Effect of precursor protein phosphorylation on import into isolated chloroplasts from *Chlamydomonas*

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Abstract In higher plants, chloroplast-destined precursor proteins are thought to be phosphorylated. Mediated by a specific 14-3-3 protein, these phosphorylated proteins bind to the chloroplast surface and are subsequently imported into the chloroplast. We demonstrate that also in the green alga *Chlamydomonas reinhardtii* the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase becomes phosphorylated by a plant protein kinase and that the phosphorylation site is located in the transit peptide. The phosphorylation status of the precursor protein regulates its import into chloroplasts especially at an early step during this process. The possible physiological function is discussed.

Key words: Chloroplast; Protein import; Phosphorylation; *Chlamydomonas*

1. Introduction

Chloroplasts are double-membraned organelles that require many proteins to maintain their structure and to perform different functions. The majority of these proteins are encoded in the nucleus and synthesised in the cytoplasm as precursors carrying an N-terminal transit sequence, which is necessary and sufficient to direct a polypeptide into the chloroplast [1–4]. The import process starts with binding of precursor proteins to the translocation complex located within the outer membrane of the chloroplast envelope (Toc), which consists of at least four subunits [5–8]. This early event involves hydrolysis of GTP [9,10]. Hydrolysis of low concentrations of ATP in the cytoplasm or intermembrane space results in the irreversible association of precursor proteins with the translocation machinery of both outer and inner envelope membranes [11–13]. The import complex of the chloroplastic inner envelope membrane (Tic) also consists of at least five subunits [14–18]. Complete translocation of precursor proteins into the chloroplast interior is accomplished via hydrolysis of ATP within the stroma [19]. During or after the import process, the transit sequence is removed by a stromal processing peptidase and the mature protein begins the process of folding and assembly [20,21].

Recently, knowledge about the components involved in the recognition of the precursor proteins on the chloroplast surface has accumulated. The heat shock protein 70 (Hsp70) family and a protein belonging to the ubiquitous 14-3-3 class of proteins might be involved in precursor binding to chloroplasts [22–24]. This has a high affinity for phosphorylated, but not for non-phosphorylated precursor proteins. Most chloroplast precursor proteins can be phosphorylated by a plant protein kinase [25]. The 14-3-3–Hsp70–precursor protein complex may be a bona fide intermediate in the in vivo protein import pathway in higher plants [24]. It is not known whether this precursor protein modification by phosphorylation occurs only in higher plants and how this phosphorylation regulates the import process. In the present study, using the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) from *Chlamydomonas reinhardtii* as a model protein, we address these issues.

2. Materials and methods

2.1. Protein binding and import assay

Intact chloroplasts from highly synchronous cultures of *Chlamydomonas* strain CC-277 (Chlamydomonas Genetics Centre, Duke University) were isolated as described previously [26]. Synthesis and radioactive labelling of *Chlamydomonas* pSS with [^{35}S]Met in a translation system were performed either with wheat germ extract (WG) prepared according to Rothen et al. [27] or with rabbit reticulocyte lysate (RL) (Roche Diagnostics, Rotkreuz, Switzerland). Chloroplast protein binding and import assay were performed according to Su et al. [28]. Electrophoretic analysis, autoradiography and evaluation of methionine incorporation using a PhosphorImager were done as before [28].

2.2. Two-dimensional gel electrophoresis and immunoblotting

Two-dimensional gel electrophoresis was performed using the method of Clemetson et al. [29]. Proteins were transferred from the gel onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by the semi-dry technique. The primary antibody was a rabbit polyclonal antiserum against SS from *C. reinhardtii* (anti-SS).

