Biochimica et Biophysica Acta, 1107 (1992) 39–43

BBAMEM 75640

Isoprenoid modification of proteins distinct from membrane acyl proteins in the prokaryote *Acholeplasma laidlawii*

Susanne Nyström and Åke Wieslander

Department of Biochemistry, University of Umeå, Umeå (Sweden)

(Received 31 December 1991)

Key words: Isoprenylation; Acyl chain; Eubacterial protein; (*A. laidlawii*)

Isoprenylation is an important posttranslational modification that affects the activity, subunit interactions and membrane anchoring of different eukaryotic proteins. The small, cell-wall-less prokaryotic *Acholeplasma laidlawii* has more than 20 membrane acyl-proteins enriched in myristoyl and palmitoyl chains. Radioactive mevalonate, a precursor to isoprenoids, was incorporated into several specific membrane proteins of 20 to 45 kDa and two soluble proteins of 23–25 kDa, respectively. No acyl proteins and none of the polar acyl lipids became labelled but these are all labelled by radioactive fatty acids. Mevalonate was incorporated mainly into a minor neutral, non-saponifiable lipid which migrated just above a C_{30}-isoprenoid (squalene) on TLC-plates. The isoprenoid chains could not be released by mild alkaline hydrolysis from most of the isoprenylated proteins, although this procedure releases acyl chains from lipids and all acylated proteins. Isoprenylated proteins were enriched in the detergent phase upon partition with the non-ionic detergent Triton X-114. This behaviour is similar to the acyl proteins of this organism and indicates that the isoprenoid chains give the proteins a hydrophobic character.

Introduction

Mycoplasmas are small, Gram-positive prokaryotes which lack a typical bacterial cell wall and are enclosed only by a single cytoplasmic membrane [1]. Most species, including *Acholeplasma laidlawii* [2,3], are found as surface parasites on eukaryotic cells. Several mycoplasmas contain a substantially larger number of lipid-modified membrane (acyl) proteins than do the common eubacteria [4–8]. In *A. laidlawii* these integral proteins are enriched in hydrophilic amino acid residues and have lower p*I* values than average for membrane proteins [9]. The extent of acyl modification is substantially less [9] than for the common acylation mechanism in prokaryotes, which yields three acyl chains per protein [10]. Furthermore, these proteins could not be labelled with a (radioactive) glycerol backbone like many other prokaryotic acyl proteins [10]. Neither do they contain sugar residues [9] nor phosphate [11] like eukaryotic protein glycophospholipid tails [12]. In the *A. laidlawii* proteins there is a strong and selective enrichment of ester-bound myristic acid (14:0) and palmitic acid (16:0) compared to the acyl chain distribution in the membrane lipids [9], similar to the chain preferences found in eukaryotic acyl proteins [13,14].

These features of the *A. laidlawii* acyl proteins are in several aspects similar to eukaryotic palmitoylated proteins. Several of the latter are also modified by isoprenoid chains [15]. The function of the isoprenylation is not clear since some modified proteins are soluble [16] while others are membrane-associated [17]. For certain isoprenylated proteins an additional attachment of acyl chains is needed for a proper membrane localization [17]. In contrast to most other mycoplasmas, *A. laidlawii* can synthesize isoprenoid pigments from acetate or mevalonate [18]. Cholesterol partially inhibits this synthesis in *A. laidlawii* [18,19], while there is a complete inhibition by cholesterol of the isoprenoid synthesis in eukaryotic cells [20].

We therefore raised the question if *A. laidlawii* proteins also contain isoprenoid chains. It is shown here that radioactive mevalonate could covalently label a unique but small set of membrane as well as cytosolic proteins. These proteins are clearly distinct from the ones modified by acyl chains. To the best of our knowledge this is the first time isoprenoid modification of eubacterial proteins have been described.

Abbreviations: 18:1c, oleic acid; 16:0, palmitic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Correspondence: S. Nyström, Department of Biochemistry, University of Umeå, S-901 87 Umeå, Sweden.

Materials and Methods

Organism and growth conditions

Acholeplasma laidlawii, strain A EF 22, was grown in a thoroughly lipid-depleted bovine serum albumin/tryptose medium [21]. 120 μM oleic acid (18:1c) and 30 μM palmitic acid (16:0) were added to this medium from sterile ethanolic stock solutions. For comparison, a completely different, not lipid-depleted medium containing horse-serum (BSR medium) [22] adjusted to pH 8.5 was used. Radioactive batches were prepared by adding 10-ml amounts of the above media to 740 kBq (20 μCi) of [2-^{14}C]mevalonic acid dibenzylethylenediamine salt (1.9 GBq/mmol) or 740 kBq of [1-^{14}C]-18:1c (2.1 Gbq/mmol) and 2.2 MBq of [9,10(n)-^{3}H]-16:0 (1.1 TBq/mmol). After 1 h of stirring the media were sterilized by filtration. Cells were grown statically at 30°C in screw-capped tubes to late-log phase. Membrane preparation was done by osmolysis as described [23]. The cytoplasmic fraction was collected as the supernatant after centifugation of the lysate at 52000 × *g* for 1 h at 5°C. 16:0 and 18:1c were from Larodan, Sweden. All radioisotopes were from Du Pont/New England Nuclear.

