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Identification and solubilization of a signal peptidase from the phototrophic bacterium *Rhodobacter capsulatus*

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In Gram-negative bacteria, exported proteins are synthesized with an amino-terminal signal sequence which is cleaved off by the signal peptidase during, or shortly after the translocation process. Here, we report the identification and solubilization of a signal peptidase from the phototrophic bacterium *Rhodobacter capsulatus* which cleaves homologous and heterologous precursor proteins at the authentic cleavage site. This signal peptidase is the first identified component of the *R. capsulatus* protein export machinery.

Signal peptidase; Protein export; *Rhodobacter capsulatus*; Phototrophic bacteria

1. INTRODUCTION

Numerous proteins in pro- and eukaryotes must be translocated across cellular membranes in order to reach their site of function. Many of these proteins carry signal sequences that are removed by specific signal peptidases during, or shortly after membrane translocation. Signal peptidases have been identified in the endoplasmic reticulum and the bacterial plasma membrane, as well as in membranes and soluble compartments of mitochondria and chloroplasts [1].

According to the endosymbiont theory, mitochondria have developed from prokaryotic ancestors. This relationship is reflected by a conservation of the principles of protein translocation across the inner membranes of bacteria and mitochondria. Thus, nuclear encoded proteins are targeted to the intermembrane space of mitochondria by the COOH-terminal part of their bipartite signal sequence, which is very similar in structure to that of mitochondrially encoded proteins destined for the same localization. Both signals are highly homologous to the classical hydrophobic signal sequence of exported bacterial proteins [2]. A structural relatedness has also been found for the signal peptidase I (leader peptidase) of the *E. coli* plasma membrane and an inner mitochondrial membrane peptidase (IMP 1) [3], which processes precursor proteins directed to the intermembrane space and inner membrane of mitochondria [4]. Moreover, an *hsp60*-type chaperone is involved in the assembly of proteins of the inner mitochondrial membrane and intermembrane space [5] and possibly in the translocation of a few proteins across the bacterial plasma membrane of *E. coli* [6,7].

Except for these similarities it is not known to what extent the molecular mechanism underlying protein export from the matrix across the inner membrane of mitochondria has been conserved during evolution from the bacterial progenitor. Based on sequence comparisons of the 16S ribosomal RNA, members of the α-subgroup of purple bacteria, such as *Rhodobacter capsulatus*, are more closely related to the ancestral endosymbiont that gave rise to the mitochondrion, than *E. coli* [8]. No details are known, however, about the molecular mechanisms of protein export in α-purple bacteria. We have recently started to analyze membrane assembly of photosynthetic proteins of *R. capsulatus* in vitro [9]. As a first step towards the elucidation of the export machinery in this organism we describe here the identification and solubilization of a signal peptidase activity from intracytoplasmic membranes.

2. MATERIALS AND METHODS

2.1. *Strains and plasmids*

The *E. coli* K12 strain used was MZ9 [10]. The *R. capsulatus* strain was 37b4 [11]. The plasmids employed for in vitro synthesis of precursor proteins were pGAH317 (preSkp) [12] and pC2P2.71 [13].

2.2. *In vitro synthesis of precursor proteins*

The membrane-free cell extract of *E. coli* used for the in vitro synthesis of precursors was prepared as described [14]. Precursors were synthesized in vitro in a coupled transcription/translation reac-

Abbreviations: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; CHAPSO, 3-[(3-cholamidopropyl) dimethylammonio]-1-hydroxy-propane-1-sulfonate; TeaOAc, triethanolamine acetate; IPTG, isopropyl-1-thio-β-D-galactoside

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tion as described [14,15]. Where indicated, IPTG and cAMP were added to final concentrations of 0.4 mM and 0.5 mM, respectively.

