EFFECTS OF CITRATE ON PHOSPHOFRUCTOKINASE FROM LACTATING RAT MAMMARY GLAND ACINI

Victor. A. ZAMMIT

Biochemistry Department, Hannah Research Institute, Glasgow University, Ayr, KA6 5HL, Scotland

Received 8 October 1979

1. Introduction

The mammary gland of the lactating rat utilizes glucose, triglycerides and non-esterified fatty acids as its main substrates for lipogenesis [1]. Ketone bodies are also used as substrates both for de novo fatty acid synthesis and for oxidation [2]. Utilization of ketone bodies by the gland inhibits glucose uptake by the gland in vivo [3] and in vitro [2]. It has been suggested [2] that a possible explanation for this interrelationship between ketone body and glucose utilization by rat mammary gland is the inhibition of glycolysis at the phosphofructokinase (PFK) (EC 2.7.1.11) step by elevated concentrations of citrate as a result of ketone body metabolism. This suggestion assumes that PFK from the secretory epithelial cells of mammary tissue is sensitive to inhibition by citrate. Although several studies have been conducted on the effects of citrate on this enzyme from other tissues (see, e.g. [4,5]) none have been performed on PFK from mammary gland and it is not known whether the enzyme from this tissue is sensitive to citrate [6].

In the present study PFK was extracted from rat mammary gland acini and found to be inhibited by concentrations of citrate that are present in the gland in vivo under conditions characterized by reduced glucose utilization.

2. Materials and methods

2.1. *Animals*

Virgin female Wistar rats were bought from A. Tuck and Son, Battlesbridge, Essex and mated ~ 10 days after arrival. They were maintained on Oxoid rat breeding diet (Oxoid Ltd., Basingstoke, Hampshire, RG24 OPW). The litter of each animal was restricted to 8 pups and the mothers were killed after 10–12 days of lactation.

2.2. *Extraction of enzyme*

PFK was extracted from acini prepared from the abdominal and inguinal mammary glands. The animals were killed by cervical fracture and the glands excised quickly. Acini were prepared according to the procedure deseribed previously. The resultant acinar preparation was homogenized using a Polytron tissue disintegrator (on setting 6 for three 30 s periods) in an ice-cooled medium containing 0.25 M sucrose, 5 mM Tris, 1 mM EDTA, 1 mM DTT at pH 8.2. The resultant homogenate was centrifuged at 50 000 × *g* for 20 min. Phosphofructokinase activity was precipitated from the supernatant with $(NH_4)_2\ SO_4$. 90% of the activity was recovered at concentrations of $(NH_4)_2SO_4$ equivalent to 30–50% saturation. The precipitation procedure was repeated. Chromatography of the protein recovered on DEAE-cellulose equilibrated with the extraction buffer (see above) and eluted with a linear gradient of $(NH_4)_2SO_4$ (0–0.4 M) resulted in all the PFK activity being recovered as a single peak. No further purification of the enzyme was carried out. Removal of NH_4^+ from the enzyme preparation was achieved by passing an aliquot through a Sephadex G-25 column equilibrated with a solution containing 0.25 M sucrose, 5 mM Tris and 0.1 mM ATP at pH 8.0. The low ATP concentration used in the absence of NH_4^+ was required to stabilize enzyme activity. Enzyme preparations were

used within 48 h of extraction from mammary gland acini.

2.3. *Assay of enzyme activity*

Phosphofructokinase activity was measured spectrophotometrically [4]. The basic reaction medium contained 100 mM KCl, 0.14 mM NADH, 5 mM $MgCl_2$, aldolase (50 μg), glycerol-3-phosphate dehydrogenase (5 μg), triosephosphate isomerase (5 μg) and either 50 mM imidazole at pH 7.1 or 50 mM Tris at pH 8.1 in 1.0 ml total vol. For studies of the effects of pH on activity imidazole buffer was used for pH 7.0–7.8 and Tris buffer was used for pH 7.6–8.8. Preliminary experiments indicated that at the same pH similar activity of the enzyme was obtained using either buffer. All coupling enzymes were dialysed against 4 changes of 1 l of a medium containing 5 mM Tris at pH 7.5 for 6 h to remove NH_4^+. Additions of fructose-6-phosphate (F6P), Mg-ATP and citrate were made as indicated in the legends to figures and tables. All assays were performed at 30°C in a Gilford spectrophotometre. Aliquots of the enzyme preparation (10–20 μl containing 3–5 mg protein/ml) were preincubated for 5 min with the reaction medium containing all components except for F6P. The reaction was started by addition of F6P and initial rates were measured. Controls from which F6P was omitted were run concurrently. Preliminary experiments indicated that similar activities were obtained using either F6P alone or a mixture of F6P, G6P and phosphoglucose isomerase [4] to start the reaction. Therefore, in all subsequent experiments F6P only was used. The rates of NADH oxidation obtained gave $\Delta A > 0.1/10$ min. Control rates were $\leqslant 5\%$ of the experimental rates.

