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PROTEIN PHOSPHORYLATION IN PEROXISOMES

Cetna Skorin, Ubaldo Soto, Cecilia Necochea,
and Federico Leighton

Department of Cell Biology, Universidad Católica de Chile
Casilla 114-D, Santiago, Chile

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SUMMARY: The possible presence of phosphorylated proteins in peroxisomes was studied in hepatocytes from nafenopin-treated and normal rats. A 63 kDa phosphorylated protein was consistently and exclusively found in the membrane of peroxisomes from hepatocytes incubated in the presence of ^{32}P-phosphate. The peroxisomes were isolated in metrizamide isopycnic gradients of postnuclear supernatants and were subfractionated by alkaline extraction to separate the membrane and the matrix proteins. Polyacrylamide gel electrophoresis, autoradiography and densitometry were employed to characterize the proteins. The 63 kDa membrane protein copurifies with peroxisomes in metrizamide gradients and apparently can be phosphorylated, in purified peroxisomes, with ATP and catalytic subunit of cAMP-dependent protein kinase.

The knowledge about peroxisomes in animal cells has consistently grown since the moment when, to illustrate their biochemical peroxidative capacity, the name was proposed (1). Many additional enzymes (2) have since been localized in the organelle, as well as some specific membrane proteins (3,4). The precise functional role of the organelle is still unknown, but much experimental evidence indicates that it might be related to several aspects of lipid metabolism (5-8). The fatty acid oxidizing capacity that peroxisomes share with mitochondria is characteristically inducible by diet (2) hypolipidemic drugs (5) and thyroxine (9) however, short-term regulatory mechanisms have not been described. The selective inhibition of peroxisomal fatty acid oxidation by phenothiazines (10) suggests that liver cells might have regulatory mechanisms acting, for example, on the channeling of fatty acids to peroxisomes. The search for peroxisomal protein phosphorylation, as an approach to evaluate possible short-term regulatory mechanisms, led to the present study. Protein phosphorylation was detected in peroxisomes, a

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finding which has several potential implications. A preliminary communication of these results has been made (11).

MATERIAL AND METHODS

Isolated hepatocytes were prepared, as previously described (10) by the method of Berry and Friend (12) modified by Krebs et al. (13). Nafenopin, 1g per kg chow for 2 to 4 weeks, was employed to induce peroxisome proliferation (14). After disaggregation, cells were suspended in collagenase-free Krebs-Henseleit bicarbonate buffer containing 2.5 mM $CaCl_2$ and 0.1 mM phosphate. Cell viability was 95.4 $\pm$ 1.5 average percent $\pm$ S.D., based on trypan blue exclusion. Preincubation with ^{32}P-phosphate was made adding 4 mCi, carrier free, to 5.3 ml of cell suspension (60-90 mg/ml). After 45 min at 37°C, in a siliconized flask, with gentle mixing, under O_2:CO_2 (95:5 v/v), the cell suspension was taken to a final volume of 13.4 ml containing 120 mM NaCl, 4.8 mM KCl, 2.0 mM $CaCl_2$, 0.1 mM KH_2PO_4, 1.2 mM $MgSO_4$,25 mM $NaHCO_3$,50 mM methanol, 10 mM semicarbazide, 0.18 mM defatted bovine serum albumin and 0.54 mM sodium laurate. The incubation was stopped after 2.5 min by cooling in ice and the addition of 2.7 ml NaF solution for a final 50 mM concentration. The cells were collected at 670 rpm for 1 min in rotor 253 International (DAMON/IEC Division) and resuspended in 2 ml of sucrose 0.25 M, imidazole 3 mM, EDTA 1 mM, pH 7.4. After washing once, in the same resuspension medium and volume, the cells were homogenized with a Dounce glass homogenizer and a postnuclear supernatant was prepared at 1800 rpm for 2 min, 4°C, in the same rotor. The nuclei-free supernatant, checked by phase contrast microscopy, was fractionated in continuous density gradients of metrizamide (15,16) 0.5 ml on top of a 1.07 - 1.27 g/ml gradient centrifuged 50 min at 40 000 rpm and 8°C in a vertical rotor (VTi-65 Beckman Instruments Inc.). Marker enzymes for peroxisomes (catalase), mitochondria (glutamate dehydrogenase), endoplasmic reticulum (NADPH:cytochrome c reductase), lysosomes (acid phosphatase) and the soluble components (phosphoglucomutase) as well as proteins, were measured as previously described (17). To isolate the membranes, peroxisomes were subfractionated by the carbonate procedure (18) applied directly to pooled peroxisomal fractions. In vitro phosphorylation was carried out with the protein kinase catalytic subunit (Sigma Chemical Co. P-2645) 40 units, 9 μCi (γ-^{32}P) ATP approximately 5 nmoles, in 0.1 ml of 35 mM KH_2PO_4, 6 mM $MgSO_4$, 0.2 mM EDTA, pH 6.8. For SDS-polyacrylamide gel electrophoresis, established procedures were employed (19,20). Membrane pellets were dissolved directly in SDS sample buffer; dilute proteins were precipitated with 10% TCA and washed with ice-cold ethyl ether (21). Autoradiography was made with Kodak XAR-5 film at -70°C, 1-4 days. Densitometry was performed with a Canalco K-II densitometer; after checking the linearity of the measurements, densitometry values were employed to characterize the fractions from the isopycnic gradients. Collagenase type I, Worthington was employed. Metrizamide was purchased from Nyegaard and Co., Oslo. Nafenopin was a gift from Ciba-Geigy. Other biochemicals were from Sigma Chemical Co. ^{32}P-Orthophosphoric acid was supplied by the Comisión Chilena de Energía Nuclear. (γ-^{32}P) ATP was a gift from P. Bull, Biochemistry Department, Universidad Católica de Chile.

