A protein inhibitor of phosphofructokinase from the mucosa of rat small intestine

A mechanism for the regulation of glycolysis that is independent of glucose

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A protein inhibitor of phosphofructokinase was detected by the chromatography on Sephadex G-100 of crude mucosal extracts prepared from the small intestine of starved rats. Addition of the protein inhibitor to extracts from fed rats increased the $K_{0.5}$ of phosphofructokinase for fructose 6-phosphate to that for starved rats. The protein inhibitor provides a mechanism for the hormonal regulation of glycolysis independently of glucose supply.

Phosphofructokinase in the small-intestinal mucosa of starved rats or streptozotocin-diabetic rats is more susceptible to inhibition by ATP than is the case for normal fed rats (Jamal & Kellett, 1983*a,b*). The mechanism responsible for the increased inhibition is unknown. A similar increase in the inhibition of rat liver phosphofructokinase where rats are starved is mediated by a decrease in the concentration of the allosteric effector fructose 2,6-bisphosphate, which is controlled by glucose and glucagon (van Schaftingen *et al.*, 1980*a,b*; Richards & Uyeda, 1980; Pilkis *et al.*, 1981; Hers & van Schaftingen, 1982). However, fructose 2,6-bisphosphate is not present in rat intestinal mucosa, so the activity of mucosal phosphofructokinase is not regulated by glucose (Jamal *et al.*, 1984), accounting for the fact that intestinal glucose utilization is not very responsive to the concentration of glucose in the lumen (Windmueller & Spaeth, 1980). Some other factor must therefore be involved in the regulation of mucosal phosphofructokinase.

We previously purified mucosal phosphofructokinase to homogeneity and noted that, in marked contrast with its properties in crude extracts, the isolated enzyme is highly activated, displaying little co-operativity with respect to fructose 6-phosphate and little inhibition by ATP (Khoja *et al.*, 1983). We suggested that these differences were caused by the separation of a tightly bound inhibitor from phosphofructokinase during purification. Moreover, because chromatography of crude mucosal extracts from starved rats on

Sephadex G-25 did not affect the regulatory properties of phosphofructokinase (Jamal *et al.*, 1984), it was apparent that the inhibitor must be a protein of M_r greater than 25000. The existence of such a protein inhibitor of mucosal phosphofructokinase is reported here.

Materials and methods

Extracts of jejunal mucosa were prepared from female CHFB Wistar rats and assayed for phosphofructokinase activity as described previously (Jamal & Kellett, 1983*a*). Regulatory properties were expressed in terms of the activity ratio, v/V , where v is the activity observed at pH 7.0 for a given concentration of fructose 6-phosphate in the presence of 2.5 mM-ATP, and V is the maximal activity at pH 8.0.

Crude mucosal extract (3 ml) was chromatographed on a column (30 cm $\times$ 2.5 cm) of Sephadex G-100 equilibrated and eluted with 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-(NH₄)₂SO₄, 30 mM-KF, 1 mM-2-mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-6-amino-n-hexanoic acid. The column was calibrated for M_r determination with bovine serum albumin (68 000), ovalbumin (43 000) and lysozyme (14 600) as standards.

Protein fractions were concentrated by ultrafiltration in a Centriflo (Amicon) membrane cone with a M_r cut-off limit of 25000. Ultrafiltrates were prepared in the same way.

Biochemicals were obtained from Sigma or Boehringer, and other reagents were of analytical grade.

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Results and discussion

The saturation curve of phosphofructokinase in crude mucosal extracts prepared from rats starved for 48 h was sigmoidal, and the concentration of fructose 6-phosphate at half-saturation, $K_{0.5}$, was 0.70 mM (Fig. 1a). When crude extract was chroma-

Fig. 1. Effect of protein inhibitor on the regulatory properties of mucosal phosphofructokinase

(a) Phosphofructokinase was separated from its protein inhibitor by chromatography on Sephadex G-100 of a crude extract prepared from the mucosa of rats starved for 48 h as shown in Fig. 2. ●, Activity of phosphofructokinase in the crude extract; ○, activity of chromatographed phosphofructokinase in fraction 22 (Fig. 2) after separation from its inhibitor; □, the activity of chromatographed phosphofructokinase after incubation of 100 μ l of fraction 22 at 0°C for 10 min with 100 μ l of concentrated inhibitor solution prepared by 5-fold concentration of the pooled fractions 27–34. The assay sample was 30 μ l. (b) Activity of phosphofructokinase in a crude mucosal extract prepared from fed rats before (○) and after (●) incubation with concentrated inhibitor solution as described for (a). For full experimental details see the text.

