München
J. KLEIN, Tübingen
J. ST. SCHELL, Köln
E. WECKER, Würzburg

Advisory Editorial Board

N. AMRHEIN, Bochum
B. A. ASKONAS, London
W. BARZ, Münster
P. BÖGER, Konstanz
G. BORNKAMM, Freiburg
D. BÜCKMANN, Ulm
K. G. GÖTZ, Tübingen
G. GOTTSCHALK, Göttingen
P. GRUSS, Heidelberg
G. ISENBERG, Köln
R. JAENICKE, Regensburg
V. TER MEULEN, Würzburg
G. F. MEYER, Tübingen
M. RAJEWSKY, Essen
H. SCHIMASSEK, Heidelberg
D. SCHULTE-FROHLINDE, Mühlheim/R.
G. SCHULZ, Freiburg
F. F. SEELIG, Tübingen
J. SEELIG, Basel
H. SIMON, München
W. STEGLICH, Bonn
H. STIEVE, Aachen
J. SUKO, Wien
A. TREBST, Bochum
G. WEISSENBÖCK, Köln
G. WICK, Innsbruck
V. ZIMMERMANN, Würzburg

EDITED IN COLLABORATION
WITH THE INSTITUTES OF THE MAX-PLANCK-GESELLSCHAFT

Volume 42c 1987

VERLAG DER ZEITSCHRIFT FÜR NATURFORSCHUNG
TÜBINGEN

Anschrift des Verlages: Postfach 26 45, D-7400 Tübingen
Satz und Druck: Allgäuer Zeitungsverlag GmbH, Kempten

Nachdruck – auch auszugsweise – nur mit schriftlicher Genehmigung des Verlages

Section a
Physics, Physical Chemistry, Cosmic Physics

Section b
Inorganic and Organic Chemistry

Contents

Contents of Number 3

Contents of Number 4

Original Communications

Contents of Number 5

Original Communications

Contents of Number 7/8

Original Communications

Notes

Report

Contents of Number 9/10

Original Communications

Notes

Contents of Number 11/12

Notes

Protein Transport in Chloroplasts: ATP is Prerequisit

Christine Schindler, Ruth Hracky, and Jürgen Soll

Botanisches Institut der Universität München, Menzinger Straße 67, D-8000 München 19, Bundesrepublik Deutschland

Z. Naturforsch. **42c**, 103–108 (1987); received August 26, 1986

Protein Transport, ATP-Hydrolysis, Chloroplasts, Envelope, *Pisum sativum*

The energy requirement for protein transport into chloroplast was assayed under conditions that permit to distinguish whether the posttranslational translocation is dependent on ATP or whether a membrane potential across the chloroplast envelope can drive this transport event.

A membrane potential is not required for translocation. ATP can support protein transport in the presence of protonophores and ionophores. Non-hydrolyzable ATP analogues and GTP, CTP, UTP cannot serve as ATP substitutes. Translocation could be observed when an ATP generating system was used to supply ATP. In contrast ATP degrading systems completely abolished translocation.

The inner envelope membrane localized ATP-ase is probably not involved in the transport event. The results suggest that ATP is needed at the outer chloroplast envelope.

Inhibition of protein transport by ADP, pyrophosphate and NaF is studied and its consequences discussed.

Introduction

Mitochondria and chloroplasts contain proteins of dual genetic origin. Some are coded for and made in the organell while others are coded for in the nucleus and made on cytosolic ribosomes as larger precursor proteins. These precursor proteins are then post-translationally taken up into the organell in an energy-dependent step [1].

In mitochondria it was shown that a membrane potential across the inner mitochondrial membrane was able to drive protein translocation. The energy requirement in chloroplasts was much less well documented and subject to debate [2, 3]. The present report is aimed to resolve this problem. We used the precursor form of the small subunit (pSSu) of ribulose-1,5-bisphosphate carboxylase (E.C. 4.1.1.39) to elucidate this question with pea chloroplasts.