2.3. Phosphorylation assay and immunoprecipitation

Phosphorylation of pSS was done during protein translation. Each 100 μl assay contained 20 μl WG or RL and 10 μCi [γ-^{32}P]ATP instead of [^{35}S]Met. After incubation at 30°C for 40 min, 2 vol of denaturing solution were added (10% sodium dodecyl sulphate (SDS); 5 mM EDTA and 40 mM Tris–HCl pH 7.4) and the mixture heated for 5 min to 95°C. It was diluted with immunoprecipitation dilution buffer (1.25% Triton X-100, 190 mM NaCl, 60 mM Tris pH 7.5, 6 mM EDTA pH 7.5, 1 mM NaF) to a final SDS concentration of 0.4%. Protein A-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) loaded with polyclonal anti-SS antiserum were

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Abbreviations: SS, small subunit of ribulose-1,5-bisphosphate carboxylase; pSS, precursor of SS; WG, wheat germ extract; RL, reticulocyte lysate; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone

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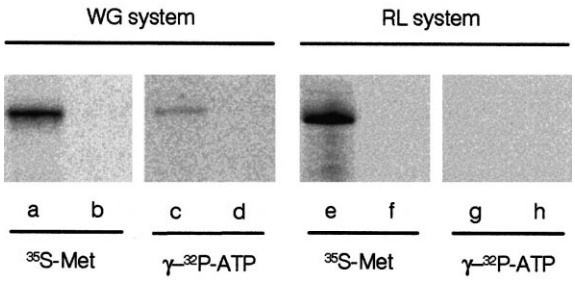

Fig. 1. Precursor protein pSS is phosphorylated in WG, but not in RL. The pSS was synthesised either by WG (lanes a–d) or RL (lanes e–h) in the presence of [^{35}S]Met (lanes a, b, e, f) or [γ-^{32}P]ATP (lanes c, d, g, h). The translation mixture was immunoprecipitated with anti-SS antiserum (lanes a, c, e, g) or pre-immunoserum (lanes b, d, f, h). The autoradiograms are shown.

added to the sample and the solution was agitated at room temperature for 2 h. The beads were separated from the mixture by centrifugation and washed five times in immunoprecipitation buffer (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 1 mM NaF). The bound proteins were eluted with Laemmli solubilisation buffer [30] and analysed by SDS–polyacrylamide gel electrophoresis (PAGE).

3. Results

3.1. pSS is phosphorylated within its transit peptide by a plant protein kinase

In earlier experiments we observed slightly different import efficiencies of pSS from *Chlamydomonas* into isolated chloroplasts depending on whether it was synthesised in WG or in RL. Therefore, we compared the phosphorylation status of pSS after its synthesis in the presence of [γ-^{32}P]ATP in these two different translation systems. The autoradiogram of immunoprecipitated pSS showed that it became phosphorylated in WG, but not in RL (Fig. 1, lanes c, g). Control experiments using [^{35}S]Met for labelling demonstrated that in both translation systems pSS was very efficiently synthesised (Fig. 1, lanes a, e). The absence of any radioactive band after precipitation with pre-immunoserum illustrated the specificity of the immunodetection (Fig. 1, lanes b, d, f, h). The results suggest that phosphorylation of the precursor protein pSS is plant specific in that this reaction occurs only in WG.

We further tried to find the phosphorylation site in the precursor protein. If the phosphorylation event is related to protein import into chloroplasts, the phosphorylation site is suspected to be located in the transit peptide. To test this hypothesis we prepared phosphorylated pSS in WG again with γ-labelled ATP and then treated it with the stromal processing peptidase-2 (SPP-2) from *Chlamydomonas*. This enzyme specifically processes pSS to mature SS by cleaving off the transit sequence in one step [23]. In our laboratory an enzyme fraction highly enriched in SPP-2 activity, and containing only a few additional protein bands on SDS–PAGE analysis, was prepared [21]. After incubation of [^{32}P]-phosphorylated pSS with this SPP-2 preparation in the presence or absence of SPP-2 inhibitor (0.5 mM *N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK]), the proteins were separated by two-dimensional gel electrophoresis, blotted to nitrocellulose and stained with anti-SS antiserum. In the assay containing the inhibitor TPCK a distinct, single immunostained spot was detected and identified as pSS (Fig. 2B). Autoradiography of the same nitrocellulose membrane revealed that this spot indeed was phosphorylated (Fig. 2A). In the assay without TPCK pSS was no longer detectable

