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Binding of an import protein to intact chloroplasts and to isolated chloroplast envelopes of *Chlamydomonas reinhardii*

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The binding affinity of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) to isolated, intact chloroplasts and to isolated chloroplast envelopes from the green alga *Chlamydomonas reinhardii* was studied under conditions where no import into chloroplasts occurred. pSS bound to both chloroplasts and envelopes with equally high affinity. The dissociation constants were $5.9 \pm 2.1 \times 10^{-9}$ M and $2.9 \pm 1.4 \times 10^{-9}$ M, respectively. The number of binding sites per chloroplast was determined to be $8.1 \pm 4.1 \times 10^{4}$. Binding of pSS to isolated envelopes or intact chloroplasts was specific with respect to the type of the membrane and the presence of the transit sequence.

Chloroplast envelope; *Chlamydomonas reinhardii*; Precursor protein; Dissociation constant

1. INTRODUCTION

Owing to their DNA and genetic system, chloroplasts and mitochondria synthesize a number of their own proteins within the organelle [1–4]. However, many of their organelle proteins are nuclear encoded, translated in the cytoplasm as precursors and imported posttranslationally into the chloroplasts or mitochondria [5–8]. The first step of this import is believed to consist of the binding of a precursor protein to a receptor on the organelle envelope. Evidence for the existence of a pertinacious receptor on the chloroplast envelope emerged from the inhibition of precursor binding after treatment of isolated pea chloroplasts with protease [9] and from the determination of a limited number of binding sites of about 3000 per isolated chloroplast of pea. [10,11].

Not much is known about the affinity and the binding mechanism of precursors for import proteins to the chloroplast envelopes. Only for the binding of pSS to pea chloroplasts a dissociation constant of 8×10^{-9} M has been published [10]. The specificity of the binding has been studied only qualitatively by competition of the import of radioactive pSS by other precursors (pPC, pFD) [12], and by inhibition of the import by chemically synthesized peptides representing a region of precursor sequences (pPC, pFD) [13]. In contrast to mitochondria, where import is dependent on ATP hydrolysis and the membrane potential, in chloroplasts protein import is only ATP driven. Binding of the precursor, however, occurs even at very low concentrations of ATP (less than 1 mM) [15]. At the present time the attempts to identify an envelope protein as a receptor protein are still controversial [15–19].

With respect to the unicellular green alga *Chlamydomonas reinhardii*, which is a well suited organism to study the molecular biology of chloroplasts, only the import of proteins in isolated chloroplasts had been demonstrated [20,21]. In view of further studies of the molecular events at the receptors during protein import, here we present measurements of some binding parameters to intact chloroplasts and also to isolated envelopes.

Abbreviations: SS, small subunit of ribulose-1,5-bisphosphate carboxylase; pSS, precursor protein of SS; pPC and pFD, precursor proteins of plastocyanine and ferredoxin, respectively; K_D: dissociation constant.

Enzyme: Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39)

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2. MATERIAL AND METHODS

2.1. *Preparation of chloroplasts and chloroplast envelopes*

Chloroplasts were isolated from synchronized cultures of *Chlamydomonas reinhardii* cw-15 (stock CC-277 from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC, USA) as described previously [22]. Chloroplast envelopes were prepared from isolated chloroplasts according to our published procedure [23].

2.2. *Preparation of pSS*

The plasmid pSP64 containing the cDNA of the gene *rbcS2*, which codes for one of the two forms of pSS present in *Chlamydomonas reinhardii*, was a generous gift of Dr. M.L. Mishkind. The two pSS in *Chlamydomonas* differ only in a few amino acids in the mature protein but not in the transit sequence [24]. The in vitro transcription of this gene was performed according to Krieg et al. [25] with some modifications: 500 μl reaction mixture containing 40 mM Tris-HCl,

pH 7.6; 6 mM $MgCl_2$; 2 mM spermidine; 1 mM each of CTP, UTP and ATP; 0.1 mM GTP; 0.5 mM m^{7}GpppG (P^1-5'-(7-methyl)-guanosine-P^3-5'-guanosine triphosphate); 0.2 U/μl SP6 RNA-polymerase; 0.2 μg/μl DNA template were incubated at 40°C. After 30 min 10 μl of 2.5 mM GTP was added and the incubation continued for another 30 min. The reaction was terminated by digestion of the template with 10 IU DNase for 10 min at 37°C. After phenol/chloroform extraction the ethanol precipitated RNA was resuspended in 250 μl water and stored at −70°C.