2.3. *Preparation and solubilization of membranes from R. capsulatus*

Intracytoplasmic membranes (ICM) were isolated from cells grown on malate-yeast extract mineral medium [11] under semiaerobic conditions. Cells were harvested at an A_{600} of 1.5, washed once with 20 mM TeaOAc, pH 8, resuspended (1 ml/g cells) in the same buffer containing 0.5 mM phenylmethylsulfonyl fluoride and disrupted by two passages through a French pressure cell at 10,000 psi. The homogenate was separated from whole cells and debris by centrifugation at 30,000 × *g* for 20 min. The cell-free extract was layered on top of a discontinous sucrose gradient (1.5; 1.2; 0.9; 0.6 M sucrose in 20 mM TeaOAc, pH 8) and centrifuged for 16 h at 150,000 × *g* in a Beckman Ti60 rotor. The broad purple band of ICM was removed, diluted 6-fold with 20 mM TeaOAc pH 8 and the membranes were pelleted by centrifugation at 300,000 × *g* for 2 h. The pellet was resuspended in 250 mM sucrose, 50 mM TeaOAc pH 7.5, 1 mM EDTA, 1 mM DTT. A light membrane fraction representing the cytoplasmic membrane of *R. capsulatus* was prepared as previously described [16].

For solubilization, membranes at a final concentration of 2.5 mg membrane protein/ml were incubated on ice for 15 min in 25 mM TeaOAc pH 7.5, 20% glycerol and 10 mM CHAPS. Alternatively, 8 mM CHAPSO, 1.25% octylglucoside, 0.5–1% Tween 20, 0.5–2% deoxycholate or 1% Triton X-100 were used in place of CHAPS. The solubilization mixture was centrifuged in an airfuge in an A 100/18 rotor (Beckman Instruments, Fullerton, CA) at 30 psi for 60 min to separate solubilized and non-solubilized material. The supernatant was collected and the pellet was resuspended in the same volume of solubilization buffer. Both fractions were tested for processing activity.

2.4. *In vitro assay of* R. capsulatus *and* E. coli *signal peptidase activity*

Precursor proteins were synthesized in vitro and protein synthesis was terminated after 30 min with 0.44 mM puromycin. To assay the *R. capsulatus* signal peptidase activity, 25 μl of the transcription/translation mixture were mixed with the desired amount of enzyme (in most cases 14 μl of the supernatant or resuspended pellet described above) and adjusted to a final volume of 50 μl and a final concentration of 20% glycerol and 4 mM CHAPS. The samples were incubated at 32°C for 30 min and the reaction was stopped by addition of 1.5 vols. of 10% TCA.

2.5. *SDS-PAGE*

SDS-PAGE was performed with some modifications of the method of King and Laemmli [17]. SDS-PAGE in the presence of urea has been described elsewhere [18]. Gels were fluorographed by a modified method of Bonner and Laskey [19] and exposed to Fuji medical X-ray films. Quantitation of the radioactivity of a single polypeptide band was performed by excising the band from the gel, incubating it overnight at 50°C in Fluorosol (National Diagnostics, Manville, NJ, USA) and counting in a liquid scintillation counter.

3. RESULTS

Signal peptidase activity was solubilized from isolated intracytoplasmic membranes (ICM) of *R. capsulatus*. These membranes develop upon induction of the photosynthetic apparatus as invaginations of the cytoplasmic membrane, from which they can be separated by sucrose density gradient centrifugation following cell breakage. ICM were treated with different detergents (deoxycholate, Triton X-100, Tween 20, octylglucoside, CHAPS, CHAPSO) and detergent extracts and extracted membranes (supernatant and pelletable material, respectively) were separated by centrifugation and were then tested for signal sequence cleavage activity.

From the nucleotide sequences of the genes encoding cytochrome c_1 [20] and c_2 [13,21] of *Rhodospirillaceae* the existence of NH_2-terminal signal sequences showing the classical anatomy of *E. coli* plasma membrane targeting signals [22] has been deduced. On the premise that the signal peptidase of *R. capsulatus* might therefore be active towards heterologous precursor proteins from *E. coli*, we initially used the in vitro-synthesized precursor of Skp (preSkp), an *E. coli* protein whose signal sequence is cleaved during translocation to the periplasm [23], as a substrate for the detergent extracts of ICM.