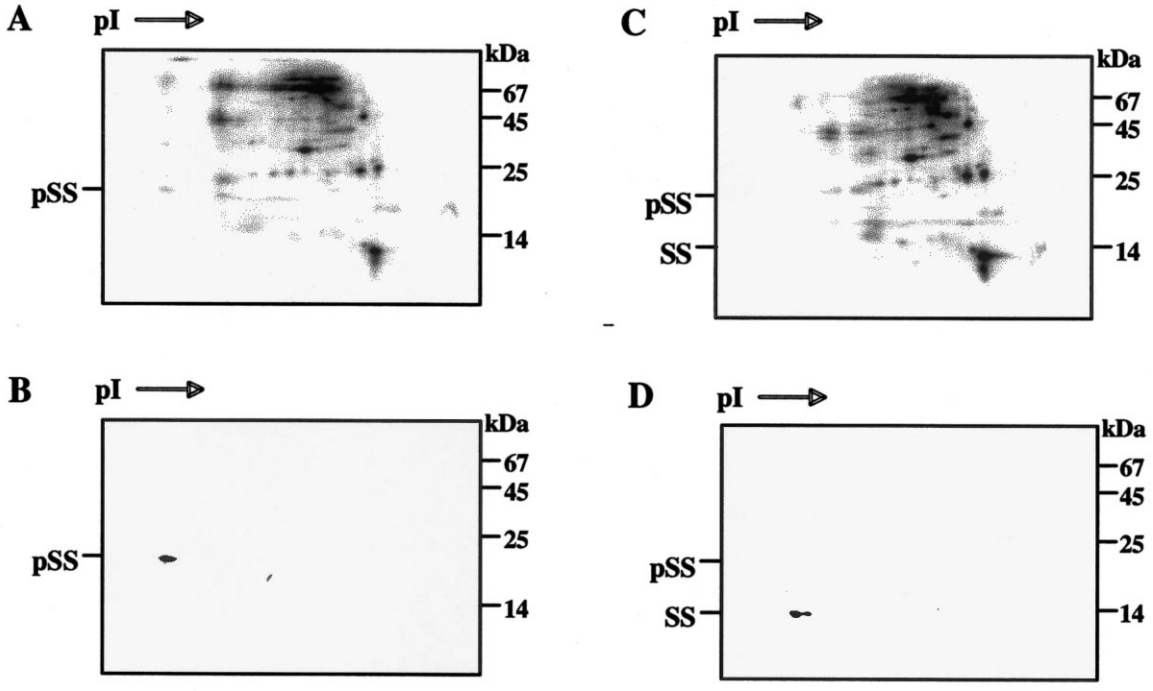

Fig. 2. Phosphorylation of pSS occurs in the transit peptide. The pSS synthesised in WG containing [γ-^{32}P]ATP was incubated at 25°C with partially purified SPP-2 in the presence (A and B) or absence (C and D) of 0.5 mM TPCK. After 60 min incubation the protein mixture was separated by two-dimensional gel electrophoresis, blotted, immunostained with anti-SS antiserum (B, D) and analysed by autoradiography (A, C).

by immunostaining, and instead a spot corresponding to mature SS could be detected clearly (Fig. 2D). However, the immunostained SS spot was not radioactive, indicating that the phosphorylation site of pSS carrying the radioactive label was located in the transit peptide, which in mature SS was cleaved off (Fig. 2C).

The transit peptide of pSS contains five Ser residues, but no Thr or Tyr. Therefore, the corresponding precursor protein kinase must belong to a Ser/Thr kinase family. In the cytoplasm of pea a similar precursor protein kinase was found with the phosphorylation consensus motif (P/G)X*n*(R/K)X*n*(S/T)X*n*(S*/T*), where $n = 0$–3 amino acids as spacer and S*/T* representing the phosphate acceptor [19]. Also in the transit peptide of pSS from *Chlamydomonas* such a motif exists: Pro-Ala-Arg-Ser-Ser_{21}, suggesting that the phosphorylation site is Ser-21 of pSS.

