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RELEASE AND ACTIVATION OF PHOSPHORYLASE PHOSPHATASE UPON RUPTURE OF ORGANELLES FROM RAT LIVER

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Summary: Liver extracts (8000 x g for 10 min) from fasted rats contain about 4 times more phosphorylase phosphatase activity when the liver was homogenized in a hypotonic medium or frozen before homogenization. This increase is caused by: (i) release of partially latent phosphatases (M_r=60 000 and 45 000 in sucrose gradient centrifugation) from ruptured organelles; (ii) rapid activation of phosphatase in the ruptured pellet by endogenous protease(s) which can be blocked by p-tosyl-L-lysine chloromethyl ketone. Only the M_r=60 000 enzyme, associated with the nuclei, can be activated proteolytically, with conversion to an M_r=45 000.

Lee and coworkers (1-3) have emphasized that vigorous homogenization of the liver increases the cytosolic phosphorylase phosphatase activity, owing to the appearance of multiple forms which would be generated proteolytically from the major cytosolic phosphatase (M_r=260 000) by liberated lysosomal cathepsins. However, it has become apparent to us that a considerable amount of enzyme is associated with the low-speed pellet and becomes activated and partially solubilized by a hypotonic shock or by freezing of the liver. We also report some physical and catalytic properties of the solubilized enzymes.

MATERIALS AND METHODS

The heat-stable protein phosphatase inhibitors (4) and particulate glycogen (5) were prepared from dog liver. ^{14}C-methylated ovalbumin was from New England Nuclear, and Triton X-100 from Packard. An Ultra-Turrax blender was obtained from Janke & Kunkel. 'Buffer A' contained 15 mM Tris, 0.025 mM EDTA and 0.33 M sucrose, pH 7.4 at 4°C. 'Buffer B' consisted of 50 mM imidazole and 1 mM dithiothreitol, pH 7.4.

Abbreviations: EGTA: ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PMSF: phenylmethylsulfonylfluoride; TLCK: p-tosyl-L-lysine chloromethyl ketone; TPCK: L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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Homogenization and low-speed centrifugation: male Wistar rats (200 g) received intraperitoneally 0.35 mg glucagon after an overnight fast, 30 min before decapitation. The liver was homogenized in 4 vol. of buffer B, with or without 0.25 M sucrose, in a Potter-Elvehjem device (3 strokes), except where indicated (see Fig. 1). To separate a pellet from the supernatant (Fig. 1 and related work), the 20% liver homogenate was centrifuged for 10 min at 8 000 x g. The pellet was suspended and recentrifuged as before, and finally resuspended in buffer B. The supernatants were pooled and 1 ml passed through a column of Sephadex G-25 (1.5 x 5 cm) equilibrated with buffer B plus 0.25 M sucrose. The pellet and the filtered supernatant were assayed for phosphorylase phosphatase at a final concentration of 0.5%.

Subfractionation of the low-speed pellet (Table 1 and related work). The liver was homogenized in 4 vol. of buffer A in a Potter-Elvehjem device. Centrifugation for 10 min at 8 000 x g yielded the post-mitochondrial supernatant, which was filtered through Sephadex G-25. The pellet was suspended in buffer A and centrifuged for 10 min at 200 x g. The latter procedure was repeated once more, yielding a final pellet (fraction I). The combined supernatants were centrifuged for 10 min at 8 000 x g and the pellet was washed once (fraction II); the corresponding supernatants were discarded. The two fractions were then subjected to a hypotonic shock by resuspension in buffer B. When indicated, the solubilized proteins were separated from a final sediment by centrifugation for 10 min at 8 000 x g. All the fractions were assayed for phosphorylase phosphatase at a final concentration of 2%.

Assays. DNA (6), glutamate dehydrogenase (7), acid phosphatase (8) and lactate dehydrogenase (9) were used as markers for nuclei, mitochondria, lysosomes and the cytosol respectively. Before assay of glutamate dehydrogenase and of acid phosphatase, the liver fractions were treated with Triton X-100 (0.1%, v/v). Glycogen synthase, glycogen phosphorylase a and 'total' phosphorylase (a + b) were measured as previously described (10,11). One unit of the above mentioned enzymes converts 1 μmol of substrate per min in the appropriate assay conditions. DNA was extracted from liver fractions by heating for 20 min at 70°C in the presence of 0.5 M $HClO_4$. Glycogen synthase phosphatase was assayed as before (10).