While deoxycholate, Triton X-100 and Tween 20 extracts showed only little cleavage of preSkp, high rates of processing were obtained when membranes were solubilized with octylglucoside, CHAPSO or CHAPS at the critical micellar concentration of these detergents in the presence of 20% glycerol. Fig. 1A shows the result obtained for CHAPS-solubilized ICM. The 17-kDa ^{35}S-labeled preSkp was obtained by cell-free transcription/translation of the *skp*-gene using an *E. coli* cell extract devoid of membranes. Post-translational incubation with the CHAPS-extract or the extracted ICM led to conversion of the 17-kDa preSkp to a 15-kDa protein which has the same molecular mass as mature Skp purified from *E. coli* [24]. The main processing activity was found in the detergent extract (57% conversion to the 15-kDa form), while some remained associated with the extracted ICM (14% 15-kDa form).

To determine whether formation of the 15-kDa form of Skp by the CHAPS-solubilized membrane protease reflected authentic cleavage of the signal sequence, preSkp was synthesized in the presence of [^{3}H]valine, incubated with the CHAPS-extract of the ICM, and the processed product, recovered from SDS-PAGE, was subjected to sequential Edman degradation. Fig. 1B shows that the peaks of radioactive material appear in cycles 7 and 16, coinciding with the position of valine residues in the mature Skp of *E. coli* [12]. Thus, the solubilized processing activity of ICM cleaves the signal sequence of preSkp at the same site as does the *E. coli* signal peptidase in vivo and therefore is a signal peptidase of *R. capsulatus*.

In this assay the processing rate of preSkp increased with increasing amounts of solubilized peptidase added, reaching saturation at the amount used in the experiment shown in Fig. 1. Complete cleavage of preSkp was not obtained even after prolonged incubation. The *R. capsulatus* peptidase activity did not depend on divalent cations. When 20 mM EDTA was added to the assay sufficient to complex Mg^{2+} ions derived from the in vitro transcription/translation reaction of preSkp, the peptidase activity remained unchanged (not shown).

We next asked whether the solubilized signal peptidase would be active also on homologous substrates. One of the few proteins of *Rhodospirillaceae* known to harbour a cleavable signal sequence is cy-

