Volume 284, number 2, 169–172 FEBS 09825 June 1991

ADONIS 0014579391005348

Subcellular distribution of enzymes involved in the biosynthesis of cyanelle murein in the protist *Cyanophora paradoxa*

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Received 11 March 1991; Revised version received 22 April 1991

Cyanelle containing organisms, notably *Cyanophora paradoxa*, the best studied among them, are unique with respect to the occurrence of peptidoglycan (murein) within an eukaryotic cell. Enzyme activities involved in the biosynthesis of UDP-N-acetyl-muramylpentapeptide could be localized within the cyanelle compartment. Some of the enzymes performing later steps of murein biosynthesis were detected in the postcyanelle supernatant rather than in the cyanelle lysate. This is taken to reflect a 'periplasmic' location of these enzymes that are partially liberated upon rupture of the cyanelle outer membrane.

Cyanophora paradoxa; Cyanelle; Peptidoglycan biosynthesis

1. INTRODUCTION

Cyanelles, the photosynthetic organelles of the biflagellated protist *Cyanophora paradoxa*, constitute an extant example for an intermediate stage in the evolution of plastids from cyanobacterial invaders according to the endosymbiotic theory [1]. The cyanelle genome corresponds in size to plastid DNAs and shows characteristics of both plastidic and cyanobacterial gene organization [2]. Notably, a surplus of at least 20 genes not encountered on higher plant plastid genomes has been found [2] (C. Michalowski et al., unpublished; D. Bryant, personal communication). The unique structural feature of the cyanelles is the retainment of a murein wall [3,4]. This is in accordance with the observed sensitivity of *C. paradoxa* against β-lactam antibiotics and with the occurrence of 7 penicillin-binding proteins (PBPs) in the cyanelle envelope [5,6]. Thus the later steps of murein biosynthesis seem to correspond to those reported for *Escherichia coli* [7]. However, concerning the earlier steps, i.e. the pathway leading to UDP-N-acetyl-muramyl-pentapeptide (UDP-mur-penta) that is localized in the cytoplasm in *E. coli* [7], two possibilities exist for *C. paradoxa*. Either the biosynthesis of UDP-mur-penta occurs within the cyanelles and the product is then translocated across the inner membrane, or this 'building block' is synthesized in the cytoplasmic space and is then bound to the lipid carrier either at the periplasmic or at the stromal face of the inner membrane. In the former case the presence in cyanelles of another 9 enzymes not found in plastids would be necessary in addition to the PBPs. This means that the respective polypeptides have to be coded for by the cyanelle genome, or nucleus-encoded precursors have to be imported from the cytoplasm. At present nothing is known about gene loci for the enzymes involved in the biosynthesis of cyanelle murein. In this paper we present data in support of UDP-mur-penta synthesis within the cyanelle, and also some results on the subcellular localization of enzymes acting on macromolecular murein.

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2. MATERIALS AND METHODS

2.1. *Growth of C. paradoxa, isolation of cyanelles and preparation of cytoplasmic and cyanelle extracts*

Cultivation of *C. paradoxa* 555 UTEX and isolation of cyanelles was as described [5], except that *Cyanophora* cells harvested in the logarithmic growth phase were opened by freeze-thawing instead of osmotic shock. The cell-free extract was subjected consecutively to low (2000 r.p.m., 2 min) and high (14000 r.p.m., 20 min) speed centrifugations in an Eppendorf desk-top centrifuge. The high speed supernatant constituted the cytoplasmic extract, i.e. the postcyanelle supernatant freed from other cell organelles. The low speed pellet (cyanelle pellet) was suspended in 50 mM Tris/HCl pH 8.0, treated for 5 min with 200 μg/ml lysozyme at 4°C, and sonicated for 10 s. After centrifugation (10000 r.p.m., 15 min) the cyanelle extract was used for assays of soluble enzymes. To test the activities of DD-carboxypeptidase I, LD-carboxypeptidase II, and DD-endopeptidase, Triton X-100 (1% final concentration) was added to the cyanelle lysate prior to centrifugation. All enzyme extracts were adjusted to a protein concentration of 20 mg/ml as determined by the method of Bradford [8].