For the in vitro translation a wheat germ extract was prepared according to Mishkind et al. [26]. To synthesize radiolabeled pSS each 50 μl of the translation mixture contained 10 μl wheat germ extract; 1 mM ATP; 0.4 mM GTP; 25 μM nonradioactive amino acids (all but methionine); 7.5 μCi [^{35}S]methionine (6.4 pmol); 8 mM creatine phosphate; 2 mM dithiothreitol; 40 μM spermine; 1.3 U/ml creatine phosphokinase; 24 mM HEPES-buffer, pH 7.5; 83.3 mM K-acetate; 2.8 mM Mg-acetate; 3 μl mRNA (ca. 2 μg). The translation was carried out during 40 min at 30°C. For the binding assays the translation mixture was centrifuged in an air-fuge (Beckman) at 30 psi and 4°C for 60 min to sediment the ribosomes.

The amount of the synthesized pSS was determined by isotope dilution. Aliquots of parallel translation assays with decreasing specific activity of [^{35}S]methionine were separated by electrophoresis on SDS-polyacrylamide gels. From the radioactivity incorporated in the pSS bands, from the specific activity of methionine and from the number of methionine residues present in pSS the amount and specific radioactivity of the synthesized pSS was graphically calculated.

2.3. *Import assay*

50 μl of chloroplasts (10^8/ml) and 50 μl translation mixture containing the radiolabeled pSS were incubated in a final volume of 300 μl import buffer (250 mM sorbitol; 50 mM HEPES-buffer, pH 7.8; 10 mM ATP) for 30 min at 25°C in white light (Osram R125, 300 W, distance 80 cm). The reaction was terminated by centrifugation through 40% Percoll in import buffer.

2.4. *Binding assay*

50 μl of chloroplasts (10^8/ml) or of isolated envelopes (350 μg/ml protein) and 10–200 μl translation mixture containing the radiolabeled pSS were incubated in a final volume of 300 μl binding buffer (250 mM sorbitol; 50 mM HEPES-buffer, pH 7.8) for 15 min at 0°C in dim green light or darkness. The binding reaction was terminated by centrifugation of the chloroplasts through 40% Percoll resulting in a 100% recovery of the chloroplasts in the pellet, or by washing the envelopes twice with binding buffer. Eventually it was followed by a proteolytic treatment with 100 μg/ml thermolysin in 1 mM $CaCl_2$ for 30 min on ice. Proteolysis was stopped by addition of 10 mM EDTA and centrifugation through Percoll. Starting from Percoll washed chloroplasts with bound pSS, import was induced by suspending the chloroplast pellet in import buffer and incubating for 15 min as described for the import assay.

2.5. *Determination of binding parameters*

The incubation mixtures were analysed by electrophoresis on SDS-polyacrylamide gels followed by fluorography. The radioactive bands were excised from the gel and the radioactivity measured by liquid scintillation counting. The specific radioactivity of pSS, the initial radioactivity in the assay, and the radioactivity recovered from the gel were used to calculate the concentrations of free and bound pSS in the binding equilibrium. The values were plotted in form of a Scatchard-graph in order to determine the dissociation constant and the number of binding sites per chloroplast. Radioactive mature SS was isolated by conventional procedures from in vivo [^{35}S]sulfate-labeled *Chlamydomonas* cells.

3. RESULTS AND DISCUSSION

Fig. 1 characterizes the experimental procedure we used to study the binding affinity of the precursor protein pSS to chloroplasts of *Chlamydomonas reinhardii*. The in vitro synthesized pSS was radioactively pure (Fig. 1, lane A). Electrophoresis of the centrifuged translation mixture on SDS-polyacrylamide gels resulted on the fluorogram in one single radioactive band with a molecular weight of 21 kDa corresponding to pSS of *Chlamydomonas*.