SDS-gel electrophoresis of proteins

Triton X-114 solubilization and temperature phase separation of *A. laidlawii* membrane or cytoplasmic fractions were performed essentially as described by Bordier [24] with minor modifications [9]. The proteins from Triton X-114 phase-partitioned *A. laidlawii* fractions were analysed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and direct autoradiography (Hyperfilm β-max, Amersham International) or fluorography as described [23]. Alkaline hydrolysis of separated proteins in SDS-PAGE gels was done as described [9].

Extraction and separation of lipids

Washed membranes were extracted twice with 20 volumes of chloroform/methanol (2:1, v/v) for 1 h at 22°C and centrifuged at 15000 × *g* for 15 min at 4°C to remove protein remains. The extracts were pooled and volumes were reduced by evaporation with N_2. Lipids were separated by thin-layer chromatography (TLC) on Silica Gel 60 plates (Merck, Germany) with chloroform/methanol/water (80:25:4, v/v) as the solvent system. Direct autoradiography was done as above.

Alkaline hydrolysis of lipids and separation by reversed phase-TLC

To follow incorporation of [^{14}C]mevalonate and labelled fatty acids into polar lipids, fatty acids and carotenoids, alkaline hydrolysis of lipids according to Kates [25] was performed. Separation of fatty acids and non-saponifiable lipids were done by reversed phase-TLC (HPTLC, RP18 plates; Merck, Germany). The developing solvents were acetone/water (9:1, v/v) or acetone/acetic acid (9:1, v/v). Isoprenoids were detected by their fluorescence under UV light and spraying with $SbCl_5$/chloroform (1:4, v/v). Geraniol (C_{10}), farnesol (C_{15}), and squalene (C_{30}) (Sigma) were used as isoprenoid standards and as fatty acid standard a mixture of commercial (^{14}C)-labelled caprylic, lauric, myristic, palmitic and stearic acids (~ 40 Bq of each) was used. Direct autoradiography was performed as above.

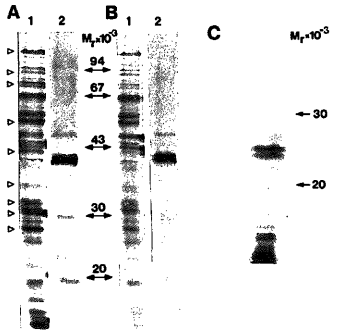

Fig. 1. SDS-PAGE of proteins from *A. laidlawii* grown with [^{14}C]mevalonate. Cells were grown with radioactive mevalonate, membranes were prepared and Triton X-114 phase partitioned as described. Delipidated proteins were separated by SDS-PAGE and gels were stained with Coomassie brilliant blue (CBB). (A) Lane 1, CBB-stained gel of lower detergent phase of Triton X-114 solubilized *A. laidlawii* membranes; Lane 2, autoradiogram of [^{14}C]mevalonate-labelled membrane proteins. (B) Lane 1 and Lane 2 are the same as in Panel A, but after treatment with methanolic 0.3 M NaOH. (C) Autoradiogram of [^{14}C]mevalonate-labelled soluble proteins. M_r arrows indicate the positions of molecular weight standards (Pharmacia-LKB, Sweden) and the open arrowheads indicate the major acyl proteins (cf. Refs. 9 and 23).

Results

By growing *A. laidlawii* in the presence of [2-^{14}C]mevalonic acid, the label was incorporated into lipids and proteins. The label in proteins was resistant to delipidation and boiling in SDS sample cocktail and was associated with a discrete set of proteins as seen on SDS-PAGE gels (Fig. 1A). There were at least five membrane proteins that became labelled; their apparent molecular masses ranged from 20 to 45 kDa. The most intensely labelled doublet band was not a major protein and had a molecular mass of ~ 40 kDa (Fig. 1A). No mevalonate was incorporated into any of the

major acylated proteins, the positions of which are also shown in Fig. 1A. In addition there were a pair of [^{14}C]mevalonate-labelled proteins in the cytoplasmic fraction; their molecular masses were 25 kDa and 23 kDa, respectively (Fig. 1C). An identical but weaker radioactive pattern was obtained from cells grown in the completely different BSR medium (data not shown), which may be attributed to its cholesterol content (cf. Ref. 19).