2.4. *Chemicals*

Biochemicals, enzymes and chromatography materials were obtained from Sigma Chemical Co., Poole, Dorset, BH17 7NH. Sucrose, ammonium sulphate and other inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, Bh12 4NN.

2.5. *Presentation of results*

In figures and tables, activity of PFK is given as a fraction (V/V_t) of the maximum PFK activity (V_t) obtained using 1 mM F6P and 0.8 mM ATP, at pH 8.1. Each set of results shown is representative of those obtained from $\geqslant 3$ similar experiments.

3. Results

The maximum activity of PFK in whole mammary gland homogenates averaged 3.54 μmol. min^{-1}. g wet wt^{-1}. The activity of PFK in acini was 10 μmol . min^{-1} . g wet wt^{-1} (the mammary glands from one animal yielded 1–2 g acini).

Phosphofructokinase from mammary secretory cells was very unstable in the absence of NH_4^+. However, 0.1 mM ATP stabilized enzyme activity (see section 2). Consequently, all NH_4^+-free enzyme preparations contained 0.1 mM ATP. (The amount of ATP added to the assay medium in the cuvette with the enzyme preparation was accounted for in calculating final ATP concentrations.)

The dependence of activity of PFK from rat mammary gland on pH was similar to that of the enzyme from other mammalian tissues [8]. At higher (inhibitory, see below) concentrations of ATP (0.8 mM) the activity of the enzyme was 32% of the maximum activity at pH 8.1. At lower, non-inhibitory concentrations of ATP (0.2 mM) the pH optimum was similar but less sharp (see table 1).

3.1. *Effects of ATP and citrate concentrations*

The effects of different concentrations of ATP and citrate on PFK activity were studied at pH 7.1 and at pH 8.1, in the absence of NH_4^+. ATP inhibited the enzyme at pH 7.1 and at pH 8.1, but the enzyme was much more sensitive to ATP inhibition at the lower

Table 1
Effect of pH on the activity of PFK at inhibitory and non-inhibitory concentrations of ATP

	Relative activity (V/V_t)	
pH	0.2 mM ATP	0.8 mM ATP
7.1	0.45	0.32
7.3	0.56	0.45
7.5	0.64	0.69
7.7	0.71	0.84
7.9	0.73	0.97
8.1	0.75	1.00
8.3	0.74	0.83
8.5	0.70	0.61

F6P was 1 mM. Activities are expressed as a fraction (V/V_t) of the maximum activity (V_t) obtained at pH 8.1 using 0.8 mM ATP and 1 mM F6P (see section 2)

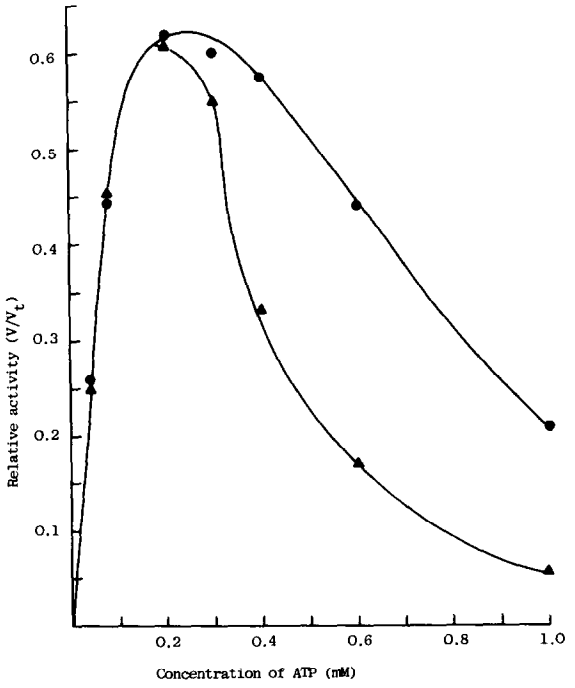

Fig.1. Effects of ATP concentration on PFK activity in the absence (●—●) and presence (▲—▲) of citrate (2 mM) at pH 7.1. F6P was 1 mM. Activities are expressed as a fraction (V/V_t) of the maximum activity (V_t) obtained at pH 8.1 using 0.8 mM ATP and 1 mM F6P (see section 2).

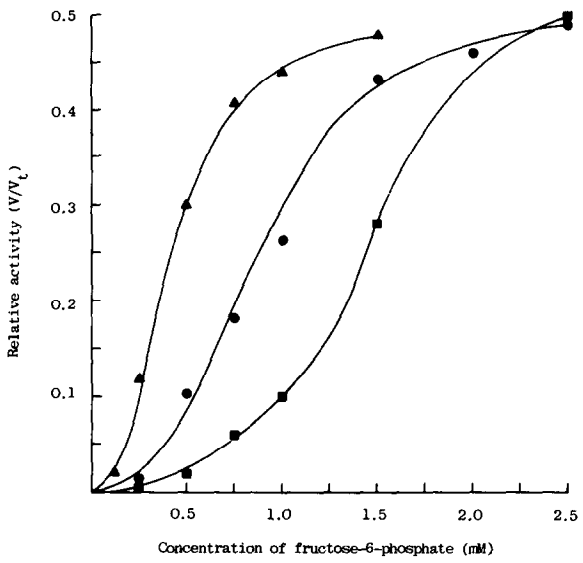

Fig.2. Effects of F6P concentration on PFK activity in the presence of 0.2 mM ATP (■—■), 0.8 mM ATP (●—●) and 0.8 mM ATP plus 2 mM citrate (▲—▲). Activities are expressed as a fraction (V/V_t) of the maximum activity (V_t) obtained at pH 8.1 using 0.8 mM ATP and 1 mM F6P (see section 2).

