Effect of polyethylene glycol on the kinetic behaviour of pyruvate kinase and other potentially regulatory liver enzymes

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Received 16 November 1984

Assay in the presence of 10% polyethylene glycol has been systematically used with potentially regulatory liver enzymes as an indirect way to induce aggregation of enzymes corresponding to that which could occur at their physiological concentrations Pyruvate kinase L was markedly affected by polyethylene glycol, as was muscle phosphorylase *a*, while pyruvate kinase M as well as glucokinase, fructose-1,6-bisphosphatase and other liver enzymes examined were not affected

Enzyme concentration *Polyethylene glycol* *Liver enzyme* *Pyruvate kinase* *Glycogen phosphorylase* a

1. INTRODUCTION

The kinetic behaviour of phosphofructokinase and pyruvate kinase studied in situ after permeabilization of erythrocytes was found to be markedly different from that observed in vitro, in contrast to other enzymes examined [1]. The behaviour of mammalian phosphofructokinase in situ can be accounted for as due to multiaggregation of the enzyme at physiological concentrations [2]. An indirect way of favouring aggregation of enzymes at the low concentrations convenient for kinetic studies is the use of polyethylene glycol (PEG) at high concentrations ([3]; unpublished observations). Liver cells cannot be effectively permeabilized for kinetic studies ([1]; unpublished results). We have used the PEG approach to explore the possibility that some potentially regulatory liver enzymes could be affected in their kinetic parameters by the concentration of the enzyme. As reported here, pyruvate kinase L was markedly affected by 10% PEG, while other liver enzymes studied were not affected.

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2. MATERIALS AND METHODS

2.1. *Enzyme preparations*

Liver obtained from fed rats (male, Wistar, 200–300 g) were homogenized in 3 vols of buffer A (50 mM Hepes, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol). The homogenate was centrifuged at 30000 × *g* for 30 min, the pellet discarded and the supernatant centrifuged again at 105000 × *g* for 30 min. The following enzyme activities were partially purified by ammonium sulfate fractionation of the supernatant, percent $(NH_4)_2SO_4$ is indicated in parentheses: pyruvate kinase L (30–40%), fructose-1,6-bisphosphatase (40–50%), glucokinase (60–75%), glyceraldehyde-3-phosphate dehydrogenase (60–80%), 6-phosphogluconate dehydrogenase (60–70%) and L-serine dehydratase (30–40%). The precipitated material was collected, dissolved

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00145793/85/$3 30

in 1–2 ml of buffer A and filtered through a column of medium Sephadex G-25 (1 × 10 cm) equilibrated with buffer A NAD^+ kinase activity was assayed in the 105000 × *g* supernatant filtered through Sephadex G-25 as described above. Rabbit muscle pyruvate kinase (Sigma, type III), glycogen phosphorylase *a* (Sigma), and bovine liver glutamate dehydrogenase (Sigma, Type II) were used after dilution in buffer A. Auxiliary enzymes were filtered through Sephadex G-25.

2.2. *Enzyme assays*

All enzyme assays were carried out at 37°C in a reaction mixture of 1 ml containing 50 mM Hepes, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$ plus specific additions as follows. Pyruvate kinase: 2 mM p-*enol*pyruvate, 1 mM ADP, 0.15 mM NADH and 1 unit of lactate dehydrogenase Fructose-1,6-bisphosphatase: 1 mM fructose-1,6-P_2, 0.25 mM $NADP^+$, 1 unit of glucose phosphate isomerase and 1 unit of glucose-6-phosphate dehydrogenase. Glucokinase: according to Viñuela and Sols [4]. Glucose-6-phosphate dehydrogenase: 0.5 mM glucose and 0.5 mM $NADP^+$ Glutamate dehydrogenase: 10 mM 2-oxoglutarate, 10 mM NH_4Cl, 1 mM EDTA and 0.15 mM NADH. Glyceraldehyde-3-phosphate dehydrogenase: by the linear method of Aragón and Sols [5]. Glycogen phosphorylase: 40 mM maltotetraose, 20 mM potassium phosphate, 0.2 mM EDTA, 1 mM dithiothreitol, 10 μM glucose-1,6-P_2, 0.5 mM $NADP^+$, 2 units of phosphoglucomutase and 1 unit of glucose-6-phosphate dehydrogenase. NAD^+ kinase: 4 mM NAD^+, 6 mM ATP and 1 mM dithiothreitol; after incubation for 30 min the reaction was terminated by addition of 3% perchloric acid; after centrifugation at 25000 × *g* for 10 min the supernatant was neutralized with a mixture of 30% potassium carbonate and 0.5 mM triethanolamine, precipitated $KClO_4$ was removed by centrifugation and the formation of $NADP^+$ was measured in the supernatant by adding 1 mM 6-phosphogluconate and 0.05 units of 6-phosphogluconate dehydrogenase. 6-Phosphogluconate dehydrogenase: 0.5 mM NAD^+ and 0.5 mM 6-phosphogluconate. L-serine dehydratase: 0.5 M L-serine, 0.1 mM pyridoxal phosphate, 0.1 mM dithiothreitol, 0.15 mM NADH and 1 unit of lactate dehydrogenase. Protein was determined by the Bradford method [6].