The non-ionic detergent Triton X-114 forms micelles in aqueous buffers at temperatures below 20°C, but above this temperature it undergoes a separation into one detergent-rich and one aqueous, detergent-poor phase. Usually integral membrane and lipid-modified proteins will be found in the bottom detergent phase, whereas proteins that do not bind detergent will remain in the aqueous phase [24]. The mevalonate-labelled membrane proteins from *A. laidlawii* partitioned into the lower detergent phase and the insoluble pellet (Fig. 1). No mevalonate-labelled protein could be detected in the aqueous phase. The two labelled cytoplasmic proteins also partitioned in the detergent phase. All *A. laidlawii* membrane acyl proteins are also found in the detergent phase [9], cf. Fig. 1A. Hence the isoprenoid chains probably give these proteins a hydrophobic character.

In many eukaryotic proteins the isoprenoid chain is attached by a thioether bond [15]. When gels of separated *A. laidlawii* [^{14}C]mevalonate-labelled proteins were subjected to alkaline hydrolysis under conditions that release 65–85% of ester-bound protein acyl chains [9], no label was released from the 40 kDa doublet or 45 kDa proteins (Fig. 1B). This indicates that the mevalonic acid derivatives were bound to these proteins through a bond that was more stable than an O- or S-ester, respectively. However, the autoradiograph shows that the labelling of the 20 and 30 kDa protein bands were less intense after the hydrolysis. Hence, the isoprenoid appears to be bound to these latter proteins by a weaker bond than a thioether.

Polar lipids constitute the majority (~95%) of the membrane lipids in *A. laidlawii*, the remainder being neutral lipids. All lipids can label the membrane acyl proteins but the neutral lipids are most efficient [9]. The [^{14}C]mevalonate was incorporated in the polar lipids to a very low extent (Fig. 2, lane 2 lower half), but appeared in the neutral lipids which consist of pigments, free fatty acids and diacylglycerols. It also appeared in an unidentified lipid which migrated just below monoacylmonoglucosyldiacylglycerol. By comparison, [^{14}C]-18:1c was incorporated into all the *A. laidlawii* polar lipids (Fig. 2, lane 1). None of the labels appeared in the cholesterol fraction. No mycoplasma can synthesize cholesterol [26].

Into which parts of the neutral lipids is mevalonate incorporated? Total lipid extracts from *A. laidlawii*

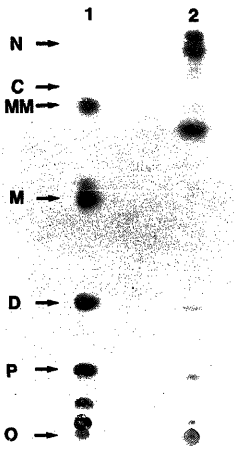

Fig. 2. Autoradiogram of lipids from *A. laidlawii*. Membrane lipids from *A. laidlawii* were separated on TLC plates and direct autoradiography was performed as described. *A. laidlawii* was grown with [^{14}C]-18:1c-plus [^{3}H]-16:0 (lane 1) or [^{14}C]mevalonate only (lane 2). The identified lipids [21] are: N, neutral lipids; C, cholesterol; MM, monoacylmonoglucosyldiacylglycerol; M, monoglucosyldiacylglycerol; D, diglucosyldiacylglycerol; P, phosphatidylglycerol; O, origin.

were hydrolyzed by methanolic 0.3 M NaOH and the non-saponifiable lipids (NSL) were extracted before acidification followed by a second extraction where saponifiable lipids (SL) were collected [25]. The different extracts were then separated by reversed phase-TLC (Fig. 3). In extracts of saponifiable lipids from *A. laidlawii*, no [^{14}C]mevalonic acid label was found (Fig. 3, SL). However, in extracts of non-saponifiable lipids from *A. laidlawii*, one major spot was observed that migrated slightly faster relative to squalene (C_{30}) (Fig. 3, NSL), and two minor spots that migrated slower.

Discussion

In this study we have investigated the metabolic fate of radioactive mevalonate in the mycoplasma *Acholeplasma laidlawii*. The results strongly indicate a covalent incorporation of mevalonate into selected membrane proteins, although the chemical nature of this modification is not yet known. Two soluble proteins with molecular masses of 23 and 25 kDa were also labelled.