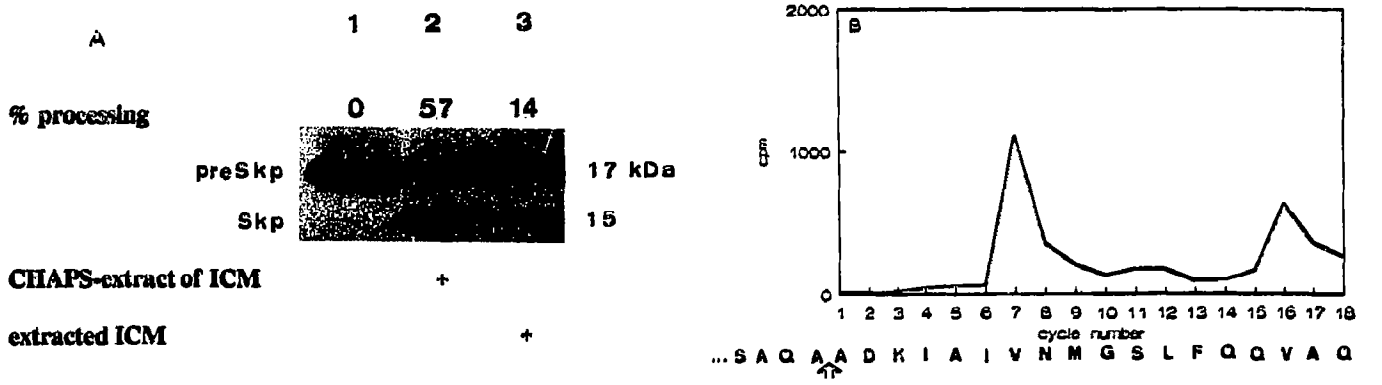

Fig. 1. Solubilization of signal-peptidase activity from intracytoplasmic membranes of *R. capsulatus*. Panel A: PreSkp (17kDa) was synthesized in vitro using a membrane-free supernatant of an *E. coli* homogenate programmed with the *skp*-gene-containing plasmid pGAH317 (lane 1). Reaction mixtures were further incubated with the supernatant (lane 2) or the pelletable material (lane 3) derived from a 135,000 × *g* centrifugation of CHAPS-treated intracytoplasmic membranes (ICM). [^{35}S]methionine-labeled translation and cleavage products were resolved by SDS-PAGE and visualized by fluorography. To determine the relative cleavage activity, bands were excised from the gel, and the radioactivity was determined. Indicated is the percentage of Skp with the sum of preSkp and Skp set at 100%. Panel B: PreSkp was synthesized in vitro in the presence of [^{3}H]valine and incubated with the CHAPS-extract of intracytoplasmic membranes. Precursor and processed Skp were separated by SDS-PAGE and blotted on a polyvinylidendifluoride-membrane (Millipore, Bedford). The processed form was localized on the membrane via autoradiography of ^{35}S-labeled marker Skp and was excised from the membrane and subjected to automated NH_2-terminal sequencing (477A protein sequencer, Applied Biosystems). Radioactivity in the products of each cycle was determined by liquid scintillation counting. Below the graph part of the amino acid sequence of preSkp is shown. The arrow indicates the cleavage site of the signal sequence.

tochrome c_2 [13,21]. Plasmid pC2P2.71 containing the cytochrome c_2-gene of *R. sphaeroides* under the control of the *lac* promoter [13] was used to program the cell-free transcription/translation system from *E. coli*. As illustrated in Fig. 2A, lane 2, two major translation products of pC2P2.71 were obtained, which exhibited apparent molecular masses of 29 kDa and 17 kDa on the SDS/urea-gels used. The 29-kDa protein is presumably the vector-encoded *bla* product. The synthesis of the 17-kDa protein was immensely stimulated by cAMP and IPTG (lane 3) indicating that it was expressed from a *lac* promoter-dependent gene. In contrast to preBla it was cleaved by the CHAPS-extract of ICM to a smaller form of 15.5 kDa (lane 4). When this 15.5-kDa protein labeled with [^{3}H]Glu was subjected to automated Edman degradation the major radioactivity was recovered from cycles 2 and 6 (Fig. 2B). These are precisely the positions where the amino acid sequence of mature cytochrome c_2 [13] predicts the occurrence of Glu. Thus, the solubilized signal peptidase also correctly processes a homologous substrate of purple bacteria. The higher values for the apparent molecular masses of precursor and mature cytochrome c_2 compared to previous reports [13] are probably due to the different gel systems used.

4. DISCUSSION

We have solubilized a signal peptidase activity from membranes of the α-purple bacterium *R. capsulatus*. The peptidase processes the in vitro synthesized precursors of the exported proteins Skp of *E. coli* and cytochrome c_2 of *R. sphaeroides* at the authentic sites. Whereas preOmpA of *E. coli* was also cleaved by the enzyme, other preproteins (Bla and LamB of *E. coli*, cytochrome c_1 and small subunit of hydrogenase of *R. capsulatus*) were not (not shown). Furthermore, among those cleaved, the extent to which cleavage occurred in vitro varied with the substrate used (cf. Figs. 1A and 2A). The different cleavage susceptibility of the precursor proteins examined most likely does not reflect a substrate specificity of the *R. capsulatus* enzyme but is rather due to a tight tertiary structure of some of the in vitro synthesized precursors not exposing the signal sequence cleavage site. The difficulty to render substrates susceptible to in vitro processing by signal peptidases has been reported by several authors studying different peptidases [4,25,26].