While this work was in progress several papers appeared describing the ATP-dependent protein translocation into *E. coli* membrane vesicles [4] and across yeast microsomal membranes [5, 6]. In accordance with these reports we present the following evidence that posttranslational protein uptake into chloroplasts is completely dependent on the hydrolysis of ATP and cannot be substituted for by a membrane potential.

Abbreviations: SSu, small subunit of ribulose-1,5-bisphosphate carboxylase; pSSu, precursor form of SSu; PEP, phosphoenolpyruvate; OAA, oxalacetate; DHAP, dihydroxyacetonphosphate; AMP-PNP, 5-adenylimidophosphate; AMP-PCP, β,γ-adenylylmethylenediphosphonate; CCCP, carbonylcyanid *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Reprint requests to Dr. J. Soll.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0100–0103 $ 01.30/0

Materials and Methods

Materials

[^{35}S]methionine (sp. act. 800 Ci/mmol), was from Amersham-Buchler (Braunschweig, FRG). Valinomycin, A 23187, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the ATP analogues 5-adenylimidodiphosphate (AMP-PNP) and β,γ-methylene-adenosine-5-triphosphate (AMP-PCP), pyruvate kinase (E.C. 2.7.1.40) and inorg. pyrophosphatase (E.C. 3.6.1.1) were from Sigma (München, FRG). Hexokinase (E.C. 2.7.1.1) was from Boehringer (Mannheim, FRG). All other chemicals were purchased from commercial sources and of reagent grade.

Plant material and growth conditions

Pea plants (*Pisum sativum*, c.v. Rosa Krone) were grown on vermiculite in the greenhouse for 12–14 days.

Chloroplast isolation

Chloroplasts were isolated in low ionic strength medium, 330 mM sorbitol, 20 mM morpholinopropane sulfonic acid-Tris (pH 7.3), 0.4 mM $MgCl_2$ [7]. The crude plastid pellet was resuspended and chloroplasts further purified through a step silica sol gradient (40% v:v/80% v:v Percoll, Pharmacia, Freiburg) [8]. Intact, purified chloroplasts were recovered from the gradient and washed free of Percoll by repeated centrifugation. A dense organell suspension (5 mg chlorophyll/ml) was used as stock solution for transport assays. Etioplasts were isolated as in [9]. Chlorophyll was determined by the method in [10].

Isolation of poly(A)RNA

Total RNA was extracted from 4–5 days old pea plants. RNA was purified by centrifugation through a CsCl gradient containing guanidinium rhodanide as described in [11]. Oligo-dT-cellulose was used to select for poly(A)RNA. Poly(A)RNA was precipitated in ethanol and stored in H_2O at −196 °C [11].

In vitro translation and uptake experiments

Poly(A)RNA was translated in a cell-free wheat germ system (prepared as in [12]), containing 20 mM Hepes-KOH (pH 7.6), 100 mM KCl, 3.5 mM Mg-acetate, 1 mM ATP, 20 μM GTP, 2 mM DTT, 8 mM creatine phosphate, 25 μM each of the amino acids (minus methionine), 4 μg creatine kinase, 100 μCi $[^{35}S]$methionine and 4 to 5 μg of poly(A)RNA in final volume of 100 μl. Translation mixtures were incubated at 25 °C for 1.5 h and then centrifuged at 140,000 × *g* for 1 h. The supernatant was carefully removed and was used for uptake experiments. To remove ATP, GTP and creatinphosphate the translation products were precipitated by adding solid $(NH_4)_2SO_4$ (52 mg/100 μl), precipitation was allowed to complete for 30 min at 4 °C. The pellet recovered after centrifugation at 140,000 × *g* for 30 min was dissolved in 50 mM Hepes-NaOH (pH 8) and dialyzed against the same buffer for 3 h.

