Catecholamines and Ca^{2+} mediate an increase in activity and reactive thiols of rat heart phosphofructokinase

Michael G. CLARK,*† Stephen RATTIGAN,* Glen S. PATTEN† and Owen H. FILSELL†

*Department of Biochemistry, University of Tasmania, Box 252C, G.P.O., Hobart, Tasmania 7001, and †Commonwealth Scientific and Industrial Research Organization, Division of Human Nutrition, Kintore Avenue, Adelaide, South Australia 5000, Australia

Catecholamines and Ca^{2+} mediate an increase in reactive-thiol status of phosphofructokinase, associated with an increase in activation of the enzyme. A correlation was observed between the number of reactive thiols and activation ($r = 0.878$; $P < 0.001$) for 14 different treatments, with approx. 1 group per 85000- M_r subunit becoming available over the range from the lowest to the highest state of activation.

INTRODUCTION

Treatment of the perfused rat heart with adrenaline leads to the activation of phosphofructokinase (EC 2.7.1.11) (Clark & Patten, 1981; Narabayashi *et al.*, 1985), which we have demonstrated is mediated predominantly by α_1 -adrenergic receptors, via a cyclic AMP-independent, Ca^{2+} -dependent, mechanism (Clark & Patten, 1984). The molecular mechanism for this activation is still largely unresolved. Studies in our laboratory failed to find an involvement of low- M_r effectors, and activation of the enzyme did not coincide with increased $[\text{P}^{32}\text{P}]_i$ incorporation, even though parallel studies indicated $[\text{P}^{32}\text{P}]$ phosphorylation of phosphorylase (Patten *et al.*, 1981). In contrast with these findings, a more recent paper from Uyeda's laboratory indicated that phosphorylation of phosphofructokinase occurred concomitant with an increase in the concentration of fructose 2,6-bisphosphate, even though increased incorporation of $[\text{P}^{32}\text{P}]_i$ was minimal (Narabayashi *et al.*, 1985). It was proposed by Uyeda's group (Narabayashi *et al.*, 1985) that phosphorylation of phosphofructokinase (which may involve protein kinase C; Hofer *et al.*, 1985) sensitizes the enzyme to activation by fructose 2,6-bisphosphate.

Even though regulation by phosphorylation is an attractive possibility, other mechanisms may control phosphofructokinase. There is increased interest in the possibility that enzymes may be regulated by disulphide-thiol exchange (Zeigler, 1985). Phosphofructokinase has been the subject of such a proposal (Gilbert, 1982). It contains 17 thiol groups per subunit, four of which are relatively highly reactive (Kemp & Forest, 1968), and the muscle enzyme is reversibly inactivated by GSSG (Luther *et al.*, 1983). Thus in the present study we have determined thiol reactivity of phosphofructokinase and its association with adrenaline/ Ca^{2+} -mediated changes in activation in perfused rat heart.

MATERIALS AND METHODS

Unless indicated otherwise, male rats (180–230 g body wt.) of the Hooded Wistar strain were used. The animals were maintained *ad libitum* on a standard

laboratory chow diet (Clark *et al.*, 1982). Hearts were perfused in the Langendorff manner with a system based on that of Williamson (1964). The perfusion medium was Krebs–Henseleit bicarbonate buffer (Clark & Patten, 1981) containing 1.27 mM- CaCl_2 (unless otherwise indicated) and 0.05 mM-EDTA, and the perfusion temperature was 37 °C. The hearts were pre-perfused at a flow rate of 5–8 ml/min for 10 min in a non-recirculating manner before the experiment commenced. Agonists were infused directly above the heart as concentrated solutions. To avoid precipitation of CaCO_3 , a concentrated solution of CaCl_2 (2.4 M) was infused into the perfusate above the heart. The rate and amount of infusion were adjusted so that the concentration of Ca^{2+} in the non-recirculating perfusate increased gradually and uniformly over 1–2 min.