Phosphorylase phosphatase was assayed at 25°C in a final volume of 0.1 ml, containing 0.7 U of [^{32}P]phosphorylase a (12), 10 mM caffeine, 0.25 M sucrose, buffer B, and a liver fraction at the appropriate final concentration. The trichloroacetic-acid soluble radioactivity was determined 4 times over a 12-min period (12). It was regularly checked by Sephadex G-15 chromatography that the released radioactivity was indeed P_i. Latent phosphorylase phosphatase activity was revealed by treatment of liver fractions with trypsin (10), and assayed at a final concentration of 0.1% unless otherwise stated. One unit of protein phosphatase dephosphorylates 1 U of phosphorylase a per min in the appropriate assay condition. Three effects account for quantitative differences between phosphorylase phosphatase activities in different experiments: (i) the specific activity of the enzyme in the post-mitochondrial supernatant increases 1.8-fold when the final concentration is lowered from 2% to 0.5%; (ii) a 50% increase of activity in the 8 000 x g supernatant was observed when the wash fluid from the pellet was included; (iii) a time-dependent proteolytic 'activation' (see results).

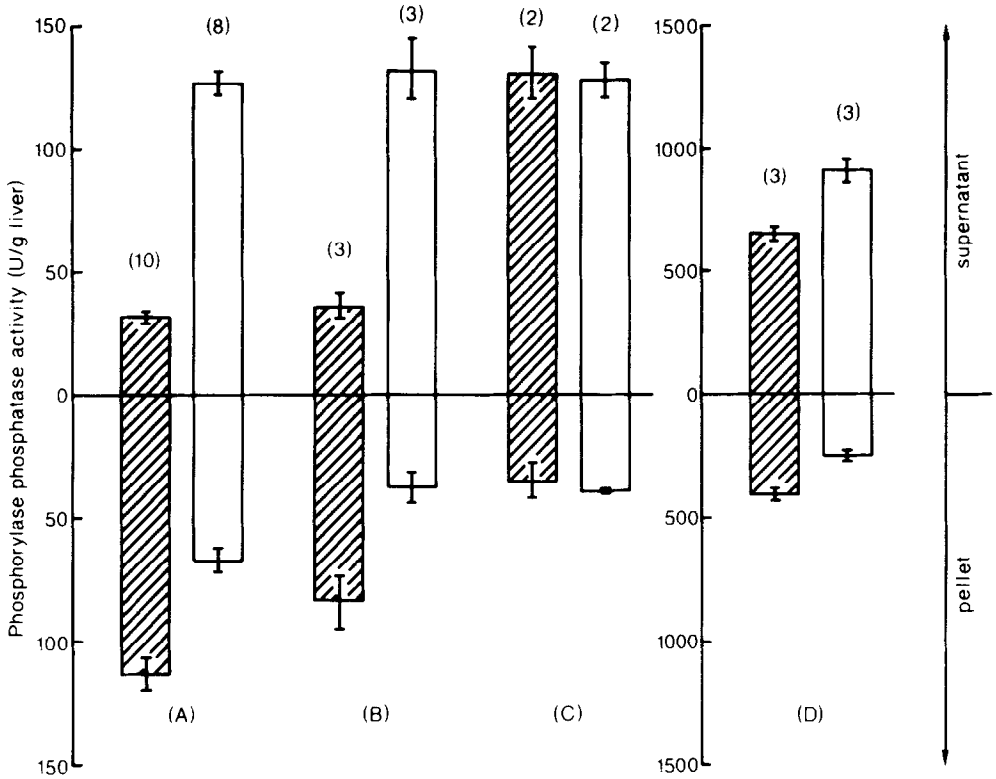

Fig. 1. *Effect of homogenization technique on the distribution of phosphorylase phosphatase activity between pellet and supernatant (10 min at 8 000 x g).* Fresh livers were homogenized with a Potter-Elvehjem tube (A,D), or an Ultra-Turrax blender twice for 15 s (B), or frozen between aluminium tongs at -196°C prior to homogenization in a Potter-Elvehjem tube (C). Homogenization was done in the presence of 0.25 M sucrose (cross-hatched) or without (open columns). Phosphorylase phosphatase was assayed in the supernatant and in the ruptured pellet either directly (A,B,C) or after incubation with trypsin (D). Vertical bars represent ± S.E.M. for the indicated number of experiments.

Protein was measured by the procedure of Lowry et al. (13). Statistics are given as means ± S.E.M., followed by the number (n) of observations.

RESULTS AND DISCUSSION

Effect of different homogenization techniques on the distribution of enzyme activity. The activity of phosphorylase phosphatase in a low-speed supernatant increased about fourfold when fresh glycogen-depleted liver was homogenized in the absence of sucrose (Fig. 1A). This increase was prevented equally well by the inclusion of either 0.1 M NaCl or 0.25 M sucrose (not illustrated). The type of homogenizer appeared much less important (Fig. 1, A and B). Freezing of the liver produced