The uptake mixture (final volume 300 μl) contained 3 mM $MgSO_4$, 10 mM methionine, 26 mM Na-gluconate, 2% bovine serum albumine, 330 mM sorbitol, 10 mM $NaHCO_3$, 6.6 mM ATP (unless otherwise indicated), 50 mM Hepes-NaOH (pH 8), 30 μl of postribosomal supernatant derived from the translation mixture and intact chloroplasts (equivalent to 100 μg chlorophyll). Experiments were carried out either in the dark or in the light at 25 °C for 30 min. Whenever uncouplers, ionophores and inhibitors were tested, a stock solution in ethanol was made. The chloroplasts were incubated 10 min with these inhibitors before starting the transport reaction by adding the translation mixture. Final ethanol concentration was less then 1% in the uptake experiment.

Following the incubation, the transport was stopped by centrifugation. To remove those plastids that had become lysed during incubation period, intact chloroplasts were reisolated by centrifugation through a 40% v:v Percoll cushion, resuspended in 50 mM Hepes-NaOH (pH 8) and treated with thermolysin (100 μg/ml) [9] in order to show protease resistent protein transport into the organell. After thermolysin treatment for 30 min on ice, the reaction was stopped by the addition of 10 mM EDTA, centrifuged for 4 min at 12,000 × *g* and the pellet was washed with 50 mM Hepes-NaOH (pH 8), 330 mM sorbitol, 5 mM EDTA. The chloroplasts were then lysed on ice by resuspending them in 10 mM Tricine-KOH (pH 7.9), 4 mM $MgCl_2$. The broken plastids were separated into a soluble extract fraction and a membrane fraction, containing both thylakoid and envelope membranes, by centrifugation for 5 min at 12,000 × *g*. The fractions were then analyzed by SDS-PAGE.

Polyacrylamide gel electrophoresis and autoradiography

All samples were analyzed by SDS-PAGE containing a 10–15% linear acrylamide gradient and 1% bisacrylamide. Proteins were solubilized in 2.5% LDS, 12% sucrose, 70 mM DTT and 60 mM Na_2CO_3. Electrophoresis was done essentially as described by Delepelaire *et al.* [13]. Stained polyacrylamide gels were fluorographed according to Bonner and Laskey [14] and autoradiographed using an intensifying screen (Agfa-Gevaert MR 600) at −80 °C.

Transport experiments were quantified by SDS-PAGE

Aliquots of posttranslational supernatant and from the transport experiments were electrophoresed on the same polyacrylamide gel. The pSSu and SSu were exactly located on the gel by autoradiography and the spots were excised from the gel, rehydrated in H_2O and the radioactivity recovered by treatment of the gel slices with 0.2 M NH_3 in 30% H_2O_2 for 48 h at 37 °C. By then the gel pieces were completely dissolved and scintillation fluid (Rotizint 22, Zinsser, FRG) was added. The radioactivity was quantitatively determined using a scintillation counter (Kontron, Betamatic). Specific activity of SSu was calculated from the published SSu-sequence in [15] and was not corrected for unlabelled methionine endogenously present in the wheat germ system.

Results

Hydrolysis of ATP is required to drive pSSu transport into pea chloroplasts. Normal transport efficiency was 20–30% uptake of the total pSSu in the transport mixture which is equivalent to about 10^5 molecules pSSu uptake per μg protein·h. Substitution of ATP by the nonhydrolyzable analogues AMP-PNP and AMP-PCP cannot restore protein transport. Re-addition of ATP to the posttranslational translocation reaction resulted in pSSu transport. In the presence of higher AMP-PNP concentrations (2–6.6 mM) ATP (6.6 mM) could only partially reconstitute pSSu transport. At lower AMP-PNP concentrations (up to 2 mM) pSSu translocation was fully restorable (data not shown). AMP-PCP did not show such an effect and using up to 6.6 mM AMP-PCP did not result in any decrease of protein transport in the presence of ATP (Fig. 1). The binding of pSSu did not change in the presence of AMP-PNP and AMP-PCP, respectively. Binding was determined in transport experiments which were not treated with thermolysin. GTP, CTP and UTP (6.6 mM) could also not restore pSSu translocation into chloroplasts and etioplasts from pea (data not shown). Exogenously added ATP could be replaced by the inclusion of ATP-synthesizing systems like pyruvate kinase in the presence of phosphoenolpyruvate (PEP) and ADP. PEP could not drive protein translocation, which was demonstrated when ADP was omitted from the uptake mixture (Fig. 2). The system described above yields ATP outside the chloroplast.