3.2. The phosphorylation status of pSS affects its import efficiency

The above data indicate that pSS is modified by phosphorylation after translation in the cytosol. To investigate the biochemical function of this kind of protein modification a series of import experiments with phosphorylated and non-phosphorylated pSS were performed. Both forms of pSS were imported into intact chloroplasts of *Chlamydomonas* (Fig. 3, lanes a–c). However, the comparison between relative radioactive intensities of the pSS and SS protein bands (Fig. 3, lane b) showed that non-phosphorylated pSS synthesised in RL was more efficiently imported and processed than phosphorylated pSS prepared in WG. The same result was obtained when the amount of imported SS protein was related to that of the precursor protein pSS added into the import experiment. The percentages of imported mature protein to total precursor protein from pSS synthesised in RL and in WL were about 12% and 10%, respectively. Both pSS preparations were successfully bound to the surface of the outer envelope membrane after 15 min incubation of pSS with chloroplasts in the dark at 4°C (Fig. 3, lanes d, e). These results suggest that pSS can bind and be imported in vitro into chloroplasts regardless of the phosphorylation status, although the import

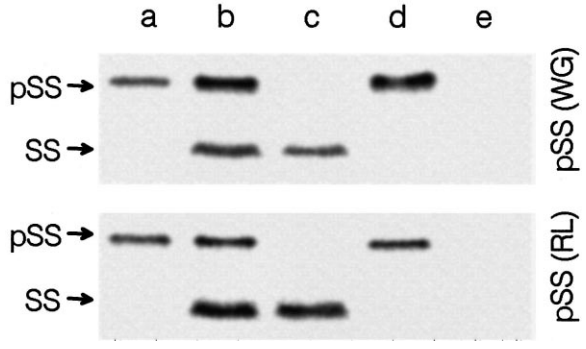

Fig. 3. Effect of phosphorylation of pSS on its binding to and import into chloroplasts. Autoradiogram of a SDS–PAGE. The pSS was synthesised and radiolabelled with [^{35}S]Met in WG or RL. Lane a, in vitro synthesised radiolabelled pSS alone; lane b, pSS imported into isolated chloroplasts and after import the chloroplasts were re-isolated by centrifugation through Percoll; lane c, as b, but the chloroplasts were treated additionally with thermolysin; lane d, pSS was bound to chloroplasts which were then re-isolated through Percoll; lane e, as d, but with thermolysin treatment after binding.