2.2. *Preparation of enzyme substrates and reference standards*

UDP-N-acetyl-muramyltripeptide and UDP-mur-penta were purified from cultures of *Bacillus cereus* treated with D-cycloserin or vancomycin, respectively, by the procedure of Flouret et al. [9] and identified by comparison on HPLC with authentic standards. [^{14}C]-D-ala-D-ala was prepared by incubation of [^{14}C]-D-ala (Amersham International, Harpenden, U.K.) with an enzyme extract obtained from *E. coli B* [10]. Tritium labelled muropeptides: disaccharide-pentapeptide, disaccharide-tetrapeptide, disaccharide-tripeptide and bis(disaccharide-tetrapeptide), were purified by HPLC [11] of muramidase digested murein from *E. coli* JE 5684, a DD-carboxypeptidase deficient strain [12], grown in the presence of [^{3}H]-*meso*-diaminopimelic acid (625 μCi/mg, 4 μg/ml) (Service des Molécules Marquées, Commissariat à l'Energie Atomique, Gif-sur-Yvette, France). [^{14}C]-UDP-N-acetyl-D-glucosamine was purchased from Amersham International. Phosphoenolpyruvate, N-acetyl-muramic acid, D-ala-D-ala, phosphonomycin, vancomycin and D-cycloserine were obtained from Sigma Chemical Co. (München, Germany).

2.3. *Enzyme assays*

UDP-N-acetyl-muramic acid (UDP-mur) biosynthesis and D-ala-D-ala adding enzyme were assayed as described [10,13], incubating 100 μl of the respective enzyme extracts with 50 nCi of [^{14}C]-UDP-N-acetyl-glucosamine (236 mCi/mmol), or 30 nCi of [^{14}C]-D-ala-D-ala (1 mCi/mmol), respectively, at 25°C (37°C for *E. coli* extracts) for 14 h unless stated otherwise. To maximize formation of UDP-mur the concentration of NADPH had to be increased to 10 mM. Activity of carboxypeptidases I and II was determined in 10 mM Tris/HCl, 10 mM $MgSO_4$, pH 7.2, containing 13 nCi of [^{3}H]disaccharide-pentapeptide (1.2 μCi/mg) or 100 nCi of [^{3}H]disaccharide-tetrapeptide (4 μCi/mg), respectively. DD-endopeptidase was assayed in 10 mM Tris/maleate, 10 mM $MgSO_4$, pH 7.25, containing 40 nCi (2 μCi/mg) of [^{3}H]bis(disaccharide-tetrapeptide). Reaction mixtures were incubated at 25°C (37°C for *E. coli* extracts) for 14 h. Reaction products and substrates were separated by paper chromatography, together with labelled standards, on Schleicher & Schüll 2043 BMGL paper developed with ethanol:water (80:20). Dried chromatograms were evaluated using a radioactivity linear analyzer (Berthold, Wildbad, Germany) and subjected to autoradiography after treatment with EN^{3}HANCE spray (NEN Research Products, Dreieich, Germany).

3. RESULTS

To date, no enzymatic activities involved in murein biogenesis have been determined for *C. paradoxa*. Cyanelle PBPs were detected by binding of a radioactive β-lactam antibiotic rather than through enzyme assays [5]. Now we investigated the activities of the first and last enzymes involved in the biogenesis of UDP-mur-penta in cyanelle extracts and cytoplasmic preparations from *C. paradoxa*, respectively. UDP-mur biosynthesis appeared to be confined to the cyanelle stroma (Table I). Elevated concentrations of phosphonomycin had an inhibitory effect on the reaction. Exclusion of NADPH from the reaction mixture also diminished the yield, obviously by reducing the conversion of the intermediate product UDP-3-enol-pyruvyl-N-acetyl-D-glucosamine.

An analogous subcellular distribution was found for the D-ala-D-ala adding enzyme in *C. paradoxa* (Fig. 1). This enzymatic activity was also localized exclusively within cyanelles. Under our test conditions 16% incorporation of D-ala-D-ala into UDP-mur-penta was observed. This activity was about half of that obtained with an *E. coli* extract (30% incorporation). D-cycloserine (1 mg/ml) had an unexpected inhibitory effect on the cyanelle enzyme reducing incorporation of D-ala-D-ala by 75%, whereas the *E. coli* enzyme was unaffected.

Table I

Synthesis of UDP-N-acetyl-muramic acid by subcellular fractions from *C. paradoxa*

Fraction	Incubation conditions	Substrate converted (%)
Cyanelle lysate	20 min	5.0
	90 min	14.5
	14 h	57.2
	14 h – NADPH	10.1
	14 h + 50 μg/ml PM[a]	50.6
	14 h + 500 μg/ml PM	19.3
	14 h, 37°C	8.2
Cytoplasm	14 h	Not detectable

Substrate saturation occurred at a concentration of phosphoenolpyruvate 4× higher than for *E. coli* extracts

[a] Phosphonomycin

The distribution of enzymes acting on macromolecular peptidoglycan in *C. paradoxa* was somewhat ambiguous. Assays were performed for DD-carboxypeptidase I and LD-carboxypeptidase II, responsible for trimming of peptide side chains of newly synthesized murein by removal of the terminal and the penultimate D-ala residues, respectively [7]. In addition, labelled bis(disaccharide-tetrapeptide) was used for the detection of DD-endopeptidase, an enzyme involved in murein degradation [7].