The hearts were freeze-clamped after 5 min exposure to either agonist or medium containing low (0–0.1 mM) or high (8–10 mM) Ca^{2+} . Cell-free extracts of frozen hearts were prepared by homogenizing 1 part (by wt.) of powdered heart with 5 vol. of N_2 -gassed homogenizing buffer (100 mM-Tris/HCl, pH 7.4, containing 50 μM -cyclic AMP, 5 mM- MgCl_2 , 50 μM - Na_2EDTA and 3 mM- NaN_3), with an Ultra-Turrax homogenizer. Under these conditions extraction of the enzyme was complete and no losses owing to inactivation occurred. A high-speed supernatant fraction was prepared (100000 g, 15 min), and 1 ml was passed through a 10 ml column of Bio-Gel P-6DG to remove low- M_r components. All operations used buffers equilibrated with N_2 . Fractions from the column containing protein were assayed for phosphofructokinase activity. The assays were conducted in disposable plastic tubes (70 mm $\times$ 10 mm) at 30 °C. For the activity ratio v_{100}/V_{max} (where v_{100} is activity at sub-saturating hexose 6-phosphate concentration), assays contained either 100 μM -glucose 6-phosphate, 3.3×10^4 d.p.m. of $[\text{P}^{32}\text{H}]$ glucose 6-phosphate, 1 mM-ATP, 6.25 mM- MgCl_2 , 62.5 μM -cyclic AMP and 50 μl of the post-column supernatant (0.1–0.2 mg of protein) in a total volume of 0.2 ml, or 1 mM-glucose 6-phosphate, 3.3×10^4 d.p.m. of $[\text{P}^{32}\text{H}]$ glucose 6-phosphate, 1 mM-ATP, 6.25 mM- MgCl_2 , 5 mM- $(\text{NH}_4)_2\text{SO}_4$, 0.085 unit of aldolase, 42 units of triose phosphate isomerase, 5 units of phosphoglucose isomerase and

† To whom correspondence and reprint requests should be addressed.

Table 1. Effect of perfusate Ca^{2+} concentration and adrenaline on the activity ratio of phosphofructokinase and its reactivity with iodo[2- ^{14}C]acetate

Isolated Langendorff-perfused rat hearts were perfused with medium containing catecholamine and/or the concentration of Ca^{2+} shown. The hearts were freeze-clamped after 4–5 min exposure to each of the conditions. Cell-free extracts of the frozen hearts were prepared and a high-speed supernatant free of low- M_r components was isolated. The activity ratio of phosphofructokinase and its reactivity with 0.2 mM-iodo[2- ^{14}C]acetate were determined as described in the text. Results for activities are given as means $\pm$ S.E.M., and results for carboxymethylation are given as means $\pm$ range, with the numbers of heart perfusions in parentheses.

Additions	Activity of phosphofructokinase (nmol/min per mg of protein)		$10^3 \times$ Activity ratio ($v_{100}/V_{\max.}$)	Iodacetate bound (pmol/pmol of phosphofructokinase subunit)		
	v_{100}	$V_{\max.}$		Total bound	Amount bound in presence of 1 mM-ATP	ATP-inhibited binding
0–0.1 mM- Ca^{2+}	0.33 $\pm$ 0.09	181.1 $\pm$ 8.2	1.77 $\pm$ 0.50 (5)	0.34 $\pm$ 0.05 (2)	0.17 $\pm$ 0.06	0.17 $\pm$ 0.01
1.22 mM- Ca^{2+}	1.02 $\pm$ 0.16	256.6 $\pm$ 18.8	4.26 $\pm$ 0.78 (7)	0.40 $\pm$ 0.06 (2)	0.18 $\pm$ 0.03	0.22 $\pm$ 0.03
8–10 mM- Ca^{2+}	2.03 $\pm$ 0.24	227.3 $\pm$ 20.7	0.01 $\pm$ 0.97 (7)	0.62 $\pm$ 0.09 (2)	0.31 $\pm$ 0.10	0.31 $\pm$ 0.01
1.22 mM- Ca^{2+} + 1 μM -adrenaline	2.66 $\pm$ 0.79	265.5 $\pm$ 28.5	10.51 $\pm$ 2.91 (5)	0.55 (1)	0.20	0.35