RESULTS

To study in situ protein phosphorylation, isolated hepatocytes were preincubated 45 min with ^{32}P-inorganic phosphate to label the intracellular pool. The preincubation was usually followed by the addition of laurate, methanol

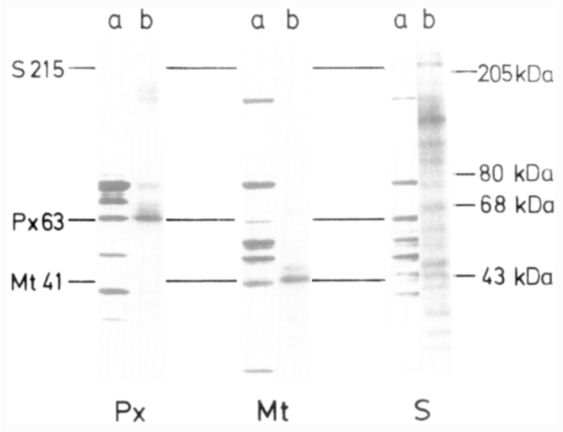

Figure 1. Phosphorylation in subcellular fractions obtained from hepatocytes preincubated with ^{32}P inorganic phosphate. Pattern from 10 µg protein per lane in SDS-polyacrylamide gel electrophoresis (7-15%) stained with Coomassie brilliant blue (a) and by autoradiography (b). The fractions shown were obtained in an isopycnic metrizamide gradient from a postnuclear supernatant of a Nafenopin treated rat. The peak of marker enzyme activity was selected for peroxisomes (Px), mitochondria (Mt) and soluble protein (S). Px63, Mt41 and S215 identify phosphorylated proteins which, in the gradient, follow the subcellular distribution or either particle-bound catalase, glutamate dehydrogenase or phosphoglucomutase. Numbers to the right are molecular mass markers. For 205, 80, 68 and 43 kDa, myosin, IgM subunit, bovine serum albumin and ovoalbumin.