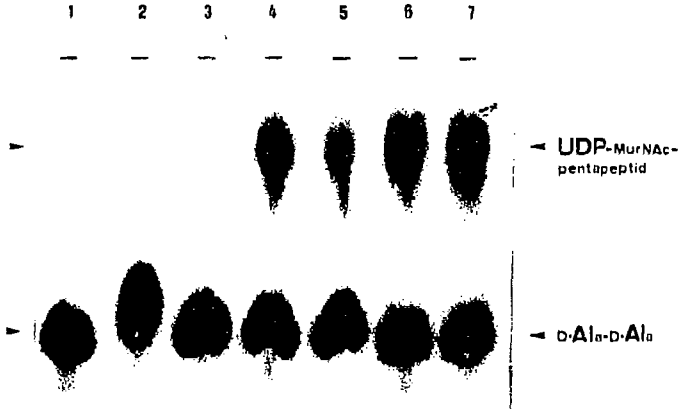

Fig. 1. Assay for D-ala-D-ala adding enzyme in subcellular fractions from *C. paradoxa*. Labelled UDP-mur-penta formed by incorporation of [^{14}C]-D-ala-D-ala was separated from the substrate by paper chromatography and visualized by autoradiography. Lanes: 1, blank; 2, cytoplasm; 3, cytoplasm + 1 mg/ml D-cycloserine; 4, cyanelle lysate; 5, cyanelle lysate + 1 mg/ml D-cycloserine; 6, *E. coli* extract; 7, *E. coli* extract + 1 mg/ml D-cycloserine.

Table II

Subcellular distribution of DD-carboxypeptidase I, LD-carboxypeptidase II, and DD-endopeptidase activities in *C. paradoxa*

Enzyme activity	Fraction	Substrate[a]	Substrate converted (%)
DD-carboxy-peptidase I	Cytoplasm		58
	Cytoplasm + PG[b]		< 5
		Penta	
	Cyanelle lysate		< 10
	Cyanelle lysate + PG		N.D.[c]
LD-carboxy-peptidase II	Cytoplasm		65
	Cytoplasm + PG		68
		Tetra	
	Cyanelle lysate		70
	Cyanelle lysate + PG		62
DD-endopep-tidase	Cytoplasm		20
	Cytoplasm + PG		N.D.
		Tetra-Tetra	
	Cyanelle lysate		N.D.
	Cyanelle lysate + PG		N.D.

[a]Substrate abbreviations: Penta, disaccharide-pentapeptide; Tetra, disaccharide-tetrapeptide; Tetra-Tetra, bis(disaccharide-tetrapeptide)
[b] PG, 2 mg/ml penicillin G
[c] N.D., not detectable

As shown in Table II, DD-carboxypeptidase I activity was higher in the cytoplasmic fraction than in the cyanelle lysate. LD-carboxypeptidase II activity was evenly distributed between both fractions. DD-endopeptidase activity appeared to be confined to the cytoplasm of *C. paradoxa*. The sensitivity to penicillin G was in accordance with that reported for the respective enzymes from *E. coli* [7].

4. DISCUSSION

The overall composition of cyanelle murein corresponds to that of Gram negative bacteria [4]. However, attempts to detect covalently bound proteins as lipoprotein, either by SDS gel electrophoresis of muramidase-treated peptidoglycan supernatants, or by analysis of muropeptides (B. Pfanzagl et al., manuscript in preparation), consistently gave negative results. This might explain why the outer membrane of cyanelles is so easily damaged during their isolation [14]. Consequently in vitro photosynthetic activity [1], protein synthesis, and protein import (M. Brandtner, J. Jakowitsch, unpublished) are completely abolished or severely impeded as compared to e.g. isolated pea chloroplasts. Nevertheless, isolated cyanelles are stabilized by the murein sacculus, retain the blue phycobiliproteins, and seem to be intact by the criterium of phase contrast microscopy.

The data presented here indicate that conversion of UDP-N-acetyl-D-glucosamine into UDP-mur-penta occurs exclusively in the cyanelle stroma, suggesting that compartmentalization of murein synthesis in cyanelles and eubacteria is identical. A peculiarity of the D-ala-D-ala adding enzyme from cyanelles is its inhibition by D-cycloserine, an antibiotic with no effect on this particular enzyme in *E. coli* [15].