50 μl of the post-column supernatant. Reactions were started by the addition of the supernatant and terminated at linear stages of the rate curves (v_{100} , 6 min; $V_{\max.}$, 2 min) by the addition of 0.5 ml of borate resin slurry as described previously (Clark & Patten, 1981). The calculated activity of phosphofructokinase in each assay was based on the specific radioactivity of the assay components.

Pooled fractions containing protein from the Bio-Gel column were also treated with iodo[2- ^{14}C]acetate. A 0.3 ml portion (0.6–1.2 mg of protein) was added to N_2 -equilibrated buffer (0.075 ml) with and without 1 mM-ATP. To each was added 0.02 ml of iodo[2- ^{14}C]acetate (final concn. 0.2 mM) and acylation was allowed to proceed for 90 min under N_2 in the dark at 0 $^\circ\text{C}$. After this time 30 mg of anti-phosphofructokinase serum (Patten *et al.*, 1981) was added and the immunoprecipitate allowed to form over 15 h at 5 $^\circ\text{C}$. The immunoprecipitates were collected by centrifugation (8000 g, 5 min), washed with 3 $\times$ 1 ml of 0.9% NaCl and solubilized by incubation for 25 min at 90 $^\circ\text{C}$ in 70 mM-Tris/HCl, pH 7.0, containing 2% (v/v) SDS, 10% (v/v) glycerol and 0.02% Bromophenol Blue. Electrophoresis (Patten *et al.*, 1981) was conducted on 7.5% -acrylamide gels (10 cm) with 3.8% stacking gels (1 cm). Non-reducing conditions gave optimal resolution of the phosphofructokinase subunit, which was cut out, dissolved and counted for radioactivity.

The amount of iodo[2- ^{14}C]acetate bound was expressed as a function of the amount ($V_{\max.}$) of phosphofructokinase applied to the gels. Conversion of enzyme activity into pmol of subunit was based on the relationship that 1 μg of heart phosphofructokinase converts 234 nmol of fructose 6-phosphate into fructose 1,6-bisphosphate/min at 30 $^\circ\text{C}$ (Mansour *et al.*, 1966) and that the enzyme is composed of four subunits, of M_r 85000.

To check that recovery of ^{14}C -labelled enzyme was quantitative, two types of experiments were conducted. In the first a known amount (approx. 6 μg) of rabbit skeletal-muscle phosphofructokinase (Sigma Chemical Co., St. Louis, MO, U.S.A.) was mixed with anti-phosphofructokinase serum, precipitated, washed, solubilized and then applied to the polyacrylamide gel. For

comparison an identical amount of phosphofructokinase was applied directly to the gel. After electrophoresis and staining, the bands corresponding to the phosphofructokinase subunit were compared by densitometry. In the second experiment a sample of iodo[2- ^{14}C]acetate-labelled rabbit skeletal-muscle phosphofructokinase was made to react with anti-phosphofructokinase serum and, after washing, the pellet was solubilized. A sample was taken for radioactivity counting and compared with the total radioactivity distributed in the gel after electrophoresis. Typical recoveries were 97–98% for protein and 99–110% for radioactivity.

To determine the amino acids labelled by iodo[2- ^{14}C]acetate, protein samples were acid-hydrolysed, and the amino acids together with labelled derivatives were applied to thin-layer plates of cellulose and resolved with butan-1-ol/acetic acid/water (12:3:5, by vol.) as solvent. Reference compounds were *S*-carboxymethylcysteine, *S*-carboxymethylmethioninesulphonium, glycollate, methylthioglycollate and the amino acids. The identity of *S*-carboxymethylcysteine was confirmed by preparing the *o*-phthaldehyde derivative and resolving by reverse-phase h.p.l.c. (Gardner & Miller, 1980).