2.3 *Chemicals and enzymes*

PEG with nominal M_r of 6000 was purchased from Scharlau (Barcelona, Spain). Fructose-2,6-P_2 was kindly donated by Professor H.-G Hers, Laboratoire de Chimie Physiologique, Université Catholique de Louvain, Brussels; maltotetraose was kindly donated by Boehringer, Tutzing. Enzymes were obtained from Sigma (St Louis, MO) and Boehringer (Mannheim, FRG) Other chemicals were from Calbiochem (San Diego, CA).

3. RESULTS

As shown in fig.1, the addition of PEG to the reaction mixture produced a significant increase in the affinity of liver pyruvate kinase L for phospho*enol*pyruvate; $S_{0.5}$ and n_H values changed from 0.4 mM and 1.4 to 0.15 mM and 0.8, respectively. This decrease in the cooperativity for phospho*enol*pyruvate led to a lesser dependence on activation by fructose-1,6-P_2, which effect on pyruvate kinase L was not modified by PEG (not shown). In contrast with the liver enzyme the kinetic behaviour of muscle pyruvate kinase M, a non-regulatory isozyme, was not affected by PEG (fig.1). As seen in fig.2, ATP inhibition of pyruvate kinase L was reduced in the presence of PEG while the inhibition by alanine was not affected by the aggregating agent.

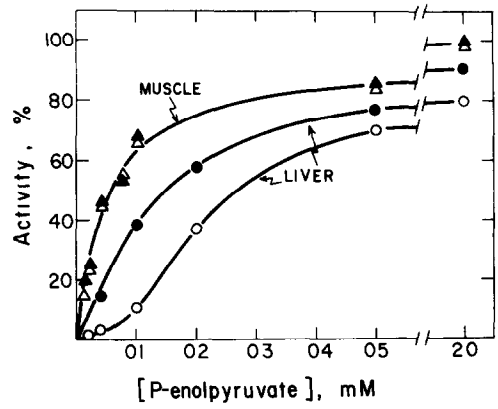

Fig 1 Effect of polyethylene glycol on the saturation curves of liver and muscle pyruvate kinase. Open symbols are control experiments and filled symbols are experiments in the presence of 10% PEG Maximal activity (100%) of liver pyruvate kinase was assayed at 2 mM P-*enol*pyruvate plus 0 1 mM fructose-1,6-P_2

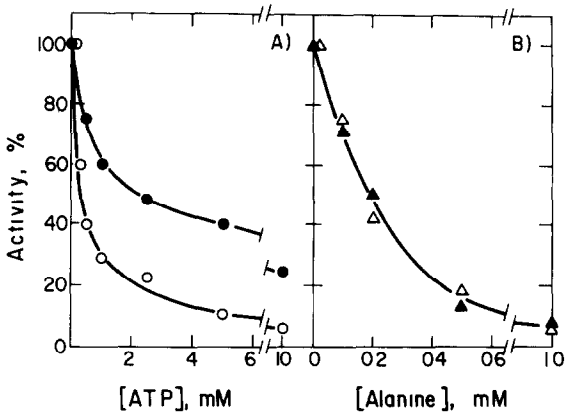

Fig.2. Effect of polyethylene glycol on the ATP (A) and alanine (B) inhibition of liver pyruvate kinase Open symbols are control experiments and filled symbols are experiments in the presence of 10% PEG.

In addition to pyruvate kinase the effect of PEG was also studied on other potentially regulatory liver enzymes, as indicated in table 1. Out of the nine enzymes examined only two were significantly affected by PEG: L-serine dehydratase, which showed an increase in affinity for L-serine and a sigmoidal saturation curve in the presence of PEG, and glycogen phosphorylase *a* from muscle that exhibited a higher $S_{0.5}$ for P_i and a smaller inhibition by P-creatine in the presence of PEG.

The levels in liver of some of the enzymes studied are known to be affected by the nutritional state of the animal. This fact opened the possibility that the liver of fasted animals could contain an isozyme, or molecular form of some of its enzymes, that could be more (or less) affected by the presence of PEG. After 18 h of fasting no significant difference was found with the following enzymes and parameters: fructose-1,6-bisphosphatase ($S_{0.5}$ and V_{max}), glucokinase ($S_{0.5}$, n_H and V_{max}), phosphofructokinase ($S_{0.5}$ and n_H for fructose-6-P, V_{max}, and fructose-2,6-P_2 activation), pyruvate kinase ($S_{0.5}$ and n_H for phospho*enol*pyruvate, V_{max}, and fructose-1,6-P_2 activation) and L-serine dehydratase ($S_{0.5}$, n_H and V_{max}).