Table 1. *Activity of phosphorylase phosphatase in subcellular fractions.* The final concentration of the fractions in the phosphorylase phosphatase assay was 2% unless otherwise stated. The figures between brackets indicate the number of observations. Fractions I and II contained 7% and 37%, respectively, of the total acid phosphatase, and less than 2% of the total lactate dehydrogenase.

	Fraction I	Fraction II	Post-mitochondrial Supernatant
Phosphorylase phosphatase (U/g liver)	20.7 ± 1.6 (9)	11.7 ± 2.1 (9)	11.7 ± 1.4 (9)
Phosphorylase phosphatase after trypsin treatment (U/g liver)	194 ± 30 (4)[a]	7.1 ± 2.1 (4)	314 ± 47 (4)[a]
Glutamate dehydrogenase (U/g liver)	1.4 ± 0.1 (9)	8.3 ± 0.7 (9)	1.2 ± 0.3 (5)
DNA (µg/g liver)	2121 ± 208 (7)	115 ± 43 (6)	97 ± 9 (5)

[a]Fractions assayed at a final concentration of 0.1%.

also a fourfold rise in soluble enzyme activity, whether or not the homogenization medium contained 0.25 M sucrose (Fig. 1C). Parallel assays of phosphorylase phosphatase in the pellets (Fig. 1, A-C) indicate that the increase in soluble enzyme activity is at least partly caused by release from ruptured organelles. This explanation is confirmed by the assay of 'total' phosphorylase phosphatase, after activation with trypsin (Fig. 1D). However, osmotic shock appears to cause activation of organelle-associated enzyme as well (see below). That the activity in the pellet was not due to contaminating unbroken cells or cytosol is shown by the fact that only 5.7 ± 1.2% (n=3) of the lactate dehydrogenase present in the homogenate was recovered in the pellet.

Subfractionation of the phosphorylase phosphatase activity associated with the low-speed pellet. Differential centrifugation yields nuclei in fraction I and mitochondria in fraction II with little cross-contamination (Table 1). In comparison to the post-mitochondrial supernatant, both fractions contained a considerable amount of phosphorylase phosphatase activity (Table 1), of which about half was solubilized by hypotonic shock. There are fundamental differences between the enzyme in fractions I

