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FIVE PALMITOYLATED POLYPEPTIDES IN THE 50 KDa RANGE ARE NOT RECOGNIZED BY AN ANTIBODY AGAINST RIBULOSE-BIPHOSPHATE-CARBOXYLASE-OXYGENASE IN *CHLAMYDOMONAS REINHARDTII*

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After incubation of *Chlamydomonas reinhardtii* cells with radioactive palmitic acid several labelled bands appeared after gel electrophoresis of delipidated protein extract. Among them, two bands (a major and a minor one) were detected in the 50 KDa range,which is the region where the LSU of the Rubisco (large sub-unit of the ribulose-biphosphate-carboxylase-oxygenase)was also found. Careful analyses by two-dimensional gel electrophoresis have shown that the five palmitate-labelled polypeptides detected in this region do not match with polypeptides immunoreacting with antibody against Rubisco. In addition, polypeptides labelled by palmitate cannot be immunoprecipitated with the same antibody further demonstrating that, in *C. reinhardtii*, the large sub-unit of Rubisco is not palmitoylated but unindentified proteins.

Post-translational protein modifications of lipidic nature are currently investigated in animal kingdom as in viruses (1, 2). The main types of such modifications have been extensively described: attachment of an extracellular protein to the cellular membrane to a phosphatidylglycerol anchor (3) or direct acylation of a protein by an acyl chain (4, 5). The two main types of direct acylations are first the myristoylation of an N-terminal glycine by an ether-amine bond described both in animals and in viruses. A protein myristoyl transferase has been purified from wheat germ (6).The second one involves an ester or thioester bond between palmitic acid to an internal or N-terminal aminoacid in a protein. In 1987, Mattoo and Edelman (7, 8) had reported that in *Spirodela oligorrhiza* two very important proteins were palmitoylated: the 32 KDa herbicide binding protein which is the first electron acceptor from the PSII and which is localized in the photosynthetic membrane, and the large sub-unit of the Rubisco (LSU). A model

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accounting for the transitory palmitoylation of the 32 KDa protein in relation with its high turnover and its susceptibility to photoinhibition was proposed.

The possibility that the large sub-unit of Rubisco could be palmitoylated opens a new field of investigation of the complex mechanisms regulating the activity of this key enzyme in plant metabolism which are currently under intensive investigation (9). We have then undertaken study of the protein palmitoylation pattern in *C. reinhardtii*. Our work demonstrates that, in our system, five polypeptides labelled with palmitate in the 50 KDa range are clearly distinct from the large sub-unit of the Rubisco and not recognized by an antibody against this enzyme.

Material and methods

The cell wall-less mutant CW15 of *Chlamydomonas reinhardtii* Dangeard was grown and incubated with [^{35}S] sulfate as previously described (10).

1 MBq of [1-^{14}C] palmitic acid from Amersham ($1.99.10^9$ Bq/mmole) was dissolved in dimethylsulfoxide and 0.1 to 0.5 MBq was added to 10 ml of cell suspension in Tris-Acetate-Phosphate medium. After incubation, cells were pelleted by centrifugation. Samples were delipidated by succesive extractions with: acetone/water (80/20), acetone, chloroform/methanol (67/33), acetone, acetone/water (80/20) and then dried under vacuum.

Proteins were analyzed by monodimensional gel electrophoresis according to Laemmli (11). Two dimensional gel electrophoresis was performed according to Rémy and Ambard-Bretteville (12): delipidated samples were suspended in 200 µl of O'Farell buffer (13) and sonicated. 25 µl of CHAPS 10%, then 100 µl of a solution containing 2% CHAPS, 9 M urea and 0.1% ampholines pH 4-9 were added to 75 µl of the sample. After centrifugation for 5 mn at 11000 x g the supernatant was layed. The first dimension was carried in glass tubes (70 mm length x 1.5 mm internal diameter) at 300 V for 16 hours. For the second dimension, IEF gels were extruded, equilibrated in the migration buffer (25 mM Tris-HCL pH 8.8 with 0.2 M glycine and 0.1% SDS) and deposited on a 12% acrylamide slab gel (90 x 80 x 1.5 mm) without stacking gel. Gels were stained by the silver staining method of Rabilloud et al. (14) and calibrated with 2D standards from Biorad. After drying, gels were autoradiographied. Proteins were matched and classified by a computer assisted system HERMES (15). Immunoprecipitation of Rubisco was carried out with purified IgG raised against tobacco Rubisco (16). Immunological detection of immobilized LSU was performed by incubating the nitrocellulose filter with the primary antibody followed by incubation with the alkaline phosphatase coupled to the secondary antibody (17). For analysis of the radioactive fatty acids, delipidated protein fraction was washed with chloroform/methanol (1/1) until no radioactivity was recovered in the chloroformic phase. Then the protein extract was saponified in 0.5 N methanolic NAOH for 30 min at 70°C. Free fatty acids were recovered in pentane after acidification of the medium, and then purified by TLC according to Mangold (18). Purified fatty acids were trans-methylated according to Metcalfe et al. (19) and then analyzed by radio-gas-chromatography in a Girdel 300 apparatus equipped with a carbowax 25 m long, a 0.5 mm diameter heated at 170°C coupled