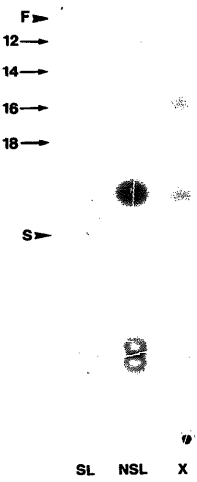

Fig. 3. Autoradiogram of non-saponifiable and saponifiable lipid fractions. Membrane lipids from *A. laidlawii* grown with [^{14}C]mevalonate were hydrolysed by alkaline methanol and the non-saponifiable lipids were collected separately from the saponifiable lipids. SL, saponifiable lipids; NSL, nonsaponifiable lipids; and X, untreated lipid extract. The solvent system was acetone/acetic acid (9:1, v/v). The positions of the isoprenoid standards are indicated by arrowheads: F, farnesol (C_{15}) and S, squalene (C_{30}). The positions of the fatty acid standards are indicated by arrows: 12, lauric acid; 14, myristic acid; 16, palmitic acid; and 18, stearic acid.

In *A. laidlawii* some of the proteins were most likely modified by an isoprenoid which could not be released by a procedure which cleaves ester-bonds. This is similar to many eukaryotic proteins where an isoprenoid is attached to a C-terminal cysteine via a thioether-bond. Our results are supported by the incorporation of [^{14}C]mevalonate into pigments but not into any of the saponifiable lipids from *A. laidlawii*. One of the labelled isoprenoid which was found in the nonsaponifiable lipids (cf. major spot in Fig. 3) might be a derivative of the carotenoid pigment in *A. laidlawii* which was formerly believed to be neurosporene (a C_{40}-carotenoid), but which now have been found by gas chromatography/mass spectrometry to be a C_{30}-apocarotenoid (P.F. Smith, University of South Dakota, personal communication). The unknown labelled lipid which migrates just below monoacylmonoglucosyldiacylglycerol (cf. Fig. 2) might be a polyprenyl-glucolipid without an acyl chain, similar to the carotenyl-glycoside found by Smith [18], or a precursor to a polyprenyl-α-D-glucolipid recently found in *A. laidlawii* under certain conditions [27]. The labelled 40 kDa membrane protein (Fig. 1A, lane 2) migrates very similarly in our gels to the β-subunit in the *A. laidlawii* membrane ($Na^+ + Mg^{2+}$)-ATPase. This ATPase is unusual since no part of it can be washed off the membrane (cf. Ref. 28). However, by the use of monospecific antibodies and Triton X-114 partitioning, these two proteins were clearly shown to be distinct (data not shown).

The isoprenoid modification of proteins seems to be very important since inhibition of the key enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase by mevinolin or compactin blocks cell growth and in many cases protein function in eukaryotes [29]. Eukaryotic protein modifications like acylation and phosphorylation very often are different from the corresponding prokaryotic ones. This also appears to be the case for isoprenylation. Protein-cysteine farnesyltransferase activities were found in crude extracts from various mammalian cells but not in *Escherichia coli* [30]. It has been shown that mevinolin does not inhibit growth of *Bacillus stearothermophilus* [31] or *E. coli*, and in *E. coli* isoprenoid biosynthesis is suggested to be different from that in eukaryotes [32]. However, in *A. laidlawii* the 3-hydroxy-3-methylglutaryl-CoA reductase is inhibited by mevinolin [33] and inhibition of polyterpene biosynthesis prevents cell growth [34]. The isoprenoid pathway has also been shown to be similar to the one in yeast [35].

To summarize the findings presented here, we have found isoprenylation of proteins in a prokaryotic organism and several results indicate that this covalently bound lipid might bear similarities to the isoprenyl-group in eukaryotic proteins. To the best of our knowledge this is the first time isoprenoid modification has been described for eubacterial proteins (cf. Ref. 36). Several questions still need to be answered. What is the nature of the isoprenoid moiety found on the proteins? Will the mechanism for isoprenylation be similar to the ones in eukaryotes? What are the functions of these proteins? The answers to these questions may have important consequences, since key signaling proteins in eukaryotes are isoprenylated and *A. laidlawii* can exchange lipid components with the eukaryotic host cell surface [37].

Acknowledgements

We thank Viola Tegman for excellent technical assistance, Dr. R.N. McElhaney, Edmonton, Canada, for a gift of ATPase and Dr. K.-E. Johansson, Uppsala, Sweden, for the preparation of ATPase antibodies. This work was supported by the Swedish Natural Science Research Council.