Total protein thiol and disulphide content was determined by the mercaptoethanol reduction method of Habeeb (1972). The total extract content of GSH and GSSG was determined by the enzymic method of Tietze (1969).

RESULTS

Table 1 shows the effect of varying the perfusate Ca^{2+} concentration and of adding 1 μM -adrenaline to hearts perfused with 1.22 mM- Ca^{2+} on the activity ratio of phosphofructokinase and its reactivity with 0.2 mM-iodo[2- ^{14}C]acetate. Increasing the perfusate Ca^{2+} concentration from approx. 0.1 mM to 8–10 mM increased the activity at 100 μM -hexose 6-phosphate by 6-fold. Adrenaline increased the activity at 100 μM -hexose 6-phosphate (1.22 mM- Ca^{2+}) by approx. 2.6-fold. The $V_{\max.}$ activity was influenced to a much lesser extent than the v_{100} by either the increase in perfusate Ca^{2+} concentration or the addition of 1 μM -adrenaline to

Fig. 1. Correlation between the activity ratio of phosphofructokinase and the acylation of the enzyme by iodo[2-¹⁴C]acetate

Experimental details are as given in Table 1. Hearts were chosen to reflect a range of activity ratios of phosphofructokinase and were: 1 and 2, hearts perfused with medium containing 0–0.1 mM-Ca²⁺; 3, heart from obese (*fa/fa*) Zucker rat perfused with 1.22 mM-Ca²⁺; 4, heart from obese (*fa/fa*) Zucker rat perfused with 1.22 mM-Ca²⁺ + 1 μ M-adrenaline; 5 and 6, hearts perfused with 1.22 mM-Ca²⁺; 7, heart perfused with 1.22 mM-Ca²⁺ + 1 μ M-isoprenaline; 8, heart perfused with 1.22 mM-Ca²⁺ + 1 μ M-adrenaline + 10 μ M-propranolol; 9, extract from heart 8 treated with 10 mM-GSSG and then gel-filtered; 10, extract from heart 8 treated with 10 mM-GSH and then gel-filtered; 11, heart perfused with 1.22 mM-Ca²⁺ + 1 μ M-adrenaline; 12, heart from lean (*Fa/?*) Zucker rat perfused with 1.22 mM-Ca²⁺; 13 and 14, hearts perfused with 8–10 mM-Ca²⁺. (a) Total groups acylated; (b) groups acylated in the presence of 1 mM-ATP; (c) groups not acylated in the presence of 1 mM-ATP (protected by ATP). The lines of best fit were compiled by the method of least squares. All values on the ordinate are in pmol/pmol of phosphofructokinase subunit.

hearts perfused with 1.22 mM-Ca²⁺. Thus the increases in activity ratio, v_{100}/V_{max} , after these perturbations were largely attributable to changes in activity determined at 100 μ M-glucose 6-phosphate. These observations were consistent with our previous findings for the adrenergic Ca²⁺-dependent activation of phosphofructokinase (Clark & Patten, 1984), where the activity ratio was determined under slightly different conditions and where total recovery of the enzyme may have been suspect (Narabayashi *et al.*, 1985). Ca²⁺ and adrenaline also increased the extent of acylation of iodo[2-¹⁴C]acetate. Increasing the perfusate [Ca²⁺] from 0–0.1 mM to 10 mM increased the total amount acylated by 1.8-fold; the acylation in the presence and absence of 1 mM-ATP were equally affected, and each was increased by approx. 1.8-fold, but only marginally increased the amount acylated in the presence of 1 mM-ATP.