to a proportional beta radiomatic flow one radioactive counter. For precise determination of the specific radioactivity, methylheptadecanoate was added in known amounts as an internal standard. Fatty acids in the lipidic phase were studied using the same method.

Results and discussion

Analysis by radio-gas-chromatography of fatty acid methyl esters from the protein fraction or from the chloroformic phase was shown in fig. 1. In the chloroformic extract, most of the radioactivity was found in palmitic acid nevertheless stearic acid was also labelled showing that palmitic acid has been partly elongated. In the protein extract, almost all the radioactivity was found in palmitic acid with only very few labelling of stearic acid. To be sure that radioactive palmitic acid found in the protein fraction cannot result from a contamination of this fraction by the traces of the highly labelled lipids in the chloroformic phase, the specific radioactivity was precisely determined in both

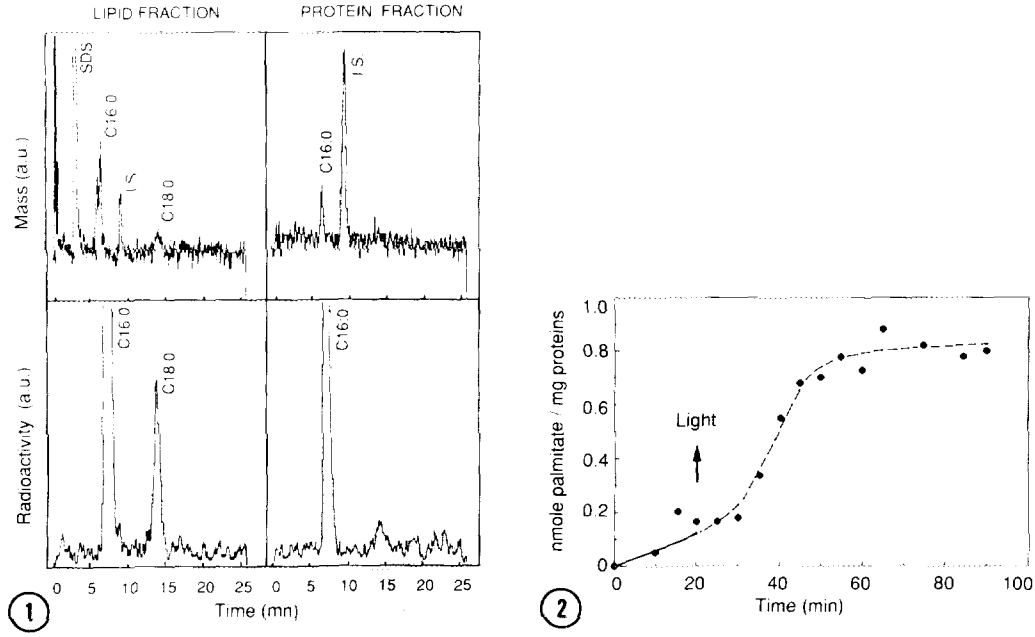

Fig.1. Mass and radio gas chromatographic analysis of fatty acids in the lipid and protein fractions after incubation of *C. reinhardtii* with radioactive palmitate for one hour in light. To determine the specific radioactivity, 5 µg of heptadecanoate (internal standard: I.S.) have been added in each sample. 1/100 of the lipid fraction extracted in chloroform but all the protein fractions have been analyzed.