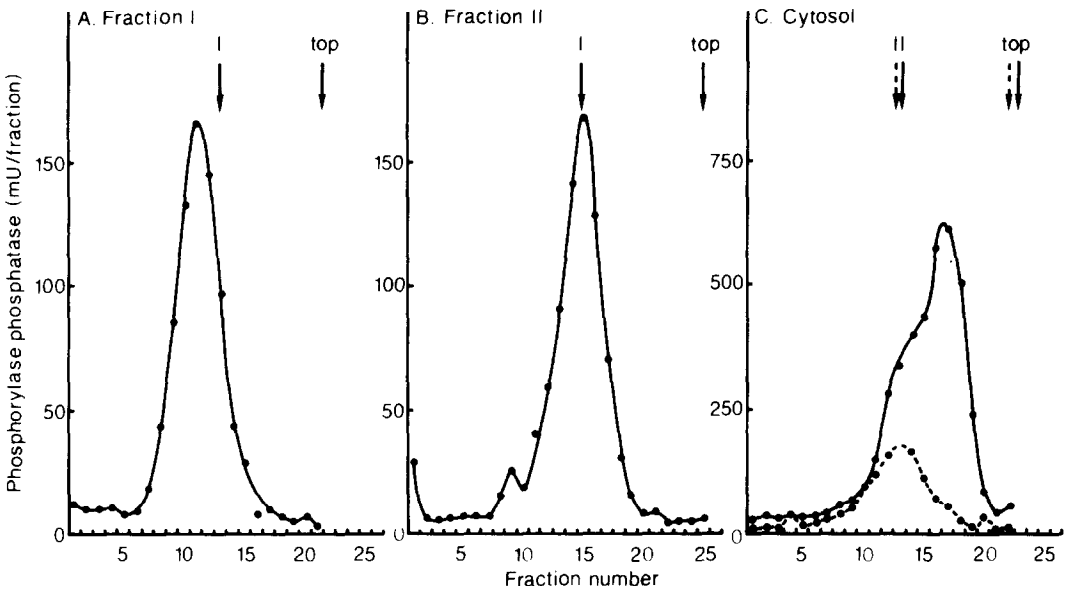

Fig. 2. *Sedimentation of the cytosolic and of the solubilized phosphorylase phosphatase from fraction I and II in sucrose gradients.* The applied samples were 0.2 ml of the solubilized enzyme from fraction I (0.3 mg protein) in (A), of the solubilized enzyme from fraction II (0.4 mg protein) in (B), and of gel-filtered cytosol (6.6 mg protein) in (C). The cytosolic fractions were the supernatants (30 min at 250 000 x g) from liver homogenates prepared either in the presence (---) or in the absence of 0.25 M sucrose (——). Markers: 1.5 µg of labeled ovalbumin (M_r= 45 000) in A & B (arrow I); endogenous lactate dehydrogenase (M_r=158 000) in C (arrow II). Centrifugation conditions: 15 h at 49 000 rev./min in a Beckman type SW 50.1 rotor at 1°C (A,B); 5 h at 65 000 rev./min in a Beckman type SW65 rotor (C). The preparation of the sucrose gradients and the calculation of the results have been described (10).

and II. Incubation with trypsin increased the phosphorylase phosphatase activity in the post-mitochondrial supernatant and in fraction I about 30-fold and 10-fold, respectively, but decreased the activity in fraction II (Table 1). By sucrose density gradient centrifugation an M_r=60 000 ± 900 (n=4) could be attributed to the solubilized enzyme from fraction I (Fig. 2A). Treatment of the enzyme with trypsin before application onto the gradient caused a 6-fold increase in phosphorylase phosphatase activity which sedimented in a single peak with an M_r=45 000 (not illustrated). The solubilized enzyme from fraction II has an M_r=45 000 ± 300 (n=3)(Fig. 2B). Trypsin treatment of this enzyme did not change its sedimentation behaviour, but simply destroyed part of the activity (not shown). Identical values were observed in one experiment where either organelle fraction was made hypotonic in the presence of 0.1 mM TLCK (see below).

These sedimentation patterns are in sharp contrast with that of a cytosolic fraction obtained from fasted rat liver: no activity was detected in the region of 60 000 - 45 000, unless the homogenate was prepared in a hypotonic medium (Fig. 2C).

Other experiments showed that neither solubilized enzyme cosedimented with added particulate glycogen (5 mg/ml), whereas a substantial part (31 ± 1%; n=4) of the cytosolic phosphorylase phosphatase activity did bind to added glycogen. Both solubilized phosphorylase phosphatases were sensitive to the heat-stable inhibitors-1 and -2. In each case the inhibition exceeded 80% at high inhibitor concentrations. An enzyme capable of activating purified hepatic glycogen synthase b could not be demonstrated in either fraction I or II. Neither fraction contained measurable phosphorylase or glycogen synthase.