ATP	6.6	-	-	-	-	-
PEP	-	5	5	-	-	-
ADP	-	-	3	-	-	-
OAA	-	-	-	1	-	1
DHAP	-	-	-	-	1	1
P_i	-	-	-	-	-	10

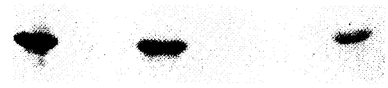

Fig. 2. ATP dependent protein uptake is driven by ATP synthesized inside or outside of the chloroplast. Pyruvate kinase (1.5 units) was added exogenously. All other concentrations (mM).

In order to see whether ATP synthesized inside the chloroplast can also support protein uptake, we incubated the protein transport system in the presence of oxalacetate (OAA), dihydroxyacetone phosphate (DHAP) and P_i. These substrates are transported *via* specific carriers into the chloroplast [16] and then can be used inside the chloroplast to yield ATP. The ATP made inside the chloroplast can also stimulate protein translocation (Fig. 2).

The role of an inner envelope bound ATP-ase in the energy requiring step in the protein translocation has been discussed [17]. We used specific inhibitors of this ATP-ase *e.g.* Na_3VO_4, $LaCl_3$ and $SbCl_3$. At concentrations known to inhibit the ATP-ase protein transport was also influenced. But Na_3VO_4 the most potent ATP-ase inhibitor [17, 18] had the least effect (Table I). On the other hand the envelope bound ATP-ase can also hydrolyze GTP and CTP at rates similar to ATP [18] which are ineffective in supporting protein uptake (see above).

Chloroplasts are surrounded by the outer envelope membrane in contact with the cytosol and responsi-

ATP	-	6.6	-	6.6	-	6.6
AMP-PNP	-	-	6.6	6.6	-	-
AMP-PCP	-	-			6.6	6.6

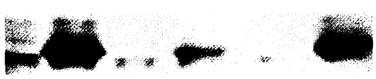

Fig. 1. Protein translocation in chloroplasts requires ATP hydrolysis. ATP was removed by $(NH_4)_2SO_4$ precipitation of the posttranslational supernatant. ATP or ATP analogues are supplied at the indicated final concentrations (mM).

Table I. Influence of chloroplast envelope bound ATP-ase inhibitors on protein uptake into chloroplasts.

Inhibitor	mM	Inhibition Protein uptake [%]	ATP-ase*
Na_3VO_4	0.1	38	98
$SbCl_3$	1	51	50
$LaCl_3$	1	20	50

* Data taken from [17].

ble for pSSu recognition and transport initiation [19] and the inner envelope membrane forming the border for the soluble chloroplast proteins. The results above demonstrated that the hydrolysis of ATP supports protein translocation across the envelope membranes. It does however not allow to conclude at which membrane the ATP is used. We tried the following approach to elucidate this question.