tographed on Sephadex G-100, phosphofructokinase was eluted quantitatively at the void volume (Fig. 2). The chromatographed enzyme (fraction 22) was far less susceptible to inhibition by ATP than in crude extract, and its $K_{0.5}$ was decreased to 0.33 mM (Fig. 1a). Thus phosphofructokinase was strongly activated by chromatography on Sephadex G-100, so that the activity ratio at 0.5 mM-fructose 6-phosphate, $v_{0.5}/V$, rose from 0.23 ± 0.04 (S.E.M.) for crude extract before to 0.64 ± 0.12 ($n = 5$, $P < 0.001$) afterwards. No change in the regulatory properties of phosphofructokinase was observed when crude extract was chromatographed on Sephadex G-25 under identical conditions (Jamal *et al.*, 1984). It was apparent therefore that the activation on Sephadex G-100 was caused not by the removal of low- M_r effectors, nor by the influence of buffer ions, but by the separation of an inhibitor of $M_r > 25000$.

Fractions from the eluate of the Sephadex G-100 column were therefore tested for the presence of an inhibitor by incubating 100 μ l of each in turn with 100 μ l of activated phosphofructokinase from the peak (fraction 22, Fig. 2) for 10 min at 0°C and then determining $v_{0.5}/V$ as usual, with an assay sample of 30 μ l. Fractions 26–34 were found to contain inhibitory activity, with maximum inhibition occurring at fraction 30 (Fig. 2). The value of $v_{0.5}/V$ observed when the latter was incubated with chromatographed phosphofructokinase was approx. 0.43, a value that is characteristic of

Fig. 2. Separation of phosphofructokinase from its protein inhibitor by the chromatography on Sephadex G-100 of a crude extract prepared from the mucosa of rats starved for 48 h

○, Total phosphofructokinase activity determined at pH 8; □, inhibitory activity as determined by the ability of each fraction to diminish the value of $v_{0.5}/V$ of phosphofructokinase from fraction 22. Portions (100 μ l) of each fraction in turn were incubated with 100 μ l of fraction 22 at 0°C for 10 min: the trough of $v_{0.5}/V$ at fraction 30 therefore represents the peak of inhibitory activity. The assay sample was 30 μ l. For full experimental details see the text.

phosphofructokinase in crude mucosal extracts prepared from fed, but not from starved, rats (Fig. 1*b*; see also Jamal & Kellett, 1983*a*; Jamal *et al.*, 1984). Fractions 27–33 were therefore pooled and concentrated 5-fold. When 100 μ l of phosphofructokinase from the Sephadex G-100 eluate (fraction 22, Fig. 2) was incubated with 100 μ l of concentrated inhibitor solution, the fructose 6-phosphate saturation curve became indistinguishable from that for the original extract applied to the column (Fig. 1*a*). Under these conditions the effect of the inhibitor on the progress curve of the pH7 assay is very marked. In the absence of inhibitor, the progress curve for chromatographed phosphofructokinase showed a slight initial acceleration to the steady state. In contrast, incubation of the enzyme with concentrated inhibitor resulted in a pronounced negative transient from about 70s to 120s (Fig. 3). Such behaviour is the converse of that described for fructose 2,6-bisphosphate (Uyeda *et al.*, 1981). With another preparation, titration of chromatographed phosphofructokinase

with concentrated inhibitor solution added directly to the assay cuvette resulted in a monotonic decrease in $v_{0.5}/V$ from 0.63 to 0.15 (Fig. 4). The titration showed a sharp end-point at 11 μ l of inhibitor solution; hence the fructose 6-phosphate curve measured with 100 μ l of concentrated inhibitor solution was determined at saturating concentrations of inhibitor. As expected on the basis of these data, when 100 μ l of crude extracts prepared from the mucosa of fed rats was incubated with 100 μ l of concentrated inhibitor solution the fructose 6-phosphate saturation curve was shifted to higher concentrations and became indistinguishable from that for a crude extract derived from a rat starved for 48h (Fig. 1*b*).