and semicarbazide for 2.5 min, to monitor peroxisomal fatty acid oxidation (10), however omission of this step does not affect the results shown. The peroxisomes were then isolated by isopycnic fractionation, in a metrizamide gradient, of a postnuclear supernatant. The results shown in Fig. 1 correspond to the fractions in which maximal purification of peroxisomes, mitochondria and soluble proteins, was obtained. Phosphate incorporation is detected in many proteins, particularly in the soluble fraction. The subcellular distribution of the labeled proteins was calculated by densitometry of the autoradiographic characterization of all the fractions from the gradient. A few proteins follow closely some of the enzyme markers employed; three are identified by their molecular mass in Fig. 1. In peroxisomes, most of the label is apparently in one protein of 63 kDa. The subcellular distribution of this protein, Px63, resembles that of particle-bound catalase, with some displacement to higher densities, Fig. 2. The radioactive label of Px63 was resistant to hot 10% TCA (22) and to chloroform: methanol-2:1 extraction

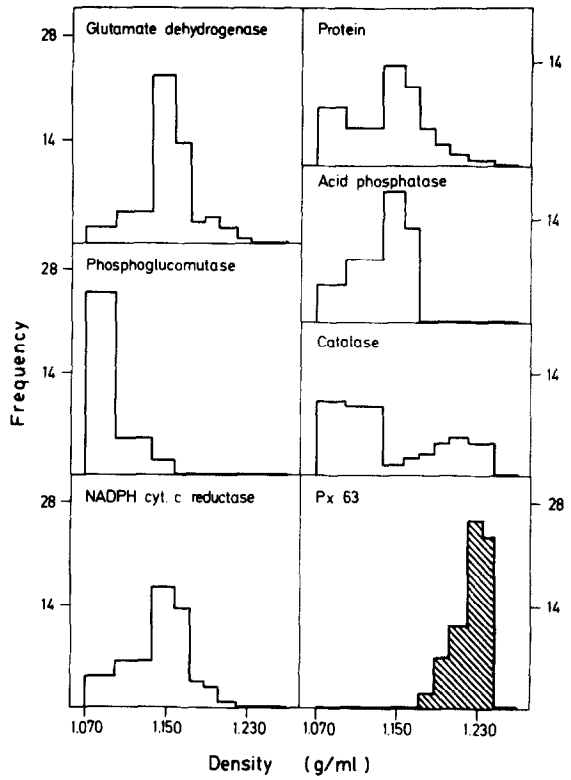

Figure 2. Subcellular distribution of the peroxisomal phosphorylated protein Px63; of total proteins and of enzyme markers for peroxisomes (catalase), lysosomes (acid phosphatase), endoplasmic reticulum (NADPH:cytochrome c reductase), mitochondria (glutamate dehydrogenase) and soluble proteins (phosphoglucomutase). Subcellular fractionation, by isopycnic equilibrium in a metrizamide gradient, of a postnuclear supernatant from hepatocytes of a nafenopin treated rat. The histograms show equilibrium density versus frequency, $Q/\Sigma Q\Delta\rho$, where Q represents the activity in a fraction and $\Delta\rho$ the increment in density between the boundaries of the fraction.

(23), but was sensitive to proteinase K. To determine the intraperoxisomal localization of Px63, a purified peroxisomal fraction, previously labeled in situ, was subfractionated with alkaline carbonate buffer. As shown in Fig. 3, Px63 is recovered exclusively in the membrane fraction from peroxisomes. In contrast, most of the peroxisomal 63 kDa protein is localized in the matrix or carbonate soluble compartment. Obviously the 63 kDa band in the electrophoresis of whole peroxisomes is heterogeneous. The membrane localization found for Px63 is also reflected in its subcellular distribution, since it is only detected bound to particles, and is not detected in the soluble protein fractions where the soluble components re-