ATP was made inside the chloroplast *via* the OAA-DHAP system, simultaneously glucose and increasing concentrations of hexokinase were added. Hexokinase should degrade the ATP which has left the chloroplast to the outside of the outer envelope and therefore hexokinase should influence the ATP dependent transport, if ATP is needed at the outer leaflet of the outer membrane. In fact, increasing concentrations of hexokinase finally completely abolished pSSu translocation (Fig. 3), thus indicating that ATP is needed at the outer envelope membrane. If the postribosomal supernatant was incubated with 6 mM ATP, glucose and hexokinase 5 min prior to the transport experiment, almost no SSu appeared in the stroma, but when glucose-6-P was included into the assay system to inhibit the hexokinase protein transport was completely restored (not shown). It is evident that ATP synthesized inside the chloroplast can support protein uptake. The chloroplast is able to supply ATP via a multitude of pathways, i) in the light *via* photophosphorylation, ii) in the dark *via* starch degradation and glycolysis. ATP made from photosynthetic electron transport can also drive protein translocation [2] (Table II). Using specific energytransfer inhibitors it was possible to demonstrate that ATP derived either from cyclic or non-cyclic electron transport is able to deliver the energy required in pSSu uptake. DCMU and antimycin A were used as inhibitors of non-cyclic and cyclic electron transport, respectively (Fig. 4, Table II).

In order to exclude a membrane potential as driving force in pSSu translocation, different classes of uncouplers were used. A potassium gradient (KCl 0–100 mM) could neither support by itself nor stimulate in the presence of ATP protein uptake into chloroplasts (Fig. 5). Valinomycin (a K specific ionophore) had no effect on pSSu uptake in the presence of varying ATP concentrations (0.6–6.6 mM)

Table II. pSSu Translocation in chloroplasts in the presence of CCCP, antimycin A and nigericin in the light or in the dark. Final ATP concentrations were 6.6 mM, where used. CCCP, antimycin A and nigericin concentrations are indicated in the table [μM].

Treatment	Transport [molecules/μg protein · h]
dark + ATP	2.4×10^4
dark + ATP + CCCP 30	2.8×10^4
dark + ATP + antimycin A 8	2.0×10^4
light	1.0×10^4
light + CCCP 2	7.4×10^3
light + CCCP 6	5.2×10^3
light + CCCP 10	3.4×10^3
light + CCCP 30	3.4×10^3
light + CCCP 30 + ATP	1.8×10^4
light + antimycin A 2	1.2×10^4
light + antimycin A 8	1.1×10^4
light + antimycin A 8 + ATP	2.4×10^4
dark + ATP	7×10^4
dark + ATP + nigericin 0.4	6.8×10^4
light	1.7×10^4
light + ATP + nigericin 0.4	8.8×10^3

ATP	-	-	-	-	6.6	-	6.6
DCMU	1.2	4	8	12	12	12	12
Antimycin A	-	-	-	-	-	8	8

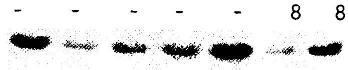

Fig. 4. Effect of cyclic and non-cyclic electron transport inhibitors on pSSu translocation. Transport was assayed in the light in the presence of DCMU and antimycin A (concentrations μM), (ATP, mM).

Hexokinase	-	-	1.7	2.3	7	14

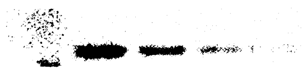

Fig. 3. ATP synthesized inside the chloroplast *via* the OAA-DHAP system is degraded by exogenously added hexokinase and thus cannot drive ATP dependent protein uptake anymore. Final concentration are OAA (1 mM), DHAP (1 mM), P_i (10 mM) and hexokinase (units) as indicated above, except in the left lane, no additions.

ATP	6.6	-	6.6	-	6.6	-	-	-
KCl	-	-	-	-	70	70	100	100
Valinomycin	-	-	1	1	1	1	-	1

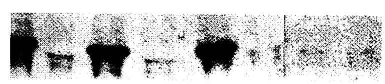

Fig. 5. Effect of the K-ionophore valinomycin on protein import in chloroplasts. Valinomycin (μM), ATP and KCl (mM) concentrations are indicated in the top panel.