A comparison of activity ratio of phosphofructokinase and the extent of acylation by iodoacetate was made for a variety of conditions (e.g. α -agonist, β -agonist, low- and high-Ca²⁺ etc.), and the data are shown in Fig. 1. Significant correlation between the extent of acylation of phosphofructokinase and activity ratio was apparent ($r = 0.878$; $P < 0.001$).

Fig. 1 shows that the highest determined activity ratio of 9 coincided with the appearance of 0.75 reactive groups per subunit. If it is assumed that maximum activation occurs at an activity ratio of 12, then maximum activation coincides with the appearance of four reactive groups per tetramer, or one per 85000- M_r subunit. Since ATP (1 mM) inhibited the reactivity of half of the available groups of both the active and the inactive forms of the enzyme, it appears possible that the activation of phosphofructokinase results in an increase in the appearance of two different types of reactive groups: ATP-inhibitable and ATP-independent.

The data of Fig. 1 also reveal two additional findings. Firstly, the perfused heart from the genetically obese Zucker (*fa/fa*) rat did not respond to adrenaline treatment in terms of either an increase in activity ratio of phosphofructokinase [this was consistent with previous observations (Patten *et al.*, 1982b, 1984)], or an increase in reactive groups for acylation. Secondly, treatment of an extract from heart perfused with medium containing 1.22 mM-Ca²⁺, 1 μ M-adrenaline and 10 μ M-propranolol with GSSG decreased both the activity ratio and the reactivity with iodoacetate. This latter observation suggested that the reactive group(s) responsible for changes in enzyme activity ratio were able to react with GSSG.

In an attempt to identify the reactive group on phosphofructokinase, iodo[2-¹⁴C]acetate-labelled enzyme was isolated as the immunoprecipitate, acid-hydrolysed, and the amino acids were resolved by t.l.c. Approx. 90% of the radioactivity was located in *S*-carboxymethylcysteine (results not shown). When ATP was included to inhibit labelling, there was a corresponding inhibition of radioactivity located in *S*-carboxymethylcysteine. These findings indicated that, under the conditions used, 0.2 mM-iodo[2-¹⁴C]acetate essentially only labelled cysteine residues. Small amounts of radioactivity were found in glycollate (4.3–4.9%) and *S*-carboxymethylmethioninesulphonium (0.4%), indicating that only a limited reaction of iodoacetate with methionine residues had occurred (Goren *et al.*, 1968).

Analysis of samples used for the experiments of Table

1 indicated that an increase in perfusate Ca^{2+} concentration from 0–0.1 mM to 8–10 mM had no effect on the total cytosolic protein thiols, which remained at 0.126 ± 0.001 ($n = 3$) nmol/ μg of protein, nor on the concentrations of GSH [7.0 ± 0.1 ($n = 3$)] and GSSG [0.20 ± 0.03 ($n = 3$)] $\mu\text{mol/g}$ dry wt. of heart].

DISCUSSION

The present study shows a correlation between activity ratio of phosphofructokinase and the apparent reactive-thiol status of the enzyme. Since conditions which gave rise to an increase in the activity ratio of the enzyme were also those which increased the apparent reactive-thiol status, it is suggested that the change in reactive-thiol status may be causal of the change in activity ratio. However, the process by which the thiol(s) on phosphofructokinase become available as a result of adrenergic/ Ca^{2+} treatment of the heart remains to be defined, and several possibilities require consideration.

Firstly, an effect of Ca^{2+} to modify directly the conformational state of the enzyme so as to expose previously buried thiols would seem unlikely. We have shown that Ca^{2+} addition to cell-free extracts does not activate phosphofructokinase (Patten *et al.*, 1982a). Thus, if a correlation between activity ratio and reactive-thiol status holds for all situations, a Ca^{2+} -mediated change in conformation of the enzyme to increase reactive-thiol availability should also be expected to increase the activity ratio. In addition, extracts were subjected to gel filtration before reaction with iodoacetate. This could be expected to remove much of the Ca^{2+} and thereby to reverse a putative Ca^{2+} -mediated conformational change.