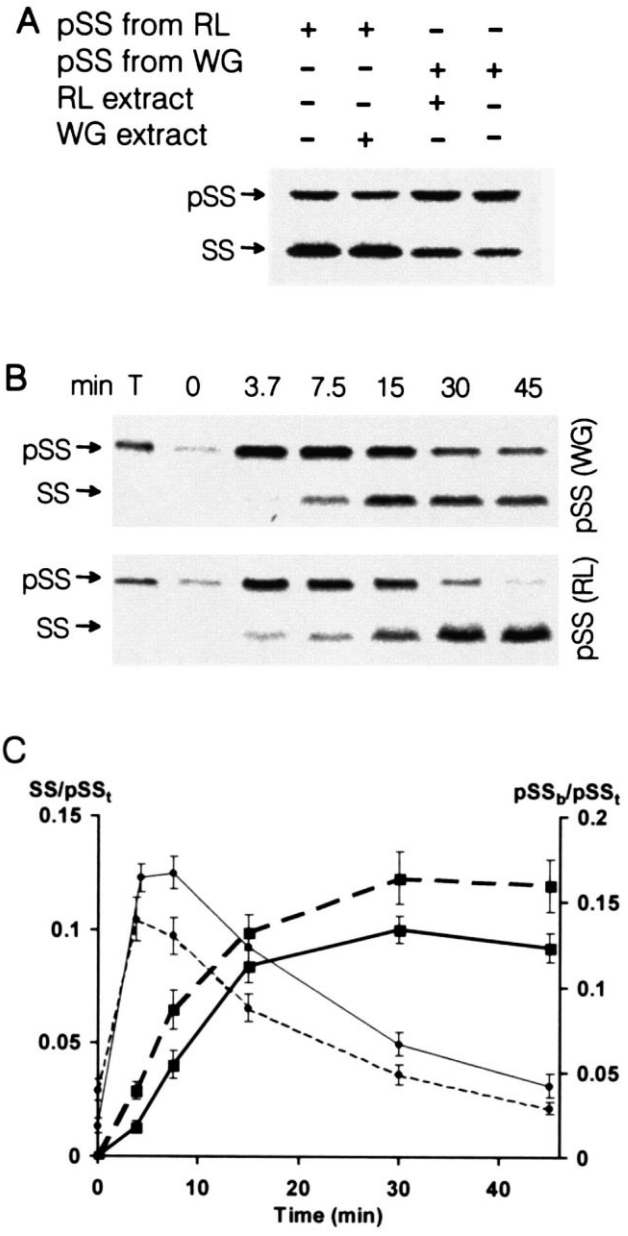

Fig. 4. Phosphorylation of pSS affects protein import into chloroplasts. A: Effect of the different translation systems on pSS import into chloroplasts. Autoradiogram of a SDS–PAGE. The pSS synthesised in RL or in WG was imported into chloroplasts with or without the addition of an aliquot of the other translation system as indicated. B and C: Time course of pSS import into chloroplasts. The pSS synthesised in WG or RL was imported into chloroplasts for varying time periods. The re-isolated, washed chloroplasts were solubilised for SDS–PAGE and autoradiography. The relative amounts of newly formed radioactive SS, pSS bound to the chloroplast surface (pSSb) and total amount of pSS added to incubation mixture (pSSt) were determined with a PhosphorImager. Lane T, in vitro synthesised radiolabelled pSS alone. Solid lines, pSS from WG; dashed lines, pSS from RL; thick lines, SS/pSSt; thin lines, pSSb/pSSt. Mean values and standard errors of three experiments are shown.

efficiencies of phosphorylated and non-phosphorylated pSS are different.

One reason for the different import efficiencies could be attributed to the different composition of the translation system still present in the import experiment. However, the im-

port efficiency of pSS into chloroplasts did not change when some WG was added to the import assay of non-phosphorylated pSS (Fig. 4A, first two lanes), or when the import of phosphorylated pSS occurred in the presence or absence of RL (Fig. 4A, last two lanes). Nevertheless, the time courses of import of pSS synthesised in RL or in WG are slightly different. Fig. 4B and C show that non-phosphorylated pSS was translocated faster across the envelope membrane than phosphorylated pSS. These data indicate that differences in pSS, most probably the different phosphorylation status of pSS, rather than different ingredients in the import assay, are the main reason for differences in the import efficiency of the two forms of pSS into chloroplasts from *Chlamydomonas*.

3.3. Phosphatase inhibitors can inhibit pSS import into chloroplasts

Since non-phosphorylated pSS was imported faster into chloroplasts than phosphorylated pSS, dephosphorylation of phosphorylated pSS during the import process seemed to be an essential step. If this is true, phosphatase inhibitors would influence import. As we did not know much about the phosphatase involved, in a preliminary experiment the effect of different phosphatase inhibitors, such as NaF, Na-vanadate and Na-molybdate, on the import of phosphorylated and non-phosphorylated pSS was tested (data not shown). NaF showed the most prominent differential effect on the import of the two forms of pSS. The import of phosphorylated pSS was reduced and completely blocked by 10 and 50 mM NaF, respectively, while a similar import inhibition of the non-phosphorylated pSS occurred only at a five-fold higher concentration, i.e. by 50 and 250 mM NaF, respectively (Fig. 5). At high inhibitor concentrations the import of both forms of pSS was blocked. At such high concentrations NaF might not only affect dephosphorylation of pSS, but also inhibit other enzymes reducing the ATP level and consequently chloroplast protein synthesis, which influences protein import. This could explain the small difference of NaF concentrations for inhibition of the phosphatase and protein import. The precursor protein phosphatase would represent a new component of the chloroplast import machinery, which remains to be identified.