The inhibitor in the concentrate of fractions 27–33 was destroyed either by incubation at 58°C for 5min or by incubation for 2h at 37°C with trypsin [0.1% (w/v) in Sephadex G-100 elution buffer], and was therefore a protein. Its M_r was determined by calibration of the Sephadex G-100 column to be $58\,000 \pm 3\,000$.

In principle, at least, there are several alternatives in consideration of the nature of the protein inhibitor. However, the possibility that it was an enzyme that destroyed a low- M_r activator could not account for the fact that phosphofructokinase was activated when crude extracts were chromatographed on Sephadex G-100, since any such activator would have been previously destroyed. In addition, the observation that the activity of phosphofructokinase was unaffected by chromato-

Fig. 3. Effect of protein inhibitor on the progress curve of phosphofructokinase assayed at pH 7

Changes in the A_{340} observed in the assay of (a) chromatographed phosphofructokinase (fraction 22, Fig. 2) and (b) chromatographed phosphofructokinase after the incubation of 100 μ l of fraction 22 with 100 μ l of concentrated protein inhibitor solution at 0°C for 10min. The assay sample was 30 μ l. In each case, 12.8munits of phosphofructokinase (maximal activity at pH8) was assayed at pH7.0 in the presence of 0.5mM-fructose 6-phosphate and 2.5mM-ATP. For full experimental details see the text.

Fig. 4. Titration of chromatographed phosphofructokinase with its protein inhibitor

$v_{0.5}/V$ was determined for chromatographed phosphofructokinase (fraction 22, Fig. 2) after the addition of a given volume of concentrated inhibitor solution directly to the assay cuvette. The enzyme assay sample was 15 μ l. For full experimental details see the text.

graphy of extracts on Sephadex G-25 effectively ruled out the involvement of low- M_r effectors. It was nevertheless perhaps conceivable that the data might still be explained if the protein inhibitor were an enzyme that generated a low- M_r inhibitor from the components of the assay mixtures. This possibility was eliminated in two ways. First, the activity of chromatographed phosphofructokinase after incubation with concentrated protein inhibitor solution in titration experiments was dependent only on the amount of protein inhibitor (Fig. 4); it was independent of the length of incubation from times as short as 5s, significantly before the negative transient (Fig. 3), to as long as 60min, about five times as long as an assay. This finding also ruled out the further possibility that the inhibitor protein was an enzyme that chemically modified phosphofructokinase in some way. Second, the assay mixture containing all components except phosphofructokinase was incubated with 30 μ l of concentrated protein inhibitor solution for different times up to 60min; ultrafiltrates of the putative reaction mixture then showed no inhibitory activity when added to the normal assay of chromatographed phosphofructokinase from which protein inhibitor was absent. The protein inhibitor had no effect on the auxiliary assay system.

Since a low- M_r effector is not concerned in the regulation of phosphofructokinase activity described above, it is apparent that the protein itself is an inhibitor of phosphofructokinase. The well-defined nature of the titration curve (Fig. 3) suggests that the inhibitor is specific for phosphofructokinase, although such a conclusion must await purification of the inhibitor and stoichiometry determination.

As we have shown above, the protein inhibitor has the ability *in vitro* to change the regulatory properties of phosphofructokinase from those characteristic of a fed rat to those of a starved rat (Fig. 1b). Whether such a change is accomplished *in vivo* either by an increase in the intrinsic strength of interaction between phosphofructokinase and its inhibitor, by, for example, a phosphorylation/dephosphorylation mechanism, or by an increase in the concentration of the latter, has yet to be established. It is nevertheless clear that the protein

inhibitor of phosphofructokinase has great potential for the regulation of glycolysis.

Much attention has been devoted to the role of fructose 2,6-bisphosphate as an intracellular signal for the regulation of glycolysis by glucose (Hers & van Schaftingen, 1982). There are, however, many situations in which glycolysis is regulated independently of glucose. Some tissues, such as erythrocytes or the present instance of intestinal mucosa, are simply not very responsive to glucose. Other tissues, although responsive to glucose, must have the facility for independent control. Obvious examples are hormonal control, such as heart by adrenaline, or neural control, especially in the contraction of skeletal muscle. In both cases, glycolysis is strongly activated, yet fructose 2,6-bisphosphate concentration is not increased (Hue *et al.*, 1981). The existence of a specific protein inhibitor of phosphofructokinase may well afford a unifying explanation for these diverse phenomena.

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