The results of experiments involving hearts from the obese Zucker (*fa/fa*) rat would also weaken the view that thiol reactivity has increased as a result of conformational change. Hearts from this strain that have a post-receptor defect for the adrenergic/ Ca^{2+} -dependent activation of phosphofructokinase showed neither a change in activity ratio nor an increase in reactive-thiol status when treated with adrenaline (Fig. 1). Since these hearts respond normally to adrenaline (Patten *et al.*, 1982b) and Ca^{2+} (Patten *et al.*, 1984) in terms of other changes, and phosphofructokinase from Zucker (*fa/fa*) hearts appears to be indistinguishable from that of control hearts, putative Ca^{2+} -mediated conformation changes would be expected to occur.

Secondly, an indirect effect of Ca^{2+} to modify the conformational state of phosphofructokinase by phosphorylation is a distinct possibility. However, we have been unable to demonstrate adrenergic/ Ca^{2+} -mediated phosphorylation of phosphofructokinase (Patten *et al.*, 1981), nor any change in activity ratio of the active form of the enzyme after treatment with phosphatase (Patten *et al.*, 1982a).

Thirdly, the catecholamine/ Ca^{2+} -mediated increase in phosphofructokinase thiol reactivity may arise from enzyme-mediated reduction. Possibilities include reduction of mixed disulphides involving low- or high- M_r donors, reduction of intra- or inter-chain disulphides or reductive cleavage of a thioester (Sulikowski-Fenton & Fahey, 1986). Regulation of enzyme activity by thiol/disulphide exchange (Zeigler, 1985) has been recognized for some time (Barron, 1951), and there are several enzymes where the activity *in vitro* is influenced by

the addition of low- M_r disulphides. Fructose-1,6-bisphosphatase, adenylate cyclase and glucose-6-phosphate dehydrogenase appear to be activated by disulphides, and phosphofructokinase, hexokinase, glycogen synthase, phosphorylase phosphatase, pyruvate kinase, hydroxymethylglutaryl-CoA reductase, 5-hydroxytryptamine *N*-acetyltransferase and pyruvate dehydrogenase appear to be inactivated by disulphides (Zeigler, 1985). For phosphofructokinase to be activated *in vivo* by reduction, it would appear necessary that a correlation between activity ratio and reactive-thiol status exists and that two forms of the enzyme can be demonstrated, one of which is the thiol form. The present study fulfils only the former of these requirements (Fig. 1). As yet, conditions for the reductive activation of phosphofructokinase in cell-free extracts from low- Ca^{2+} hearts have not been possible (results not shown), nor have we been able to identify the structure contributing to the low-activity form of the enzyme (mixed disulphide, thioester etc.). However, the Ca^{2+} -mediated appearance of two types of thiol (ATP-protected and non-ATP-protected) in approximately equal proportions (Fig. 1) may provide evidence for reductive cleavage of a disulphide. Thus, if Ca^{2+} -mediated activation of phosphofructokinase results from reduction of an intrapolypeptide disulphide, the reductive cleavage of a disulphide between one class I (non-ATP-protected) and one class II (ATP-protected) thiol (Kemp & Forest, 1968) in two of the four subunits may account for the observed increase in thiol reactivity of Fig. 1. The relationship between the thiols appears to be less clear for adrenaline + propranolol-, isoprenaline- and adrenaline-mediated activation, where the generation of a class II thiol predominates (Table 1, Fig. 1).

In summary, a hormone-mediated change in activity ratio of an enzyme associated with a change in reactive-thiol status is reported. The data are consistent with the view that it is the shift in reactive-thiol status to a more reduced state that has led to an increase in activity ratio of phosphofructokinase. The overall change in thiol content mediated by Ca^{2+} appears to be small, representing 6% of the total thiol content of phosphofructokinase, and the change occurred in conditions where the total protein thiol content and glutathione redox ratio were apparently unaffected.

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