References

1 Barile, M.F. and Razin, S. (eds.) (1979) The Mycoplasmas, Vol. 1, Academic Press, New York.
2 Neupert, G. and Sterba, T. (1983) Exp. Pathol. 24, 207–211.
3 McGarrity, G.J., Sarama, J. and Vanaman, V. (1985) ASM News 51, 170–183.
4 Dahl, C.E., Sacktor, N.C. and Dahl, J.S. (1985) J. Bacteriol. 162, 445–447.
5 Dahl, C.E., Dahl, J.S. and Bloch K. (1983) J. Biol. Chem. 258, 11814–11818.
6 Boyer, M.J. and Wise, K.S. (1989) Infect. Immun. 57, 245–254.
7 Ruuth, E. (1988) Ph. D. Thesis. University of Umeå.
8 Wroblewski, H., Nyström, S., Blanchard, A. and Wieslander, Å. (1989) J. Bacteriol. 171, 5039–5047.
9 Nyström, S., Wallbrandt, P. and Wieslander, Å. (1992) Eur. J. Biochem., 204, 231–240.
10 Hayashi, S. and Wu, H.C. (1990) J. Bioenerg. Biomembr. 22, 451–471.
11 Johansson, K.-E. (1983) in Electroimmunochemical Analysis of Membrane Proteins (Bjerrum, O.J., ed.), pp. 322–346, Elsevier, Amsterdam.
12 Thomas, J.R., Dwek, R.A. and Rademacher, T.W. (1990) Biochemistry 29, 5413–5422.
13 Schmidt, M.F.G. (1989) Biochim. Biophys. Acta 988, 411–426.
14 McIlhinney, R.A.J. (1990) Trends Biochem. Sci. 15, 387–391.
15 Maltese, W.A. (1990) FASEB J. 4, 3319–3328.
16 Maltese, W.A. and Sheridan, K.M. (1990) J. Biol. Chem. 265, 17883–17890.
17 Hancock, J.F., Magee, A.I., Childs, J.E. and Marshall, C.J. (1989) Cell 57, 1167–1177.
18 Smith, P.F. (1963) J. Gen. Microbiol. 32, 307–319.
19 Smith, P.F. and Smith, M.R. (1970) J. Bacteriol. 103, 27–31.
20 Goldstein, J.L. and Brown, M.S. (1990) Nature 343, 425–430.
21 Christiansson, A. and Wieslander, Å. (1980) Biochim. Biophys. Acta 595, 189–199.
22 Whitcomb, R.F. (1983) in Methods in Mycoplasmology, Vol. 1 (Razin, S. and Tully, J.G., eds.), pp. 147–158, Academic Press, New York.
23 Nyström, S., Johansson, K.-E. and Wieslander, Å. (1986) Eur. J. Biochem. 156, 85–94.
24 Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607.
25 Kates, M. (1972) Lab. Tech. Biochem. Mol. Biol. 3, 269–610.
26 Smith, P.F. (1979) in The Mycoplasmas, Vol. 1 (Barile, M.F. and Razin, S., eds.), pp. 231–259, Academic Press, New York.
27 Bhakoo, M., Lewis, R.N.A.H. and McElhaney, R.N. (1987) Biochim. Biophys. Acta 922, 34–45.
28 Lewis, R.N.A.H. and McElhaney, R.N. (1983) Biochim. Biophys. Acta 735, 113–122.
29 Brown, M.S. and Goldstein, J.L. (1980) J. Lipid. Res. 21, 505–517.
30 Manne, V., Roberts, D., Tobin, A., O'Rourke, E., De Virgilio, M., Meyers, C., Ahmed, N., Kurz, B., Resh, M., Kung, H.-F. and Barbacid, M. (1990) Proc. Natl. Acad. Sci. USA 87, 7541–7545.
31 Huser, B.A., Patel, B.K.C., Daniel, R.M. and Morgan, H.W. (1986) FEMS Microbiol. Lett. 37, 121–127.
32 Zhou, D. and White, H. (1991) Biochem. J. 273, 627–634.
33 Glasfeld, A., Leanz, G.F. and Benner, S.A. (1990) J. Biol. Chem. 265, 11692–11699.
34 Smith, P.F. and Henrikson, C.V. (1966) J. Bacteriol. 91, 1854–1858.
35 Henrikson, C.V. and Smith, P.F. (1966) J. Bacteriol. 92, 701–706.
36 Epstein, W.W., Lever, D., Leining, L.M., Brenager, E. and Rilling, H.C. (1991) Proc. Natl. Acad. Sci USA 88, 9668–9670.
37 Tarshis, M.A., Ladygina, V.G., Migoushina, V.L., Klebanov, G.I. and Rakovskaya, I.V. (1981) Zbl. Bakt. Hyg. I. Abt. Orig. A. 250, 153–166.