Fig. 2. Kinetic of palmitate incorporation in whole protein fraction during a dark-light transition. Conditions of incubation as in methods.

fractions for stearic and palmitic acids. The specific radioactivity of stearic acid was found very close in the protein fraction as in the chloroformic phase (respectively $2.4.10^6$ and $3.0.10^6$ Bq/mmole) but specific radioactivity of palmitic acid was found three times higher (20.10^6 Bq/mmole) in the protein fraction than in the chloroformic phase ($7.7.10^6$ Bq/mmole).

Fig. 2 presents the kinetic of incorporation of radioactive palmitate in the protein fraction during a dark-light transition. A marked stimulation of incorporation by light was observed after a lag of few minutes.

When cells were incubated in the presence of cycloheximide (50 µg/ml) in conditions where protein synthesis was inhibited at 80%, or in the presence of chloramphenicol (500 µg/ml) in conditions where protein synthesis was inhibited at 20%, only a weak reduction of the rate of protein palmitoylation was observed. These findings are in agreement with previous reports showing that if myristoylation is a co-translational process, palmitoylation seems largely independent from the protein synthesis (20).

As seen in fig. 3, after incubation in light of whole cells of *C. reinhardtii* with radioactive palmitic acid, and analysis of the whole delipidated protein extract by mono-dimensional gel electrophoresis, several bands were labelled. Among a dozen of bands, two of them where repeatedly largely more highly labelled: one with a low molecular weight (at about) which 10 KDa could be the acyl-carrier-protein. The second one with an apparent molecular weight of 43 KDa. One band appears also slightly labelled in the 52 KDa region. In addition, a labelled band is always found in the 32 KDa region. In reference to the work of Mattoo and Edelman (7) who have suggested that the LSU of Rubisco can be palmitoylated, a more careful analysis of the labelled polypeptides in the 50 KDa

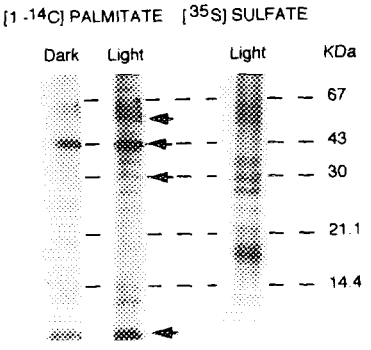

Fig. 3. Pattern of protein labelling by radioactive palmitate or sulfate in *C. reinhardtii*. After incubation of cells with [1-^{14}C] palmitate or [^{35}S] sulfate for one hour, in light. Whole delipidated protein extracts were submitted to mono-dimensional gel electrophoresis and the gels were autoradiographied. 10, 32, 43 and 52 KDa bands were indicated by arrows.

region was undertaken.*C. reinhardtii* cells were first incubated in the light with radioactive palmitic acid, then the cells were broken by osmotic shock and membrane and soluble fractions were separated by centrifugation. The labelled 43 KDa and 52 KDa bands were recovered in the supernatant.

In fig. 4 is presented analysis by two dimensional gel electrophoresis of the soluble protein fraction from cells incubated with [^{35}S] sulfate (A) or with [1-^{14}C] palmitate (B) for one hour in the light. As seen in (B), the bands at 43 KDa are resolved by two dimensional gel electrophoresis in four labelled polypeptides while only one polypeptide was found in the region of 52 KDa.

To see if these polypeptides could be identified as polypeptides from the LSU of Rubisco, a soluble protein fraction labelled with radioactive palmitate was immunoprecipitated using a serum raised against tobacco Rubisco holoenzyme but no palmitate labelled proteins were recovered in the pellet. It was verified by gel electrophoresis that non labelled Rubisco has been specifically immunoprecipated .

To definitively prove that the palmitoylated polypeptides are not LSU of Rubisco, a soluble protein fraction labelled with radioactive palmitate was