The isolated chloroplasts of *Chlamydomonas reinhardii* which were able to perform light-driven protein synthesis and photosynthetic CO_2 fixation [27,28] were also able to process pSS to mature SS (Fig. 1, lane B). Although the chloroplasts had been reisolated by centrifugation through Percoll some unprocessed pSS remained bound to the chloroplast surface. After treatment of the incubation mixture (Fig. 1, lane B) with the protease thermolysin, which cannot penetrate envelopes, and after reisolation of the intact chloroplasts, all of the pSS, but not the processed labeled SS, had been degraded and lost (Fig. 1, lane C). Obviously the newly formed SS had been internalised into the chloroplasts and protected from degradation. When chloroplasts were treated with thermolysin before the addition of pSS, much less radioactive pSS was bound or imported pointing to the pertinacious nature of the presumptive receptor (data not shown).

This processing and import of precursor protein could be uncoupled from the event of binding of the precursor to chloroplasts. In the dark and at 0°C the radioactively labeled pSS was bound, but not processed. After reisolation by centrifugation through a Percoll gradient, the chloroplasts from such an incubation mixture still retained their bound pSS (Fig. 1, lane D), which however was removed from the chloroplast surface by thermolysin treatment (Fig. 1, lane E). When washed chloroplasts containing bound pSS were subjected to a second incubation of 15 min at 25°C in the light, some of the pSS was again processed (Fig. 1, lane F). By longer incubation still more SS was produced and the amount of bound pSS was reduced further (not shown). Therefore and for reasons discussed below, the uncompleted processing seemed essentially not to be due to unspecific adsorption of pSS to sites on the envelope other than the receptor. Slow import as compared to the binding kinetics, or receptors with damaged import activity but still intact binding affinity may account for the failure to process all bound pSS.

When isolated chlorophyll-free chloroplast envelopes instead of intact active chloroplasts were incubated with radiolabeled pSS, these membranes also bound pSS (Fig. 1, lane G). The pSS was firmly bound and not removed by reisolation of envelopes.

The experimental procedure described for the binding experiments (Fig. 1, lanes D and G) were modified to determine the binding affinity of pSS to the presumed receptor on the chloroplast surface. In a serial assay a fixed number of chloroplasts were incubated with in-

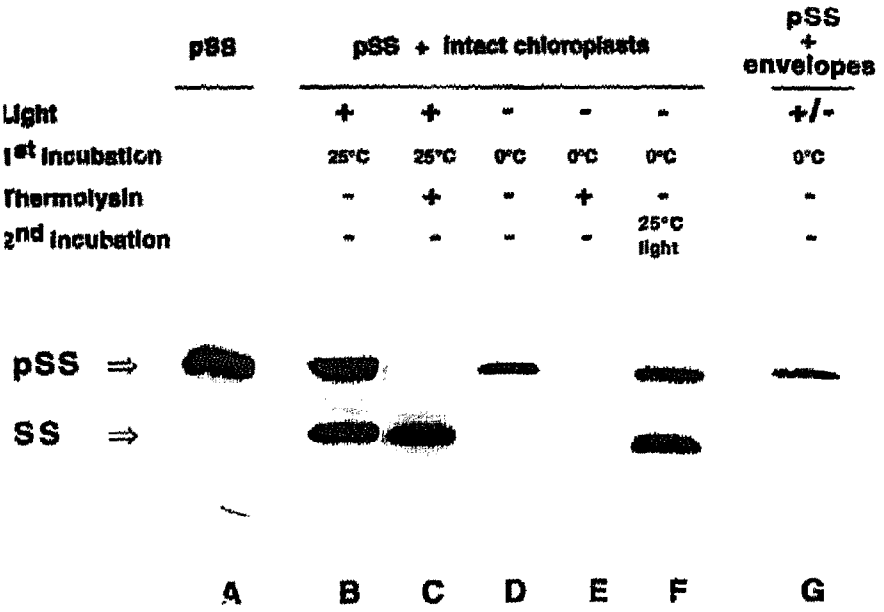

Fig. 1. Characterization of the binding and import experiments. Fluorogram of SDS-polyacrylamide gels with electrophoretically separated incubation mixtures. Lane A: wheat germ translation mixture containing in vitro synthesized, radiolabeled pSS. Lane B: import assay; after incubation the chloroplasts were centrifuged through Percoll. Lane C: as B, then treated with thermolysin. Lane D: binding assay with intact chloroplasts; after incubation the chloroplasts were washed by centrifugation through Percoll. Lane E: as D, then treated with thermolysin. Lane F: as D, then incubated under import conditions for 15 min. Lane G: binding of pSS to isolated chloroplast envelopes.