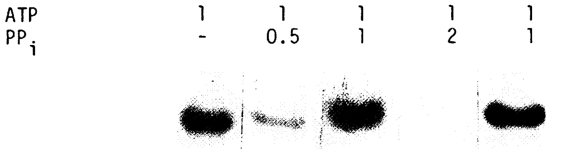

Fig. 6. Protein uptake in pea etioplasts was studied in the presence of sodium pyrophosphate and its reversibility assayed by the addition of pyrophosphatase (10 units, right lane) (concentrations in mM).

(Fig. 5). Nigericin [3] was also ineffective in protein transport inhibition in the presence of ATP (Table II). The ionophore A 23187 specific for Ca^{2+} and divalent cations had no effect on pSSu uptake in the presence of ATP (A 23187 1–10 μM, Mg 10 mM).

Protonophores like CCCP do inhibit protein uptake in the light, by uncoupling photosynthetic electron transport, but CCCP does not influence pSSu translocation in the presence of exogenous ATP (Table II).

Finally the influence of several other substrates on protein translocation into plastids was determined which were thought to alter ATP hydrolysis. Sodiumpyrophosphate inhibited pSSu transport into etioplasts and chloroplasts (Fig. 6). Simultaneous inclusion of pyrophosphatase completely reversed this effect. At lower ATP concentrations ADP is also able to inhibit transport into plastids (data not shown).

NaF, a phosphatase inhibitor leads to a strong decrease in transport while binding is increased (Fig. 7). Similar data have been obtained with etioplasts [9].

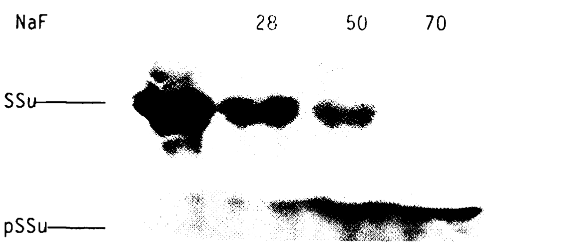

Fig. 7. pSSu translocation across chloroplast envelope membranes is influenced by NaF. After completion of the uptake experiment, chloroplasts were not treated as usually by thermolysin but were separated into a membrane and a soluble fraction which contains pSSu and SSu, respectively (see also Materials and Methods).

Discussion

Posttranslational protein transport into mitochondria, chloroplasts and other organells and membranes is an energy dependent process [1, 2]. In mitochondria it was clearly demonstrated that a membrane potential across the inner mitochondrial membrane can drive the translocation [1]. The data presented above however show that in chloroplasts and etioplasts [9] the hydrolysis of ATP is necessary to stimulate this process.

Uncouplers such as valinomycin, CCCP or A 23187 had no effect. But when ATP was depleted *via* an enzymatic reaction, transport was completely abolished. Moreover the energy-rich nucleosidetriphosphates GTP, CTP and UTP were unable to support pSSu transport. Hydrolysis of ATP is prerequisite for translocation. The nonhydrolyzable ATP analogues AMP-PNP and AMP-PCP did not stimulate protein transport, readdition of ATP however resulted in a restoration of translocation.

The ATP required in the pSSu translocation event can be generated inside and outside of the chloroplast. The manipulation we used to generate ATP inside the chloroplasts *via* the OAA-DHAP system or by light was as successful in driving protein translocation as the PEP-pyruvatekinase system outside the chloroplast. ATP can derive either from cyclic or non-cyclic electron transport as was demonstrated using DCMU and antimycin A as specific inhibitors. This demonstrates that plastids have a very versatile system to provide ATP for protein uptake and are not fixed to one ATP source. This is especially important in plastid development, when for example in etioplasts and proplastids ATP cannot derive from photosynthetic electron flow. While the outer envelope membrane is probably permeable to ATP the inner envelope membrane has a specific carrier system, which is more active in pea than in spinach chloroplasts. This would allow newly synthesized ATP to move to the place of utilization. We have obtained some evidence that the ATP is needed at the outer envelope or at least at the outside of the inner envelope membrane by using hexokinase as an external ATP depleting system. Hexokinase which cannot penetrate the envelope membranes, abolishes protein transport when the internal chloroplast ATP synthesizing system *via* OAA-DHAP is used. The inner envelope membrane-bound ATP-ase is probably not involved in the hydrolysis of ATP necessary for pSSu translocation as was demonstrated using specific inhibitors. And also by the fact that this ATP-ase can hydrolyze GTP and CTP to a similar extent as ATP while GTP and CTP are not able to support pSSu uptake. The question now arises which is the ATP requiring step and what is the mechanistic background. The data presented in this paper and in [9] agree with the idea that ATP is needed to phosphorylate a protein which is required for translocation [6, 20].