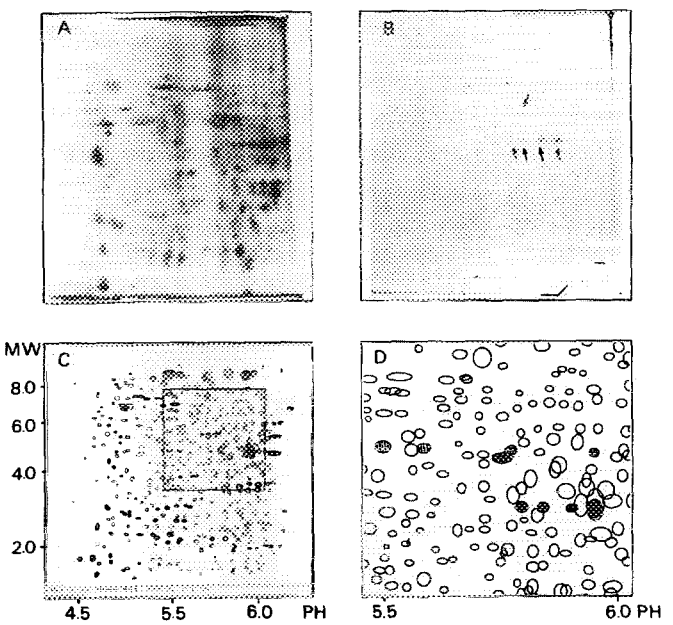

Fig. 4. A and B: Two dimensional gel analysis of soluble protein fraction labelled or with [^{35}S] sulfate (A) or [1-^{14}C] palmitate (B).The five palmitate labelled polypeptides are indicated by arrows in B.

C and D: Localization of LSU polypeptides and palmitate labelled polypeptides on the two dimensional map of ^{35}S labelled polypeptides of the soluble protein fraction by computer analysis. In (D)The four polypeptides reacting with anti Rubisco are indicated in grey and the five palmitate labelled polypeptides are indicated in black. (D) represents four time magnification of the upper right part of the whole map presented in (C).

submitted to two dimensional gel electrophoresis, transferred on nitrocellulose filter, immunoblot was performed using anti tobacco Rubisco and the filter was autoradiographied. As seen in fig. 4, the four polypeptides reacting with the anti Rubisco do not match with the five palmitate labelled polypeptides.

Our work shows that, in *C. reinhardtii* and in our conditions, the set of five polypeptides which are labelled by palmitic acid in the region of 50 KDa are not the LSU of Rubisco but other unidentified polypeptides migrating very near the LSU. Identification of these polypeptides are under investigation.

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References

1. Schmidt, M.F.G. (1989) Biochim. Biophys. Acta 988, 411-426.
2. Mc Ilhinney, R. (1990) Trends Biochem. Sci. 15, 387-391.
3. Ferguson, M.A.J. (1991) Current Opinion in Structural Biology 1, 522-529.
4. Olson, E.N., and Spizz, G. (1986) J. Biol. Chem. 261, 2458-2466.
5. Grand, J.A. (1989) Biochem. J. 258, 625-638.
6. Towler, D.A., Adams, S.P., Eubanks, S.R., Towery, D.S., Jackson-Machelski, E., Glaser, L. and Gordon, J.I. (1987) Proc. Natl. Acad. Sci. USA 262, 1030-1036.
7. Mattoo, A., and Edelman, M. (1987) Proc. Natl. Acad. Sci. USA 84, 1497-1501.
8. Mattoo, A., Norman, H., Callahan, F., StJohn, J., and Edelman, M. (1988) In Post-translational modification of proteins by lipids, a laboratory manual (U. Brodbeck, Ed.), pp. 82-87, Spinger Verlag.
9. Portis, A. (1990) Biochem. Biophys. Acta 1015,15-28.
10. Picaud, A., Creach, A., and Trémolières, A. (1991) Plant Physiol. Biochem. 29, 441-448.
11. Laemmli, U.K. (1970) Nature 227, 680-685.
12. Rémy, R., and Ambard-Bretteville, F. (1987) In Methods Enzymol. (R. Douce and L. Packer, Eds) 148, 623-632, Academic Press.
13. O'Farell, P. (1975) J. Biol. Chem. 250, 4007-4021.
14. Rabilloud, T., Carpentier, G., and Tarroux, P. (1988) Electrophoresis 9, 288-291.
15. Vincens, P., and Tarroux, P. (1987) Electrophoresis 8, 100-107.
16. Firestone, G., and Winguth, S. (1990) In Methods Enzymol. (M. Deutscher, Ed.) 182,688-700, Academic Press.
17. Shambrock, J., Fritsch, E.F., and Maniatis, T. (1989) In Molecular Cloning. Cold Spring harbor laboratory Press.
18. Mangold, H.K. (1964) J. Am. Oil Chem. Soc. 41, 762-773.
19. Metcalfe, L.D., Schmitz, A.A., and Pelka, J.R. (1966) Anal. Chem. 38, 514-515.
20. Giroud, C., and Eichenberger, W. (1989) Plant Cell Physiol. 30, 121-128.