pH (fig.1), the activity at 1.2 mM ATP being 30% of that at 0.2 mM ATP. At pH 8.1 maximal activity was obtained at higher concentrations of ATP (0.8 mM) and inhibition (10%) only occured at > 2.4 mM ATP. Addition of citrate potentiated the ATP-induced inhibition at pH 7.1. The app. K_i for citrate inhibition (at 1 mM–F6P and 0.6 mM ATP) was 0.2 mM. However, citrate had no effect on enzyme activity at pH 8.1 (data not shown). The inhibitory effects of ATP and of citrate were due to a decrease in affinity of the enzyme for F6P (fig.2). Thus, in the presence of non-inhibitory concentrations of ATP and in the absence of citrate the app. K_m for F6P was 1.0 mM whereas in the presence of 0.8 mM ATP plus citrate (2 mM) it was 1.5 and 2.2 mM, respectively.

4. Discussion

The mammary gland contains a number of different tissue types (e.g., secretory, muscle, adipose cells). Since utilization of substrates for lipogenesis of milk-fat occurs exclusively in the secretory cells it was considered necessary to extract and study the properties of PFK from these cells. Consequently acini were prepared from 10–12 day lactating rat mammary glands. These preparations were judged to be substantially free of cell types other than secretory cells by microscopic examination [9].

In the present study a partially purified preparation of PFK was used; consequently precise quantitative interpretation of the data has to be made with caution. However, it is possible to ascertain that the mechanism of inhibition by citrate (potentiation of ATP-induced inhibition and a rise in K_m for F6P) is similar to that found for citrate inhibition of PFK from other tissues [5,10]. The K_i for citrate inhibition (0.2 mM) is similar to that for PFK from rabbit muscle and brain tissues and ~ 10-fold lower than for PFK from rat and rabbit liver [4,5]. Therefore PFK from mammary gland is relatively very sensitive to citrate inhibition. Moreover, the K_i observed is similar to the concentrations of citrate which were found in mam-

mary gland slices and in vivo. These range from 0.12–0.26 mM in the normal animals [2,11] and increase to 0.39 mM in lactating rats injected with acetoacetate [3]. It is suggested that the effects of citrate on phosphofructokinase observed in vitro are of physiological significance and support the suggestions that the reduced rate of glucose utilization by rat mammary gland by ketone bodies may be due to inhibition of glycolysis at the PFK reaction by elevated concentrations of citrate in the gland. This mechanism would spare glucose during periods of increased fat fuel oxidation by the gland (e.g., short-term starvation). However, because of the high rates of lipogenesis in the lactating rat mammary gland, oxidation of non-esterified fatty acids may be limited in the gland. Hence, the formation of citrate due to ketone body metabolism may be of major importance in regulating phosphofructokinase activity and, consequently, glucose utilization by the gland, even if ketones are not an alternative lipogenic substrate to glucose [12].

Acknowledgements

I thank Professor J.A.F. Rook for his interest and encouragement, and Mr N. Lambert for excellent assistance.

References

[1] Hawkins, R. A. and Williamson, D. H. (1972) Biochem. J. 129, 1171–1173.
[2] Williamson, D. H., McKeown, S. R. and Ilic, V. (1974) Biochem. J. 150, 145–152.
[3] Robinson, A. M. and Williamson, D. H. (1977) Biochem. J. 164, 749–752.
[4] Underwood, A. H. and Newsholme, E. A. (1965) Biochem. J. 95, 868–875.
[5] Tsai, M. Y. and Kemp, R. G. (1974) J. Biol. Chem. 249, 6590–6596.
[6] Kuhn, N. J. (1978) Biochem. Soc. Trans. 6, 539–543.
[7] Robinson, A. M. and Williamson, D. H. (1978a) in: Biochemical and Clinical Aspects of Ketone Body Metabolism (Soling, H. D. and Seufert, C.-D. eds) Georg Thieme, Stuttgart.
[8] Kemp, R. G. (1971) J. Biol. Chem. 246, 245–252.
[9] Katz, J. Wals, P. A. and Van de Velde, R. L. (1974) J. Biol. Chem. 249, 7348–7357.
[10] Newsholme, E. A. and Start, C. (1973) Regulation in Metabolism, John Wiley and Sons, London, New York.
[11] Gumaa, K. A., Greenbaum, A. L. and McLean, P. (1971) in: Lactation (Falconer, I. R. ed) Butterworth, London.
[12] Robinson, A. M. and Williamson, D. H. (1978b) Biochem. J. 176, 635–638.