The close relationship between *Rhodobacter* and mitochondria is indicated by the high structural homology of electron carriers such as the cytochrome bc_1 complex and cytochrome c_2 [2]. It is therefore conceivable that the signal peptidase of *R. capsulatus* resembles the signal peptidase of the inner mitochondrial membrane (IMP1). IMP1 differs from the *E. coli* signal peptidase in that it appears to have more than one subunit [4]. On the other hand, both enzymes have significant sequence homology [3]. Since in mutants lacking IMP1 the precursor of the mitochondrially encoded cytochrome c_1 is still correctly processed [27] it is likely that the mitochondrial inner membrane possesses a second signal peptidase activity. Further analysis of the *R. capsulatus* peptidase and the signal peptidases of the mitochondrial inner membrane should make it possible to address the question of whether the mitochondrial peptidases have close bacterial analogues or whether they further

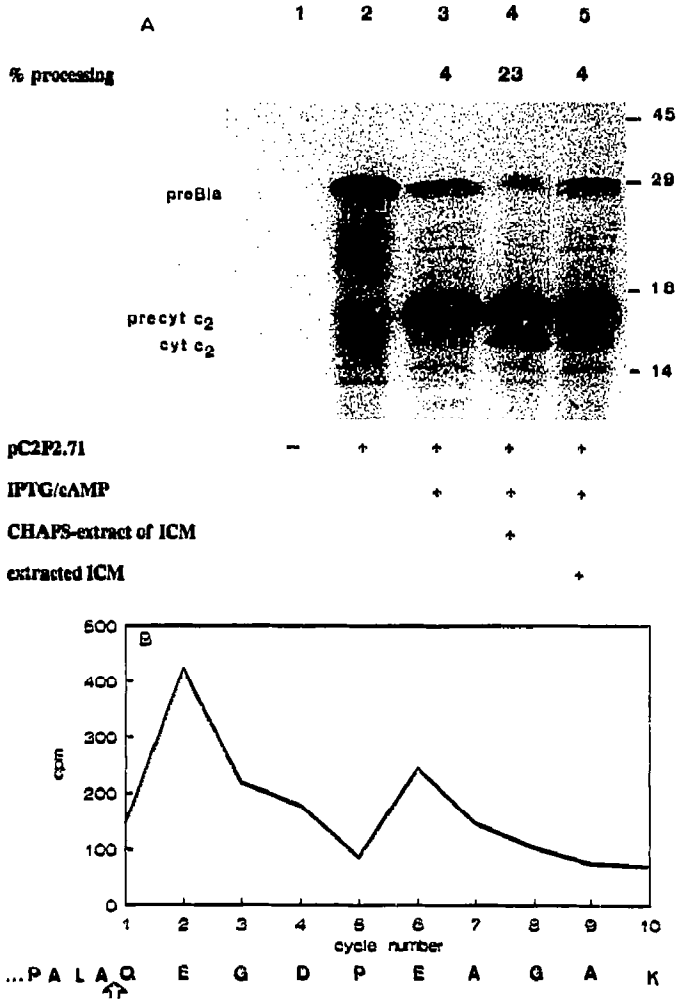

Fig. 2. The precursor of *R. sphaeroides* cytochrome c_2 is correctly processed by *R. capsulatus* signal peptidase. Panel A: plasmid pC2P2.71 encoding the precursor of β-lactamase (preBla) and cytochrome c_2 (precyt c_2) was used to program the cell-free transcription/translation system from *E. coli*. Expression of precyt c_2 was significantly enhanced by addition of cAMP and IPTG. This precursor was partially processed by the CHAPS-extract of ICM. Proteins were resolved by SDS-PAGE in the presence of urea. Numbers to the right indicate the molecular masses of marker proteins. Panel B: radiosequencing of [^{3}H]Glu-labeled processed cyt c_2. For experimental details see legend to Fig. 1.

evolved into new proteins after the mitochondrial ancestors populated the eukaryotic progenitor.