An outer envelope membrane-bound protein kinase has been described [20, 21] which shows similar inhibition responses to ADP and PP_i as does the pSSu transport. In addition NaF, a known phosphatase inhibitor effectively blocks protein import, while binding is concomittantly increased. Though this data are not direct evidence, they second a very interesting model which should be elucidated in more detail.

Acknowledgements

We like to thank Dr. U. J. Flügge, University of Göttingen, for his continuous support and critical discussion. This work was in part supported by the Deutsche Forschungsgemeinschaft, Bonn.

[1] R. Hay, P. Böhni, and S. Gasser, Biochim. Biophys. Acta **779,** 65–87 (1984).
[2] A. Grossman, S. Bartlett, and N. H. Chua, Nature **285,** 625–628 (1980).
[3] K. Cline, M. Werner-Washburne, T. H. Lubben, and K. Keegstra, J. Biol. Chem. **260,** 3691–3696 (1985).
[4] L. Chen and P. C. Tai, Proc. Natl. Acad. Sci. U.S.A. **82,** 4384–4388 (1985).
[5] J. A. Rothblatt and D. J. Meyer, Embo J. **5,** 1031–1036 (1986).
[6] M. G. Waters and G. Blobel, J. Cell Biology **102,** 1534–1540 (1986).
[7] H. Y. Nakatani and J. Barber, Biochim. Biophys. Acta **461,** 510–512 (1977).
[8] G. Mourioux and R. Douce, Plant Physiol. **67,** 470–473 (1981).
[9] C. Schindler and J. Soll, Arch. Biochem. Biophys. **247,** 211–220 (1986).
[10] D. J. Arnon, Plant Physiol. **24,** 1–15 (1949).
[11] T. Maniatis, E. F. Fritsch, and J. Sambrock, Molecular cloning, pp. 196–197, Cold Spring Harbor, N.Y. 1982.
[12] B. E. Roberts and B. C. Paterson, Proc. Natl. Acad. Sci. **70,** 2330–2334 (1973).
[13] P. Delepelaire and N. H. Chua, Proc. Natl. Acad. Sci. **76,** 111–115 (1979).
[14] W. M. Bonner and R. A. Laskey, Eur. J. Biochem. **46,** 83–88 (1974).
[15] G. Coruzzi, R. Broglie, A. Cashmore, and N. H. Chua, J. Biol. Chem. **258,** 1399–1402 (1983).
[16] M. D. Hatch, L. Dröscher, U. J. Flügge, and H. W. Heldt, FEBS Letters **178,** 15–19 (1984).
[17] T. D. Nguyen and P. A. Siegenthaler, FEBS Letters **164,** 211–220 (1986).
[18] D. R. McCarthy and B. R. Selman, Plant Physiol. **80,** 908–912 (1986).
[19] A. Bitsch and K. Kloppstech, Eur. J. Cell Biol. **40,** 160–166 (1986).
[20] J. Soll and B. B. Buchanan, J. Biol. Chem. **258,** 6686–6689 (1983).
[21] J. Soll, Planta **166,** 394–400 (1985).