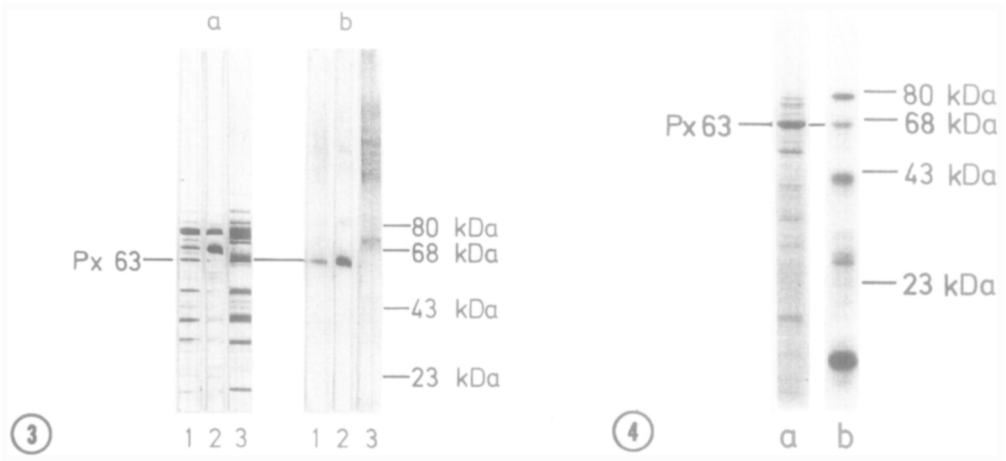

Figure 3. Subfractionation of peroxisomes from hepatocytes preincubated with ^{32}P inorganic phosphate. Peroxisomal fraction from a nafenopin treated rat, lane 1, subfractionated into membranes, lane 2, and matrix contents, lane 3. Protein pattern in SDS-polyacrylamide gel electrophoresis (7-13%) stained with Coomassie brilliant blue (a) and by autoradiography (b). In lane 1, 10 µg of protein, and in lanes 2 and 3, the protein recovered in the subfractionation of peroxisomes containing 120 µg of protein. Numbers to the right are molecular mass markers.

Figure 4. Phosphorylation of a peroxisomal fraction with catalytic subunit of cAMP-dependent protein kinase. Pattern from 10 µg protein in SDS-polyacrylamide gel electrophoresis (12%) stained with Coomassie brilliant blue (a) and by autoradiography (b). Numbers to the right are molecular mass markers.

leased from peroxisomes are found. To further characterize the phosphate acceptor, a purified peroxisomal fraction from a normal rat was phosphorylated in vitro with ATP and catalytic subunit of cAMP-dependent protein kinase. A normal rat was used since Px63 is detected both in control and nafenopin-treated rats. As shown in Fig. 4, a 63 kDa protein, presumably corresponding to Px63, is phosphorylated as well as several other bands heavily labeled. The pattern observed is very different from that seen after in situ phosphorylation, except for the presence of the band tentatively identified as Px63.

DISCUSSION

We have found one major phosphorylated protein in the peroxisomes from hepatocytes under conditions in which several other cell proteins are phosphorylated. The study relies only on slab gel electrophoresis and autoradiography to detect the protein, therefore further characterization is

required to establish if Px63 is a polypeptide that corresponds to a single molecular species and to identify the nature of the phosphate bond. On a protein basis Px63 is only a minor peroxisomal membrane polypeptide, different from the main peptides already described (3,4). In whole peroxisomes the major protein band at or close to 63 kDa is catalase (24) but the subfractionation data indicates that the phosphate acceptor does not behave as expected for a catalase subunit. The polypeptidic nature of Px63 is supported by its behaviour under treatment with acid, organic solvents and protease. The peroxisomal localization of Px63 is based in the correlation of its pattern of subcellular distribution with that of particle-bound catalase. The slight difference from particle-bound catalase might be expected for a protein localized in different compartments in the organelle (25). Although the presence of another unidentified cell component containing only a small amount of protein and with equilibrium properties similar to peroxisomes in metrizamide gradients, can not be excluded, variations in the centrifugation conditions did not dissociate Px63 from peroxisomes (data not shown). Furthermore, the intensity of the label, per mg protein, is similar in peroxisomal fractions from normal or nafenopin treated rats, in spite of large differences in the protein content of the peroxisomal fractions; it is very unlikely that the concentration of the contaminating constituent would parallel the change in peroxisomal concentration. The question of the role played by Px63 in peroxisomes remains open. It is in the membrane and therefore, among other possibilities, it could be involved in the translocation of peroxisomal substrates and products or in determining the specific properties of the peroxisomal membrane surface.

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