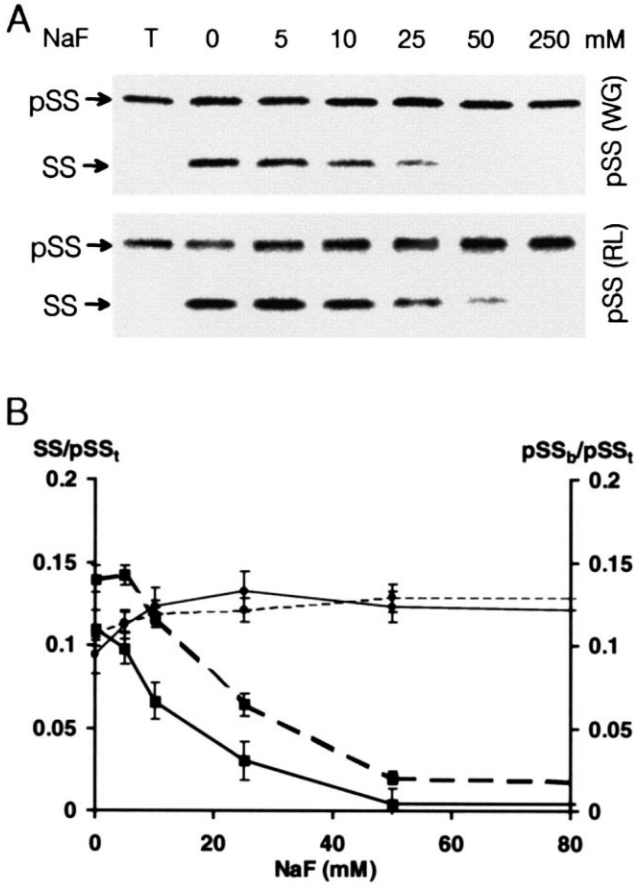

Fig. 5. Effect of phosphatase inhibitor NaF on import of phosphorylated and non-phosphorylated pSS into chloroplasts. The pSS synthesised in WG or RL was imported into chloroplasts in the presence of different concentrations of NaF as indicated. After incubation the chloroplasts were re-isolated, solubilised and analysed by SDS–PAGE and autoradiography. The relative amounts of newly formed radioactive SS, pSS bound to the chloroplast surface (pSSb) and total amount of pSS added to incubation mixture (pSSt) were determined with a PhosphorImager. Lane T, in vitro synthesised radiolabelled pSS alone. Solid lines, pSS from WG; dashed lines, pSS from RL; thick lines, SS/pSSt; thin lines, pSSb/pSSt. Mean values and standard errors of three experiments are shown.

4. Discussion

Up to now few common features have been detected in different plastid precursor proteins from the same or different species. In the present work we provide evidence that pSS from *Chlamydomonas* can be specifically phosphorylated within its transit peptide. In pea, it was found that phosphorylated precursor proteins can bind to the chloroplast import machinery, but must become dephosphorylated before being translocated across the envelope membranes. The protein kinase was detected in the cytosol of pea mesophyll cells and in WG, but not in RL. When pSS from tobacco was synthesised in WG, it formed an oligomeric guidance complex with other proteins, two of which were Hsp70 and a 14-3-3 protein [24]. The formation of the guidance complex required an intact phosphorylation site in the chloroplastic precursor protein. The pSS present in this complex was imported at a four-fold higher rate into chloroplasts than the non-bound free precursor form. These findings, together with the present results, suggest that the phosphorylation of precursor proteins is one of the common features for protein import into plastids. Phosphorylation, by inducing the complex formation of the precursor protein with a specific 14-3-3 protein, confers the specific binding of the precursor protein to the import machinery in the chloroplast envelope.

The physiological meaning of precursor phosphorylation/dephosphorylation is not clear yet. Since both forms of pSS bind to and are imported into chloroplasts, at a first glance the phosphorylation status of a precursor protein seems not to be of much importance, at least in vitro. Nevertheless, the present results indicate that import efficiency is modulated by the phosphorylation status and may reflect a regulation mechanism. This regulation may operate via the interaction of the 14-3-3 protein with the phosphorylated precursor protein, favouring the binding of precursors to the import receptor. Alternatively, the dephosphorylation could be rate limiting so that precursor proteins have to wait for import until they are dephosphorylated. If the activity of the precursor protein kinase or the phosphatase depends on the physiological state of the cells or chloroplasts, such a mechanism could

explain the cell cycle dependence of the protein import capacity in *Chlamydomonas* [31].

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