The association of phosphorylase phosphatase with plasma membranes has been reported (14). Therefore a more detailed analysis of fraction I was undertaken, which showed an association with nuclei rather than with plasma membranes: (i) the ratio of phosphorylase phosphatase/DNA in the nuclear fraction remained unchanged when nuclei were purified according to Widnell and Tata (15); (ii) when fraction I was subjected to centrifugation in an aqueous two-phase polymer system, used for isolating plasma membranes (16), 84% of the activity remained associated with the nuclei, and less than 1% was recovered at the interphase. Fraction II contains mitochondria, a substantial part of the lysosomes (Table 1) and probably other organelles. The location of phosphorylase phosphatase within the latter fraction has not yet been investigated.

The association of phosphorylase phosphatases with nuclei and with fraction II cannot be explained by mere adsorption of cytosolic enzyme. They have distinct properties, and are released by osmotic shock, though not at all by 0.1 M NaCl (see above).

Activation of phosphorylase phosphatase in the low-speed pellet. When the post-mitochondrial supernatant was subjected to a hypotonic shock, there was no indication for a time-dependent proteolytic activation of phosphorylase phosphatase (Table 2), although this fraction contained the bulk of the lysosomes. In contrast, hypotonic rupture of the complete nucleo-mitochondrial pellet caused a more than twofold increase in activity after 2 h at 0°C (Table 2). A trial with a mixture of

Table 2. *Activation of phosphorylase phosphatase in the low-speed pellet.*
A pellet and a post-mitochondrial supernatant were prepared as described, except that the homogenization was done with 1 vol. of buffer B (plus 0.25 M sucrose), and that the wash fluid was discarded. The washed pellet was resuspended in the same medium to a concentration equivalent to a 50% liver homogenate. Either fraction was diluted with 4 vol. of water, with or without protease inhibitors. After 5 min, 15 vol. of buffer B containing 0.33 M sucrose and the same inhibitor concentration were added and the first assay started.

Cell fraction	Phosphorylase phosphatase activity (U/g liver)	
	assayed at once	after 2h at 0°C
Post-mitochondrial supernatant	16.9	17.3
Low-speed pellet (10 min at 8 000 x g)		
without inhibitor	69.9	145.0
+ 0.5 mM benzamidine	71.6	134.1
+ 0.3 mM PMSF	68.6	134.1
+ 0.1 mM TPCK	61.2	74.0
+ 0.1 mM TLCK	17.4	14.7
+ 5 mM EGTA	75.1	152.4
+ inhibitor mixture	19.0	14.4

protease inhibitors revealed that there was furthermore a rapid 4-fold activation that previously had gone unnoticed because a period of at least 5 min at 0°C was systematically allowed to ensure a complete membrane rupture. The early inhibitory effect was to be attributed entirely to TLCK, but TPCK also inhibited the slower second-phase activation (Table 2). In subsequent experiments TLCK was added at various times after hypotonic shock; the compound did not inhibit phosphorylase phosphatase activity that was already revealed, but blocked any further increase of the activity. Since the activation was not affected by EGTA (Table 2), it appears distinct from the action of Ca^{2+}-dependent proteases from skeletal muscle, which degrade the cytosolic phosphatase to smaller species (17).

CONCLUSIONS

'Activation' of phosphorylase phosphatase after hypotonic homogenization of the liver cannot be attributed to an attack of the cytosolic enzyme(s) by liberated lysosomal cathepsins (Table 2). Release of phosphorylase phosphatase from disrupted organelles (Fig. 1, Table 1) is a partial explanation. Activa-

tion of released enzyme by a TLCK-sensitive protease contributes significantly as well (Table 2).

It is not clear at present which role, if any, the organelle-associated protein phosphatases have in glycogen metabolism. However, in much previous research the real amount of phosphorylase phosphatase activity has been grossly overestimated by the use of hypotonic media and/or frozen liver.

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