creasing amounts of centrifuged translation mixture containing [^{35}S]methionine-labeled pSS. The concentration of [^{35}S]pSS in this translation mixture had been determined by isotopic dilution. So the absolute amount of pSS added to each assay could be calculated. After reaching the binding equilibrium the chloroplasts were reisolated and washed. The amount of bound pSS was determined by electrophoresis on SDS-polyacrylamide gels and liquid scintillation counting of the excised protein bands. Fig. 2a shows that this direct measurement of bound pSS resulted in a rather smooth adsorption isotherm. By calculating the relatively small amount of bound pSS from the difference of the high initial amount and the still high amount of free pSS remaining in the supernatant after binding, what is experimentally much easier to do, the values were less consistent due to the much higher statistical error. From the Scatchard-plots (Fig. 2b) of 4 independent experiments a dissociation constant of $K_D = 5.9 \pm 2.1 \times 10^{-9}$ M could be calculated, indicating a high affinity binding of pSS to the receptor with a rather negative standard free enthalpy of $\Delta G^\circ = -43.0$ kJ/mol for binding. The K_D determined here for *Chlamydomonas* chloroplasts and pSS is comparable to that for the binding of pSS from pea to pea chloroplasts [10].

From Scatchard graphs as exemplified in Fig. 2b the number of binding sites per chloroplast can also be calculated. By evaluating 4 experiments we found that $8.1 \pm 4.1 \times 10^4$ molecules pSS were bound per *Chlamydomonas* chloroplast. This value is more than 10 times higher than that found on pea chloroplasts. The difference may be due on the one hand to the greater surface and bigger size of the single, cup-shaped, branched chloroplast of *Chlamydomonas* and on the other hand to the physiological state of the cells from which the chloroplasts have been isolated. The synchronized cell cultures were harvested at the stage when protein synthesis, and consequently protein import into the chloroplasts, was at the maximum [29].

In an analogous set of experiments the binding of [^{35}S]pSS to isolated envelope membranes of *Chlamydomonas* was studied (Fig. 3). A dissociation constant K_D of $2.9 \pm 1.4 \times 10^{-9}$ M was calculated from 5 independent determinations. This value is very close to the K_D for intact chloroplasts indicating that the binding of pSS to the receptor is independent on any physiological chloroplast activity. Binding occurred even when ATP and GTP have been eliminated from the pSS-solution by gelchromatography.

Although from a physical point of view high affinity does not necessarily imply a highly specific binding, in biological systems a low dissociation constant may be the result of the formation of a specialised receptor-ligand pair during evolution. Therefore, the very low K_D we measured may be an indication for a highly specific binding of the precursor protein pSS to chloroplast envelopes. Furthermore, the tendency of the adsorption isotherms (Fig. 2a) to reach saturation at a limited number of binding sites per chloroplast is also indicative for a specific precursor binding. The fact that the adsorption isotherms can be linearised by double reciprocal plotting (not shown) and by the Scatchard