Signal peptidase activity was also solubilized from a light membrane fraction which is resolved on sucrose density gradients from ICM and which represents the cytoplasmic membrane of the *R. capsulatus* membrane system (not shown). Roughly the same preSkp cleavage activity was extracted from equivalent amounts of cytoplasmic membranes and ICM (based on protein content). For the closely related organism *R. sphaeroides* it has been shown that newly synthesized B875 reaction center cores of the photosynthetic apparatus predominantly incorporate into a distinct cytoplasmic membrane fraction from which they are then transferred into the ICM [28]. Dierstein et al. [16] also found preferential integration of the reaction center polypeptides into a light, non-ICM membrane fraction in *E. capsulatus*. Nevertheless, the finding that the *R. capsulatus* signal peptidase was solubilized from different fractions of the *R. capsulatus* membrane system suggest that the protein translocation machinery is located both in the cytoplasmic membrane and in the ICM. The solubilization of the signal peptidase from *R. capsulatus* is a first step in the analysis of the protein export process of phototrophic bacteria.

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REFERENCES

[1] Müller, M. (1992) Experientia, in press.
[2] Hartl, F.-U. and Neupert, W. (1990) Science 247, 930–938.
[3] Behrens, M., Michaelis, G. and Pratje, E. (1991) Mol. Gen. Genet. 228, 167–176.
[4] Schneider, A., Behrens, M., Scherer, P., Pratje, E., Michaelis, G. and Schatz, G. (1991) EMBO J. 10, 247–254.
[5] Cheng, M.Y., Hartl, F.-U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L. and Horwich, A.L. (1989) Nature 337, 620–625.
[6] Bochkareva, E.S., Lissin, N.M. and Girshovich, A.S. (1988) Nature 336, 254–257.
[7] Kusukawa, N., Yura, T., Ueguchi, C., Akiyama, Y. and Ito, K. (1989) EMBO J. 8, 3517–3521.
[8] Woese, C.R. (1987) Microbiol. Rev. 51, 221–271.
[9] Troschel, D. and Müller, M. (1990) J. Cell. Biol. 111, 87–94.
[10] Swidersky, U.E., Hoffschulte, H.K. and Müller, M. (1990) EMBO J. 9, 1777–1785.
[11] Dierstein, R.A. (1984) Eur. J. Biochem. 138, 1–11.
[12] Holck, A. and Kleppe, K. (1988) Gene 67, 117–124.
[13] Donohue, T.J., McEwan, A.G. and Kaplan, S. (1986) J. Bacteriol. 168, 962–972.
[14] Müller, M. and Blobel, G. (1984) Proc. Natl. Acad. Sci. USA 81, 7421–7425.
[15] Müller, M., Fisher, R.P., Rienhöfer-Schweer, A. and Hoffschulte, H.K. (1987) EMBO J. 6, 3855–3861.
[16] Dierstein, R., Schumacher, A. and Drews, G. (1981) Arch. Microbiol. 128, 376–383.
[17] King, J. and Laemmli, U.K. (1971) J. Mol. Biol. 62, 465–477.
[18] Krishnabhakdi, S.S. and Müller, M. (1988) FEBS Lett. 231, 99–101.
[19] Bonner, W.M. and Lasky, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[20] Gabellini, N. and Sebald, W. (1986) Eur. J. Biochem. 154, 569–579.
[21] Daldal, F., Cheng, S., Applebaum, J., Davidson, E. and Prince, R.C. (1986) Proc. Natl. Acad. Sci. USA 83, 2012–2016.
[22] von Heijne, G. (1990) J. Membr. Biol. 115, 195–201.
[23] Thome, B.M. and Müller, M. (1991) Mol. Microbiol. 5, 2815–2821.
[24] Thome, B.M., Hoffschulte, H.K., Schiltz, E. and Müller, M. (1990) FEBS Lett. 269, 113–116.
[25] Lively, M.O. and Walsh, K.A. (1981) Proc. Fed. Soc. Exp. Biol. 40, 70–75.
[26] YaDeau, J.T. and Blobel, G. (1989) J. Biol. Chem. 264, 2928–2934.
[27] Pratje, E. and Guiard, B. (1986) EMBO J. 5, 1313–1317.
[28] Reilly, P.A. and Niederman, R.A. (1986) J. Bacteriol. 167, 153–159.