The interpretation of results on the subcellular distribution of carboxypeptidases I and II, and DD-endopeptidase was hampered by technical difficulties to isolate cyanelles with an intact outer membrane, suitable to prevent leakage of periplasmic enzymes into the cytoplasmic fraction. Nevertheless, our results might give an idea about the degree of association of these enzymes to the membranes of the cyanelle envelope. Both DD-enzymes, carboxypeptidase I as well as endopeptidase, seem to be only loosely associated to the membranes, whereas LD-carboxypeptidase II apparently is bound more firmly. In our opinion, presence of these enzymes in the cytoplasmic compartment of *C. paradoxa* could be caused by leakage of periplasmic material from the cyanelles following fragmentation of their outer membrane. This situation is just the opposite to that most frequently found in eubacteria where the DD-activities, normally connected with PBPs, are membrane-bound and LD-carboxypeptidase II is a soluble, periplasmic protein [7]. However, PBPs not associated with membranes have been described in some organisms, notably in *Caulobacter crescentus* [16].

If the DD-enzymes studied were PBPs, as seems most likely in view of the inhibitory effect of penicillin G, they would have to be either soluble, or rather weakly bound to the cyanelle membranes. Preliminary evidence from in vitro studies on cyanelle PBPs is in support of this hypothesis [5].

In total a complement of about 20 enzymes is necessary for the synthesis of the cyanelle wall. In the absence of any knowledge on the location of the respective genes, it seems nevertheless improbable that all of them are nuclear. If a cyanelle gene product has to be targeted to the periplasm a protein translocation apparatus in the inner envelope membrane is required. The recent finding of a cyanelle gene with homology to eubacterial *secY* (C. Michalowski et al., manuscript in preparation) supports this speculation.

Acknowledgements: We thank Drs. J.-V. Höltje (M.P.I., Tübingen) and J. van Heijenoort (Univ. Paris-Sud) for providing us with HPLC standards. This work was supported by grants from the 'Fonds zur Förderung der Wissenschaftlichen Forschung' (P7832-CHE, W.L.) and from CICYT (BIO88-0251-C03-3, M.A.P.). We are most grateful for travel money obtained through the 'Wissenschaftlich-Technische Kooperation 'Osterreich-Spanien' (Project A.2).

REFERENCES

[1] Wasmann, C.C., Löffelhardt, W. and Bohnert, H.J. (1987) in: The Cyanobacteria (Fay, P. and van Baalen, C., eds) pp. 303-324. Elsevier, Amsterdam.
[2] Bohnert, H.J. and Löffelhardt, W. (1991) in: Algal Symbiosis (Reisser, W., ed) Springer, Heidelberg, in press.
[3] Schenk, H.E.A. (1970) Z. Naturforsch. 25b, 656.
[4] Aitken, A. and Stanier, R.Y. (1979) J. Gen. Microbiol. 212, 218-223.
[5] Berenguer, J., Rojo, F., De Pedro, M.A., Pfanzagl, B. and Löffelhardt, W. (1987) FEBS Lett. 224, 401-405.
[6] Kies, L. (1988) Endocyt. C. Res. 5, 361-372.
[7] Höltje, J.-V. and Schwarz, U. (1985) in: Molecular Cytology of *Escherichia coli* (Nanninga, N. ed.) pp. 77-119. Academic Press, London.
[8] Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
[9] Flouret, B., Mengin-Lecreulx, D. and Van Heijenoort, J. (1981) Anal. Biochem. 114, 59-63.
[10] Mengin-Lecreulx, D., Flouret, B. and Van Heijenoort, J. (1982) J. Bacteriol. 151, 1109-1117.
[11] Glauner, B., Höltje, J.-V. and Schwarz, U. (1988) J. Biol. Chem. 263, 10088-10095.
[12] De Pedro, M.A., Schwarz, U., Nishimura, Y. and Hirota, Y. (1980) FEMS Microbiol. Lett. 9, 219-221.
[13] Mengin-Lecreulx, D., Flouret, B. and Van Heijenoort, J. (1983) J. Bacteriol. 154, 1284-1290.
[14] Giddings, Jr., T.H., Wasmann, C.C. and Staehelin, A.R. (1983) Plant Physiol. 71, 409-419.
[15] Gale, E.F., Cundliffe, E., Reynolds, P.E., Richmond, M.H. and Waring, M.G. (1981) The molecular basis of antibiotic action. Wiley, London.
[16] Koyasu, S., Fukuda, A. and Okada, Y. (1982) J. Gen. Microbiol. 128, 1117-1124.