Table 1
Other enzymes studied in the presence of PEG

Enzyme	Kinetic parameters ± 10% PEG[a]		
	$S_{0.5}$ (mM)		Other observations
Fructose-1,6-bisphosphatase		0.07	5 μM Fru-2,6-P_2 inhibited 50% 5 μM + 0.1 μM AMP inhibited 85%
Glucokinase	glucose	8	n_H 1.3
Glucose-6-phosphate dehydrogenase	Glc-6-P $NADP^+$	0.025 0 015	 K_i NADPH 0.11 mM
Glutamate dehydrogenase	2-oxoglut.	0.3	K_i GTP 0.02 mM
Glyceraldehyde-3-phosphate dehydrogenase	NAD^+	0.03	
Glycogen phosphorylase *a*[b]	P_i	15 (25)	40 mM P-creat. inhibited 90% (30%)
NAD^+ kinase	NAD^+	1.0	
6-Phosphogluconate dehydrogenase	$NADP^+$	0.01	K_i NADPH 0 08 mM
L-Serine dehydratase		600 (140)	n_H 1 (1.7)

[a] When the results obtained in the presence of PEG were different from those in its absence the value with PEG is given in parentheses. V_{max} was affected by PEG only in the case of phosphorylase, which was 150% of the control value
[b] From muscle

4. DISCUSSION

The addition of PEG to the assay for liver pyruvate kinase L leads to a more active form of enzyme that shows higher affinity for phospho*enol*pyruvate and less sensitivity to ATP inhibition (figs 1,2). This change is presumably mediated by enzyme aggregation induced by PEG. A similar decrease in the cooperativity for phospho*enol*pyruvate has been reported when pyruvate kinase L was studied in situ in permeabilized erythrocytes [1]. This suggests that the regulatory behaviour of pyruvate kinase L is influenced by its own concentration, as it has also been reported for phosphofructokinase [2]. The failure of Flory et al. [7] to detect marked changes in the behaviour of pyruvate kinase L assayed at cellular concentration by the stopped-flow approach could be due to the use of rather high concentrations of P-*enol*pyruvate in addition to the hysteresis problems. Most other enzymes studied in the presence of PEG (table 1), as well as by the in situ approach [1], did not show a significant change in their kinetic behaviour. This indicates that most enzymes may not be affected by their concentrations in vivo. The exception seems to be the case of some regulatory enzymes such as phosphofructokinase and pyruvate kinase L. Note that muscle pyruvate kinase, a non-allosteric isozyme, was not affected by PEG, suggesting that the effect of enzyme concentration, when it occurs, may work mainly by adjusting some of the properties of regulatory enzymes. Eagle and Scopes [8] had reported that muscle phosphorylase *a* assayed at high enzyme concentration (2 $mg \cdot ml^{-1}$) had a K_m for P_i higher than that observed with diluted enzyme. Our results with PEG suggest that multiaggregation of the enzyme is likely to be involved.

Results so far confirm the value of PEG to mimic high concentrations of enzyme for kinetic studies and also that artifacts in vitro due to disaggregation of diluted enzymes, although uncommon, occur with some enzymes. The overcrowding of proteins in the cytoplasm has recently been emphasized [9].

ACKNOWLEDGEMENTS

This work was supported by a grant from the Comisión Asesora para la Investigación Científica y Técnica as well as personal support to R.M. from Cooperación Iberoamericana del Ministerio de Educación y Ciencia and from the Fondo de Investigaciones Sanitarias

REFERENCES

[1] Aragón, J J , Felíu, J E , Frenkel, R A and Sols, A. (1980) Proc. Natl. Acad Sci USA 77, 6324–6328.
[2] Boscá, L , Aragón, J J and Sols, A. (1985) J Biol Chem , in press.
[3] Reinhart, G.D (1980) J. Biol. Chem. 255, 10576–10578
[4] Viñuela, E. and Sols, A. (1963) J Biol. Chem. 238, 1175–1177
[5] Aragón, J.J. and Sols, A. (1978) Biochem. Biophys. Res. Commun. 82, 1098–1103.
[6] Bradford, M. (1976) Anal. Chem. 72, 248–254
[7] Flory, W., Peczoch, B.D , Koeppe, R E. and Spirey, H.O (1974) Biochem. J 141, 127–131.
[8] Eagle, G.R and Scopes, R.K (1981) Arch Biochem. Biophys. 210, 540–548
[9] Fulton, A B. (1982) Cell 30, 345–347