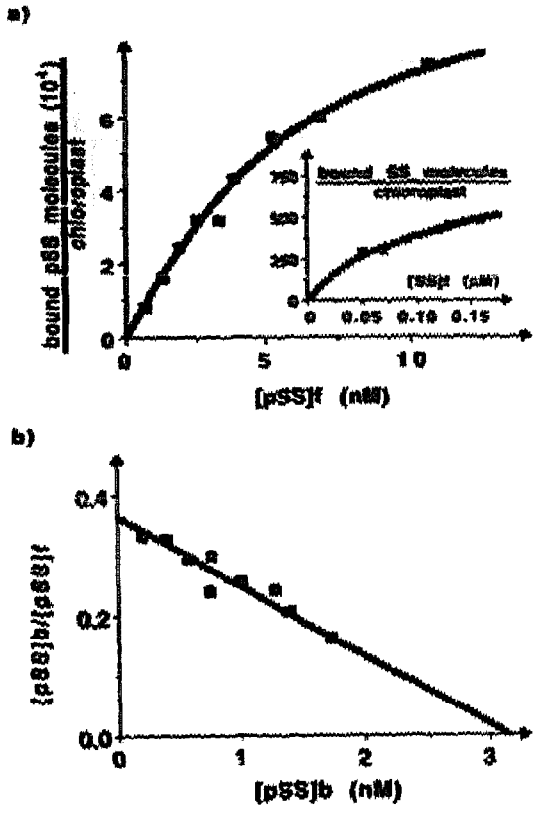

Fig. 2. Binding of pSS to intact chloroplasts of *Chlamydomonas reinhardii*. (Data from a single experiment out of four.) (a) Adsorption isotherm for binding of pSS to chloroplasts. Constant amounts of chloroplasts were incubated with increasing concentrations of [^{3}S]pSS in binding assays. The concentration of free pSS ($[pSS]_f$) was measured in the supernatant. The concentration of bound pSS ($[pSS]_b$) was determined via the radioactivity in the pSS-band after electrophoresis of the washed, solubilised chloroplasts on SDS-polyacrylamide gel. Inset: same experiment but with mature [^{3}S]SS instead of pSS. Note the different scales of the graph. (b) The above data could be linearised in form of a Scatchard plot for determination of K_D and the number of binding sites.

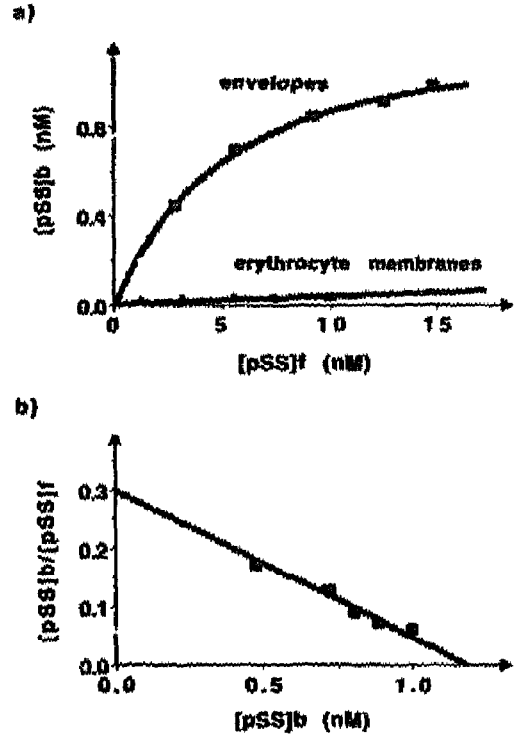

Fig. 3. Binding of pSS to isolated chloroplast envelopes of *Chlamydomonas reinhardii*. (Data from a single experiment out of five.) (a) Adsorption isotherm for binding of pSS to envelopes and to erythrocyte membranes. The concentrations of free ($[pSS]_f$) and bound pSS ($[pSS]_b$) were determined as in Fig. 2. (b) The data for the binding of pSS to envelopes could be linearised in form of a Scatchard plot for the determination of K_D.

formalism (Figs. 2b and 3b) demonstrates that each receptor contains only one binding site and that pSS is not bound in comparable amounts unspecifically or to other receptors with somehow lower binding affinities.

For the binding of pSS to chloroplasts the transit sequence is necessary. The inset in Fig. 2a shows that the binding of radioactively labeled mature SS, i.e. pSS without the transit sequence, was negligible as compared to the binding of pSS. The positively charged transit sequence (6 basic and no acidic residues) does not bind unspecifically to lipids. Even isolated erythrocyte membranes containing a high percentage of charged phospholipids did not bind pSS to a significant degree (Fig. 3a). The question whether other precursor proteins bind to the same receptor has not been answered yet in *Chlamydomonas*. In *Silene*, however, displacement studies indicate that pPC and pFD are competing with pSS for the same receptor [17].

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